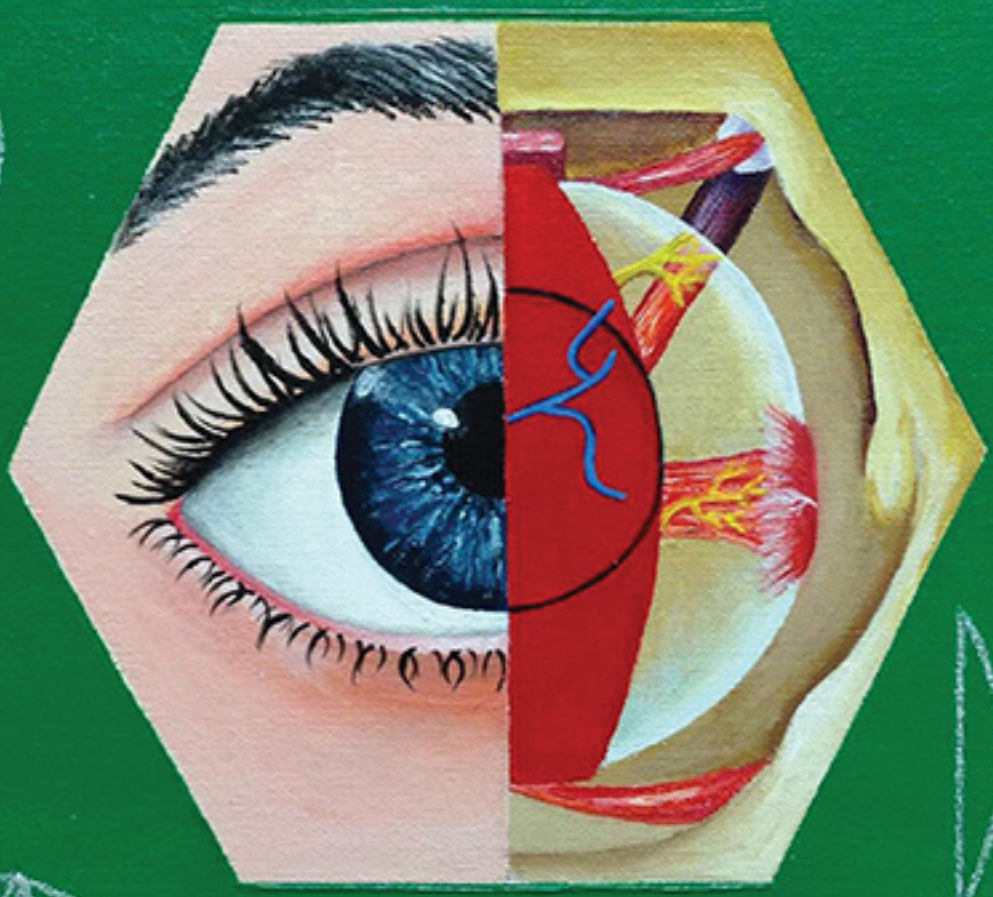
Science Journal

of the

Lander College of Arts and Sciences – Flatbush

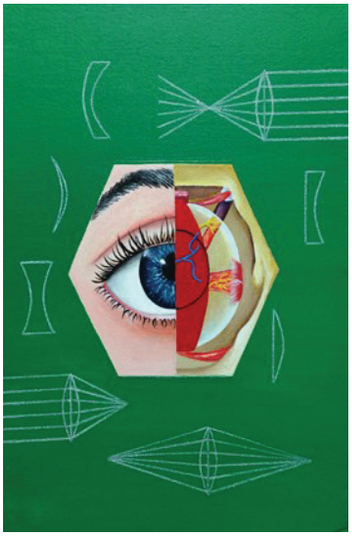
A Division of Touro College



Volume VII • Number 2 • Spring 2014

About The Cover

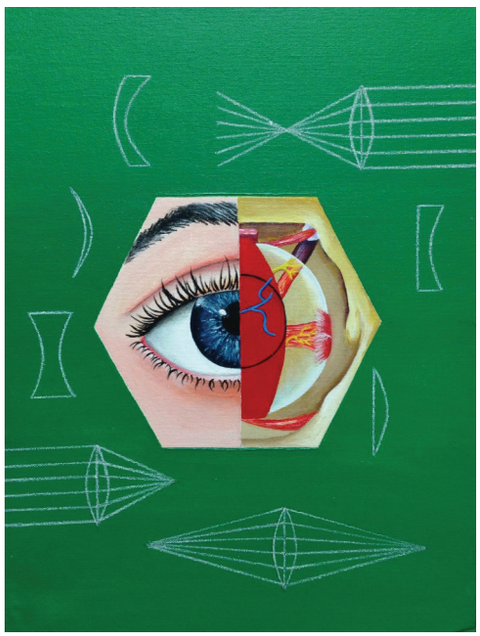
This issue has several articles about the nervous system and one on Myopia. Art student Karen Bleich prepared an original painting of the eye and several muscles that are associated with it. If you look carefully you will see the nerves (yellow) on the muscles. The addition of Karen to our staff continues the tradition of making "The Science Journal of the Lander College of Arts and Sciences – Flatbush" a publication that is truly of the students. All writing, reviewing, editing, layout design and cover illustration are performed by those students on the Masthead with the exception of the additional reviews provided by the faculty reviewers and the oversight of the project by the faculty Advisor, Dr. Robert S. Bressler.



This issue completes Volume 7 and marks seven years of publishing this journal. We appreciate our readership. Any comments, manuscripts for consideration, and other communications may be sent to touroscience-journal@gmail.com

Lander College of Arts and Sciences
A Division of Touro College
Flatbush Campus
1602 Ave J
Brooklyn, NY 11230
718.252.7800
tourosciencejournal@gmail.com

Contents



Environmental Factors and Progressive Myopia: A Global Health Problem

Jeffrey Weissman

Abstract

Myopia, or nearsightedness, is a refractive error whose prevalence has increased over the past three decades, leading to a growing concern and interest among both the public and scientific communities. For years, the only explanation and basis for myopia has been genetic factors. However, the genetic model does not explain the dramatic increase in prevalence. Current research suggests that the increase is also due to environmental factors, such as fewer hours of outdoor activities, early educational pressures requiring intense close work, as well as a lack of exposure to sunlight. One study compared the prevalence and risk factors for myopia in 6 and 7-year old children of Chinese ethnicity in Sydney and Singapore. In another study, a diffuser was placed over the eyes of chicks which caused the eyes to grow excessively myopic. This increased myopia was due to the lack of dopamine which originates from cells in the eye when stimulated by sunlight. One additional study suggested that formula milk, unlike breast milk, lacks DHA and can also result in myopia. The results of these studies suggest that progressive myopia is due not only to hereditary factors but also due to environmental factors. Recognition of these factors may be useful in developing future treatments.

Introduction

Nearsightedness, or myopia, as it is medically termed, is a vision condition in which close objects are seen clearly, but objects farther away appear blurred. Myopia generally occurs when the eyeball becomes elongated, or when the cornea, the clear front cover of the eye, has increased curvature. In myopia, light entering the eye does not focus directly on the retina rather in front of the retina, hence distant objects appear blurred. The more elongated the eye, the greater the myopia.

Myopia is a common refractive condition affecting approximately 100 million people in the United States (Vitale, et al., 2009). Its prevalence has increased over the past three decades, leading to a growing concern and interest in both the public and scientific communities. Myopia today is emerging as a global health problem, not only because of the costs associated with correcting refractive errors, but also because of the pathology associated with higher levels of myopia, such as retinal tears, retinal detachments, and macular degeneration. The prevalence of myopia varies in different parts of the world. Generally speaking, myopia is much more prevalent in industrialized countries and cities compared to rural areas (Uzma, et al., 2009). In 2009, a study showed that the prevalence of myopia in the United States, for people between the ages of 12 and 54, surged from 25% in the early 1970's to 42% by 2000. In Taiwan and Singapore, myopia is found in approximately 30% of all children 6 and 7 years old, and increases to 80% in young adults (Saw, et al., 2002). The rapid increase in the prevalence of myopia strongly suggests that environmental factors are having a considerable influence on the development of myopia not explainable by the genetic model. The cause of myopia has been debated for decades, and the exact mechanism responsible for the development of progressive myopia still remains unclear. There is significant evidence that many people inherit nearsightedness, or at least the tendency to develop nearsightedness. If one or both parents are nearsighted, there is an increased likelihood that their children will be nearsighted (Kurtz, et al., 2007). However, heredity alone does not explain why today there is such a dramatic increase in myopia. The dramatic increase in nearsightedness strongly suggests that, on top of the genetic model, environmental factors must be having a considerable influence on the development of myopia. Numerous studies support this hypothesis. This paper will review some of the recent research that supports the theory that environmental factors are contributing to the increase in progressive myopia, and will briefly review some of the solutions that may help slow down this progression.

Jeffrey Weissman will be graduating in January 2015 with a B.S. in Biology.

Discussion

A study carried out by Rose et al. (2008), showed differences in the prevalence of myopia in 6 and 7-year old children of Chinese ethnicity living in Sydney Australia vs. those living in Singapore. The study discovered that the prevalence of myopia was much greater for children living in Singapore (29.1%) than similarly aged children living in Sydney (3.3%). The range of spherical equivalents was −6.70 to +4.85 diopters for Singapore vs. −2.88 to +3.50 diopters for Sidney. The mean spherical equivalent was −0.16 diopters for the former vs. + 0.86 diopters for the latter. Consistent with these differences in refraction, the axial lengths and anterior chamber depths, two additional markers of myopia, were also significantly greater in Chinese children living in Singapore vs. those living in Sydney (Table 1). (Rose, et al. 2008)

Certainly, one factor that could possibly contribute to these large differences is parental myopia, which when present, has always been associated with a greater likelihood of myopia developing in children (Mutti, et al., 2002). However, in this study, there were no differences in the proportion of children with 0, 1, or 2 myopic parents between the two cities. In the Sydney sample, 32% of children had no parents with myopia, 43% had one myopic parent, and 25% had two myopic parents. This is comparable to the Singapore sample where there were 29% with no myopic parents, 43% with one myopic parent, and 28% with two myopic parents. The genetic differences related to myopia in the two populations is not significant, hence environmental factors must be playing a role.

Lifestyle factors that could possibly be contributing to the differences are outlined in Table 2. The children of Chinese origin living in Sydney actually read slightly more books, spent more time reading, writing, using computers outside of school, and watched less television than did the Chinese children living in Singapore. The cumulative measure of near-work activity was greater in the Sidney children

Table 1. Distribution of Refractive Error and Ocular Biometry Values in the Right Eyes of Children of Chinese Origin Living in Singapore and Sydney

	Sydney		Singapore		
	Children, No. (n=124)	Mean (SD)[a]	Children, No. (n=628)	Mean (SD)[a]	*P* Value
Female sex, %		53.2		50.3	.60
Myopia (spherical equivalent of ≤−0.5 D), %		3.3		29.1	<.001
Age, y	124	6.41 (0.35)	628	7.16 (0.39)	<.001
Spherical equivalent refraction, D	124	0.86 (0.78)	628	−0.16 (1.43)	<.001
Axial length, mm	123	22.60 (0.67)	613	23.13 (0.90)	<.001
Anterior chamber depth, mm	124	3.27 (0.22)	613	3.58 (0.27)	<.001
Corneal radius of curvature, mm	124	7.87 (0.25)	626	7.73 (0.25)	<.001
Axial length to corneal radius ratio	123	2.87 (0.07)	611	2.99 (0.10)	<.001

Abbreviation: D, diopter.
[a] Except where noted otherwise.

Table 2. Myopia Risk Factors in Children of Chinese Origin Living in Singapore and Sydney

	Sydney		Singapore		
Activity Outside School	Children, No. (n=124)	Mean (SD)	Children, No. (n=628)	Mean (SD)	*P* Value
Books read, No./wk	119	4.44 (2.46)	628	2.39 (2.27)	<.001
Reading and writing, h/wk	109	20.81 (13.88)	611	17.76 (8.78)	.03
Computer use, including computer games, h/wk	108	4.65 (6.62)	625	3.55 (4.48)	.10
Total near-work activity, h/wk[a]	106	29.93 (20.09)	608	23.54 (11.84)	.002
Coaching classes, h/wk	118	1.21 (1.75)	622	1.74 (2.02)	.007
Television viewing, h/wk	113	11.32 (6.47)	627	12.65 (7.37)	.07
Outdoor activities and sports, h/wk	102	13.75 (1.02)	586	3.05 (0.12)	<.001

[a] Includes reading, writing, computer use, crafts, and playing musical instruments.

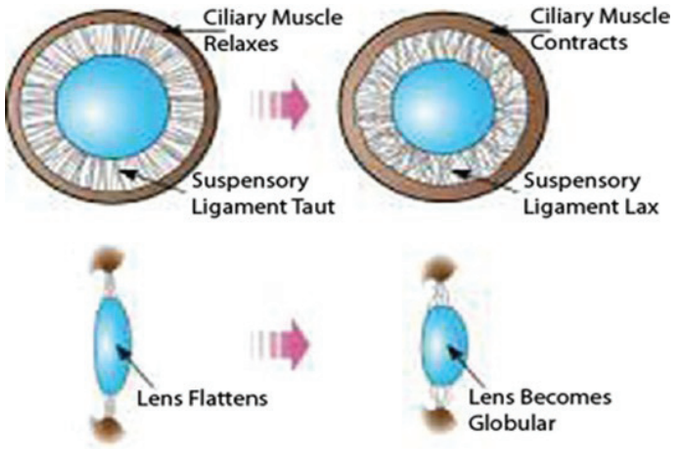
vs. the Singapore children, but the differences were small in magnitude, not statistically significant, and do not account for the increased myopia in the Singapore children. The largest statistically significant difference observed was that Chinese children living in Sydney spent nearly 14 hours per week in outdoor activities compared with just over 3 hours per week in Singapore. (Rose, et al., 2008) The authors offered two theories as to why the greater time spent outdoor by the children from Sydney may have resulted in less myopia. The first theory is that when outdoors, children require less accommodation in their vision, since their focus is not on near objects. Viewing near objects such as reading requires an accommodative response from within the eye. Viewing distant objects does not require a similar response. The second theory is that outdoor activity results in more exposure to brighter sunlight, which stimulates the release of dopamine, a known growth inhibitor within the retina.

Accomodation

The first explanation to account for the differences between the Singapore and Sydney children is related to the different styles of education within the two countries. In Singapore, most students are enrolled in a structured 3-year preschool program, with the aim of ensuring that children read fluently by the time they start school. In Sydney, most children attend a one year part-time preschool program, which is largely concerned with social development. This is followed by enrollment in a full-time kindergarten year before 1st grade, again with an emphasis on social development (Singapore Ministry of Education, 2004). Differences in the educational intensity at such an early stage can certainly have an impact on the early appearance of myopia in Singapore. The higher levels of myopia in Singapore is a result from Singapore's competitive and academically oriented schooling system, where there is an emphasis on educational achievements (Saw, et al., 2007). Continuous close work requires increased accommodation which can start the children in Singapore on a trajectory toward developing myopia from a very early age.

Why does intense education and competitive educational achievement increase the prevalence of myopia in Singapore? When one views distant images, parallel rays of light enter the eye and converge at a focal point on the retina. However, when viewing objects from near, instead of parallel rays entering the eye, the rays are diverging. The diverging rays activate an internal ocular mechanism called accommodation, which stimulates the circular ciliary muscles causing the lenses to change their curvature to a more convex shape (Figure 1). This change in curvature allows the diverging rays to now focus on the retina. The authors postulated that the constant contracting and relaxing of the ciliary muscles will eventually result in an increase in the axial length and a greater depth of the anterior chamber.

Figure 1: Accommodation (Elkington et al., 1999)



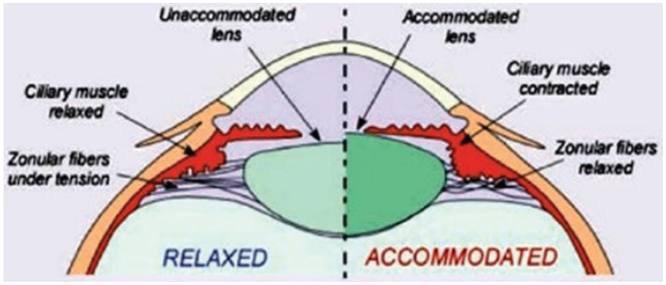
A more in depth understanding of the mechanism of accommodation will show how accommodative stress can result in axial elongation. Since small or near objects are typically focused at a further distance because of their diverging light rays, the eye accommodates by assuming a lens shape that has a shorter focal length. This reduction in focal length will cause more refraction of light and serve to focus the images on the retinal surface.

For near objects, the circular ciliary muscles contracts, allowing the lenses to assume a more convex shape. The increase in the lens curvature corresponds to a shorter focal length. On the other hand, a distant object is typically focused at a closer distance because the light rays entering the eye are parallel rays. The eye accommodates these parallel rays by assuming a lens shape that has a longer focal length. Hence, for distant objects the ciliary muscles relax and the lens returns to a flatter shape. This decrease

in the curvature of the lens corresponds to a longer focal length.

As outlined in Figure 2, the ciliary muscles adjust the shape of the lens since the muscles are attached to the zonules of Zinn, which in turn are attached to the lenses. Contraction of the ciliary muscles slackens the zonules, so that they do not pull as much on the lenses. The lenses become rounder, and the eyes can now focus on near objects. When the ciliary muscles relax, the zonules pull the edges of the lenses so that they become flatter and thinner to accommodate viewing distant objects.

Figure 2: Accommodation Mechanism (Dortonne, 2011)



The intense contraction of the ciliary muscle is believed to be the basis for the abnormal elongation of the myopic eye. The constant focusing on near objects causes a spasm of the ciliary muscle, and traction on the sclera, to which the ciliary muscle is attached on its external side. As the muscle contracts and pulls on the sclera, it compresses and increases the pressure within the vitreous cavity or larger chamber of the eye. Over time, with continuous contraction on the outer sclera, the sclera stretches and elongates, resulting in an enlarged eye. The body produces more aqueous liquid to fill the increased aqueous and vitreous cavity volumes. This increased elongation of the eye results in a progressive myopic state.

This explains why in Singapore the prevalence of myopia is higher. Since there is a greater amount of continuous intense accommodative stress at a younger age, involving reading, continuous computer viewing and other intense close visual work, the Singapore children may be more likely to develop nearsightedness. The assumption is that the continuous and intense accommodative mechanism of contracting and relaxing at a young age when the eye is in its formative years of growth is responsible for axial length elongation. The constant viewing of objects at 16-26 inches causes the focusing system to contract and get stuck at the near reading distance, thus stimulating the ciliary muscle leading to eye elongation and myopia.

Additional evidence supporting this hypothesis comes from Dr. Roger Zylberman, from the department of Ophthalmology at Shaare Zedek Medical Center, Jerusalem, Israel (Zylbermann, et al., 1993). He examined Jewish teenagers attending school in Jerusalem. He took 870 students: 175 males and 224 females from general schools, and 193 males and 278 females from Orthodox schools. The students' ages ranged from 14 to 18 years, as outlined in Table 3 (Zylbermann, et al., 1993) .

The distribution of the degree of myopia among the teenage students in Zylberman's study is outlined in Table 4. The prevalence of myopia was 31.7% in females attending general schools, and 36.2% in females from Orthodox schools. However, it was 27.4% in males from gen-

Table 3. Age Distribution of Subjects

	Female, No. [%]		Male, No. [%]		
Age (Years)	General Schools	Orthodox Schools	General Schools	Orthodox schools	Total
14	40 (17.9)	64 (23.0)	56 (32.0)	43 (22.3)	203 (23.3)
15	61 (27.2)	61 (21.9)	45 (25.7)	37 (19.2)	204 (23.4)
16	42 (18.8)	46 (16.5)	22 (12.6)	37 (19.2)	147 (16.9)
17	39 (17.4)	57 (20.5)	24 (13.7)	30 (15.5)	150 (17.2)
18	42 (18.8)	50 (18.0)	28 (16.0)	46 (23.8)	166 (19.1)
Total	224 (25.7)	278 (32.0)	175 (20.1)	193 (22.2)	870 (100.0)

eral schools, and 81.3% in males attending Orthodox schools. The difference in the prevalence of myopia between Orthodox males and all the other groups was statistically significant Table 4 (Zylbermann, et al., 1993) .

Table 4. Distribution of the Degree of Myopia

Diopters Needed to correct Myopia	% of Females		% of Males	
	General Schools	Orthodox Schools	General Schools	Orthodox Schools
- 0.50 to - 1.75	36.6	36.8	58.3	22.9
- 2.00 to - 3.75	38.0	41.9	31.3	33.8
- 4.00 to - 5.75	18.3	17.1	6.3	22.9
≥ - 6.00	7.0	4.3	4.2	20.4
Total Myopes	31.7	36.2	27.4	81.3

The authors explained that the reason the incidence of myopia was much higher in Orthodox Jewish males, was due to differences in their education systems. The curriculum and study methods in the Orthodox schools are distinctly different from secular schools. A moderate amount of accommodative eye use is required of male and female students in general schools, and of female students in Orthodox schools. Males in Orthodox schools, however, differed from all three other groups by their uncommon study habits characterized by sustained near vision, and frequent changes in accommodation due to their habitual swaying while studying. The rocking habit, by its constant defocusing and refocusing action, the variety of print size, and the need for accurate accommodation when reading the very tiny print in the Talmud, all require more intense accommodation. Overall, there is a very heavy accommodative stress in the young Orthodox males. The high degree and increased prevalence of myopia observed in the Orthodox male group is presumed to be due to their heavy accommodative needs, resulting from their unusual study habits. The higher accommodative needs of Singapore youth, could account as well for their increased myopia.

Sunlight Effect

The second explanation for the differences in myopia between Singapore and Sydney children, in regard to time outdoors, is related to sunlight exposure. Brighter light may reduce the development of myopia through the release of dopamine from the retina, which is known to act as an eye growth inhibitor (Stone, et al., 1989).

Dopamine is a neurotransmitter that plays a number of important roles in the brains and bodies of animals. In the brain, dopamine functions as a neurotransmitter, a chemical released by nerve cells to send signals to other cells in the brain. Outside the nervous system, dopamine functions in several parts of the body as a local chemical transmitter. It has a paracrine function, which means it is synthesized locally and it affects cells near the cells that release it. For example, in blood vessels it's a vasodilator. In kidneys, it increases sodium excretion and urine output. In the pancreas it reduces insulin production. In the digestive system it reduces gastrointestinal motility, and in the immune system it reduces lymphocyte activity.

In the eye, dopamine is released by a set of amacrine cells which then activate D1 and D2 dopamine receptors distributed throughout the retina (Rohrer, 1993). Dopamine plays a role in light adaptation. A reduction in retinal dopamine is known to occur in parkinsonian patients, resulting in reduced contrast sensitivity. Dopamine is also essential for eye cell survival, and for controlling normal eye growth (Witkovsky, 2004).

A study from the Australian National University showed that increased dopamine release, resulting from light exposure, stimulates D2 receptors within the eyes of chickens, resulting in suppression of axial elongation, or eye growth (McCarthy, et al., 2007).

The study was conducted as follows. When the eyelids of young chicks were sutured or when diffusers were put on the eyes of the young chickens, there was axi-

al elongation of the eyes, resulting in form deprivation myopia. However, if during the day the diffusers were removed for short periods, allowing normal exposure to light, the young chicks did not develop elongation and myopia. The authors concluded that light deprivation resulted in myopia because of a decrease in retinal dopamine. The authors proved that by suturing the eyelids or using diffusers on the young chicks there was impaired contrast sensitivity. This led to decreased dopamine release, decreased D2 dopamine receptor stimulation and finally increased eye growth.

In a second experiment when the diffuser was not removed, injecting dopamine during total darkness also prevented myopia in the young chicks. And finally when the authors injected a dopamine antagonist before removing the eye diffuser, there was again increased myopia, even though the eyes were exposed to light, since the dopamine stimulation of the D2 receptor was now blocked (Boelen, et al., 1994).

To further support his hypothesis, McCarthy citied a similar study that showed that by removing the diffuser for three hours there was an increase in measurable dopamine and less myopia in young chicks (Napper, et al., 1995).

We see from these studies that normal vision and the prevention of myopia are related to the stimulation of dopamine release and activation of D2 dopamine receptors. Since dopamine is necessary to maintain normal eye growth and prevent myopia, we can now explain why the children in Sydney who were exposed to more sunlight had less myopia, since their dopamine levels were higher than the Singapore children who spent most of their time indoors.

Breastfed Children

A third theory as to why Singapore children are more myopic has been advanced. This theory is based on a retrospective study from Singapore, which showed that breastfed children were 50% less likely to be nearsighted (Chong, et al., 2005, Williams, et al., 2001).

They studied 797 children, aged 10 to 12 as part of the Singapore Cohort Study of the Risk Factors of Myopia. There was no significant difference with the participants as regards to sex, age, or race. A total of 418 of the 797 children were breastfed and 379 were not. The degree of myopia was measured using cycloplegic autorefraction. Cycloplegia temporarily paralyzes the accommodative mechanism, allowing for a precise measurement of the degree of myopia. Myopia was defined as any individual with a spherical equivalent of at least a -0.5 diopters. All the study participants were given medical tests and also answered a series of questions including the number of books they read per week.

The results showed that children who were breastfed had a lower prevalence of myopia. Only 259 out of the 418 or 62.0% were myopic. Of the children who were not breastfed, 262 out of the 379 or 69.1% were myopic. The authors concluded that since these differences were statistically significant, breastfeeding is independently associated with a decreased likelihood of myopia.

They believed that docosahexaenoic acid also known as (DHA) is the main element responsible for early visual development in babies. DHA is found at very high concentrations in the cell membranes of the retina, and plays an important role in the regeneration of the visual pigment rhodopsin, and in the visual transduction system that converts light hitting the retina to visual images in the brain (SanGiovanni and Chew, 2005). Since breast milk is the main source of DHA in newborns, Chong et. al. (2005) concluded that reduced DHA in non-breastfed infants can result in an impairment of normal ordered eyeball growth, which can then lead to the development and severity of myopia. They recommended infant breastfeeding as a protective measure to lower the probability of the development of myopia.

In an article regarding the association between breastfeeding and myopia, it was shown that infant feeding did not influence visual development (Rudnicka, et al., 2008). Their findings were contrary to the previous study linking myopia with breastfeeding rather than formula feeding, and they concluded that other environmental factors were important for visual development and myopia in early life, and not breastfeeding.

Environmental Modifications

Based on the three environmental theories leading to myopia, society should consider the use of DHA supplementation in bottled milk especially in preterm infants, insistence on more outdoor or brighter light exposure for young children, and finally, based on the accommodative theory, the use of cycloplegic drugs or reading glasses in myopic children to reduce accommodative stress. These environmental modifications may reduce the risk of progressive myopia in young children (Gross, et al., 2006).

Drug Therapy

The use of a cycloplegic eye drop to reduce accommodation in children has been the most controversial of the proposed modifications. Numerous drug studies, requiring the use of atropine, or atropine-like drugs have concentrated on the role of accommodation in progressive myopia. The most convincing information was documented in the Atropine in the Treatment of Myopia (ATOM) study, which is the largest randomized controlled trial of its kind to date (Chua, et al., 2006). The ATOM study followed 400 eligible children between the ages of six and 12 for two years. After two years, in the placebo-treated eyes not receiving atropine, the mean progression of myopia was -1.20±0.69D with axial elongation of 0.38±0.38mm. In the atropine-treated eyes, myopia progression was only -0.28±0.92D with the axial length essentially unchanged (-0.02±0.35mm).

Despite the efficacy of atropine in reducing childhood myopia progression, atropine therapy is not accepted as a standard treatment. Although no serious adverse events related to atropine were reported in the ATOM study, side effects include increased light sensitivity due to mydriasis of the dilated pupil, which can impair a child's ability to perform well in school and athletics. The cosmetic issues of pupil dilation caused by atropine can also be awkward for children during the critical periods of social development, when they seek the acceptance of their peers.

While atropine therapy may not be appropriate for most children, the ATOM study suggests that pharmaceutical management has potential for reducing myopia, and that other atropine-like drugs, including pirenzepine and cyclopentolate, may be options. These drugs are weaker and not as long acting and have fewer side effects. One study found that 2% pirenzepine gel slowed childhood myopia progression by almost half after a year of treatment; however, 11% of subjects still withdrew from the study because of minor side effects (Tan, et al., 2005, Siatkowski, et al., 2008). Hence, there is no simple answer.

Conclusion

This paper has highlighted the fact that today there is an increased prevalence of myopia not explainable on the basis of the genetic model. Numerous environmental factors have been advanced including intense near activity resulting in accommodative stress, diminished exposure to outdoor light resulting in dopamine expression within the eye, and finally the reduced intake of DHA in non-breastfed babies. Finally, we have suggested that recognizing the significance of these environmental factors may help prevent some of the devastating complications associated with progressive myopia.

References

Boelen, M.K., Wellard, J., Dowton, M., Morgan, I.G., (1994). Endogenous dopamine inhibits the release of enkephalin-like immunoreactivity from amacrine cells of the chicken retina in the light. Brain Res., 645(1-2), 240 -246.

Chong, Y.S., Liang, Y., Tan, D., Gazzard, G., Stone, R.A., Saw, S.M. (2005). Association between breastfeeding and likelihood of myopia in children. JAMA, 293(24), 3001-2.

Chua, W.H., Balakrishnan, V., Chan, Y.H., (2006). Atropine for the treatment of childhood myopia. Ophthalmology, 113(12), 228-91.

Dortonne, I., (2011). Biomechanical response of the in situ primate lens. The Harvard Undergraduate Journal., 2:1

Elkington, A.R., Frank, H.J., Greaney, M.J., (1999). Clinical Optics, 3rd Ed. London: Blackwell Science, LTD, 107-112.

Gross, D.A., Grosvenor, T.P., Keller J. T., (2001). Optometric clinical practice guideline care of the patient with myopia. Reference Guide for Clinicians, Prepared by American Optometric Association Consensus Panel, St. Louis, Online Available: http://71.153.51.135/documents/CPG-15.pdf

Kurtz, D., Hyman, L., Gwiazda, J.E., Manny, R., Dong, L.M., Wang, Y., Scheiman M., (2007). Role of parental myopia in the progression of myopia and its interaction with treatment in COMET children. Invest. Ophthalmol. Vis. Sci., 48(2), 562-70.

McCarthy, C.S., Megaw, P., Devadas, M., Morgan, I.G. (2007). Dopaminergic agents affect the ability of brief periods of normal vision to prevent form-deprivation myopia. Exp. Eye Res., 84(1), 100-7.

Mutti, D.O. Mitchell, G.L., Moeschberger, M.L., Jones, L.A., Zadnik, K.

(2002). Parental myopia, near work, school achievement, and children's refractive error. Invest. Ophthalmology Vis. Sci., 43(12), 3633-3640.

Napper, G.A., Brennan, N.A., Barrington, M., Squires, M.A., Vessey, G.A., Vingrys, A.J., (1995). The duration of normal visual exposure necessary to prevent form deprivation myopia in chicks. Vision Res., 35(9), 1337-1344.

Rohrer, B., Spira, A.W., Stell, W.K. (1993). Apomorphine blocks form-deprivation myopia in chickens by a dopamine D2-receptor mechanism acting in retina or pigmented epithelium. Vis. Neurosci., 10(3), 447- 453.

Rose, K.A., Morgan, I.G., Smith, W., Burlutsky, G., Mitchell, P., Saw, S.M., (2008). Myopia, lifestyle, and schooling in students of Chinese ethnicity in Singapore and Sydney. Arch. Ophthalmol., 126(4), 527-530.

Rudnicka, A.R., Owen, C.G., Richards, M., Wadsworth, M.E.J., Strachan, D.P. (2008). Effect of breastfeeding and sociodemographic factors on visual outcome in childhood and adolescence. Am. J. Clin. Nutr., 87,1392–9.

SanGiovanni, J.P., Chew, E.Y. (2005). The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. Prog. Retin. Eye Res., 24(1), 87-138.

Saw, S.M., Carkeet, A., Chia, K.S., Stone, R.A., Tan, D.T. (2002). Component dependent risk factors for ocular parameters in Singapore Chinese children. Ophthalmology, 109(11), 2065-71.

Saw, S.M., Cheng, A., Fong, A., Tan, D., Morgan, I. (2007). School grades and myopia. Ophthalmic Physiol. Opt., 27(2), 126-129.

Siatkowski, R.M., Cotter, S.A., Crockett, R.S., Miller, J.M., Novack, G.D., Zadnik, K. (2008). Two-year multicenter, randomized, double-masked placebo-controlled, parallel safety and efficacy study of 2% pirenzepine ophthalmic gel in children with myopia. J. AAPOS, 12(4), 332-9.

Singapore Ministry of Education. (2004). Education Statistics Digest. Singapore Ministry of Education

Stone, R.A., Lin, T., Laties, A.M., Iuvone, P.M., (1989). Retinal dopamine and form-deprivation myopia. Proc. Natl. Acad. Sci. U.S.A., 86(2), 704-706.

Tan, D.T., Lam, D.S., Chua, W.H., Shu-Ping, D.F., Crockett, R.S., (2005). One-year multicenter, double-masked, placebo-controlled, parallel safety and efficacy study of 2% pirenzepine ophthalmic gel in children with myopia. Ophthalmology, 112(1), 84-91.

Uzma, N., Kumar, B.S., Khaja Moinuddin Salar, B.M., Zafar, M.A., Reddy, V.D. (2009). A comparative clinical survey of the prevalence of refractive errors and eye diseases in urban and rural school children. Can J. Ophthalmol., 44(3), 328-33.

Vitale, S., Sperduto, R.D., Ferris, F.L., 3rd. (2009). Increased prevalence of myopia in the United States between 1971-1972 and 1999-2004. Arch. Ophthalmol., 127(12), 1632-9.

Williams, C., Birch, E.E., Emmett, P.M., Northstone, K., Avon Longitudinal Study of Pregnancy and Childhood Study Team. (2001). Stereoacuity at age 3.5 y in children born full-term is associated with prenatal and postnatal dietary factors: a report from a population-based cohort study. Am. J. Clin. Nutr., 73(2), 316-22.

Witkovsky, P. (2004). Dopamine and retinal function. Doc Ophthalmol, 108(1), 17-40.

Zylbermann, R., Landau, D., Berson, D. (1993). The influence of study habits on myopia in Jewish teenagers. J. Pediatr. Ophthalmol. Strabismus, 30, 319–22.

The Viability of Organ Printing

Estie Schick

Abstract

Organ printing is an emerging technology that can potentially replace the need for human organ transplants altogether. Organ printing uses bioprinting methods to create three-dimensional biological constructs. Although it has not yet been implemented successfully, with nearly two decades of research devoted to this area, much progress has been made. This article outlines the various aspects of the organ printing process, describes both the accomplishments and challenges of bioprinting, and discusses the feasibility of bioprinting as a viable method for organ replacement.

Introduction

The cutting-edge principles of organ printing technology have been compared to the age-old properties of Johannes Guttenberg's printing press (Mironov et. al., 2008). The essential elements necessary for printing a book include a printing press, ink, paper, movable type, and a written text to be printed. These very same components can be applied to the up-and-coming field of bioprinting. Bioprinting is literally biological printing and utilizes the technology of a bioprinter to build a three-dimensional biological construct. This incredible feat is performed by the printer placing cells, bioink, layer-by-layer in specific locations onto a biopaper suitable for sustaining cell life. Printing biomaterials is obviously much more complex, but at its most basic levels it is analogous to the printing methods of a simple printing press. The necessary components for bioprinting are a bioprinter, bioink, biopaper, a method for depositing the biomaterials in set locations, and a model of the tissue or organ to be printed.

Organ printing technology has emerged as the topic of much research and discussion because of the shortage of organs for transplantation. There are other options besides for human organ donation such as xenotransplantation as well as artificial or mechanical organs (Boland et. al., 2003). But these options are the source of deleterious side effects, causing many to look to bioprinting as the future method for organ replacement.

That is not to say that there are not many roadblocks in the way of organ printing. The entire idea of 3D printing is somewhat reminiscent of science fiction and that is even before live human organs enter the picture. Because this is such a new field of study, there has not yet been much success in actually printing an organ. There are important steps and milestones that must be met along the way. In fact, there is no hope of an organ being printed successfully before tissue can be printed flawlessly. And there is certainly a long way to go before organs will be printed on an industrial scale. So it is important to ask: will bioprinting be a viable method for replacing damaged organs?

Materials and Methods

In order to answer the question proposed above, many journal articles relating to this topic have been read. Touro College's library database was also used to search for relevant studies and reviews. The next step taken was to look for articles that were referenced by those obtained through the Touro College Library search engines that seemed pertinent. All of the articles accumulated through this research have been used in an attempt to conclusively answer the question of the viability of organ printing.

Results

The Basics of Bioprinting: Bioprinters

Bioprinting uses computer-aided printing technology to deposit cells layer-by-layer in specific locations and form three-dimensional biological constructs. Many factors play a part in determining the efficacy of the bioprinting method, and a major one is the bioprinter. A decade ago, one of the first studies detailing the transformation of an ordinary commercial inkjet printer into a bioprinter was published. Inkjet printing was chosen specifically because the cells in the bioink were kept more hydrated than could

Estie Schick is currently pursuing a B.S. in Biology and will graduate in January 2015.

be obtained using any other printing method. In addition, inkjet printers are a significantly more economical choice than a more complex 3D printer. Inkjet printers are able to deposit tiny ink drops onto a substrate upon demand using thermal inkjet technology (Wilson, Boland, 2003). A small air bubble is heated until it expands and then collapses. When it collapses, that air bubble serves as the pressure pulse that forces a very tiny droplet of bioink out of the nozzle. The temperature can reach as high as 300° C, but the entire process is so instantaneous that pulse lasts only a couple of microseconds. Consequently the heightened temperatures do not permeate the bioink, and living cells can thereby be printed. With the advantage of inkjet based bioprinting, living cells can be printed at the same time as nutrients, drugs and growth factors, as well as gels and scaffolds (Cui, Boland, 2009).

Multiple printers were studied and each one was optimized for a specific application. The Cannon BJ2200 printer was modified so that cells could be printed onto very thin samples- as thin as 1 mm. Temperature controls were also added so that none of the living cell samples could be denatured by a heat above 100° F. The printer software drivers of an HP 550C were adapted so that solutions with different electrical charges and different viscosities could be printed. In order for pH, charge and viscosity of the cell sample not to affect the printing, new software was written that adjusts voltages constantly. Using the designs for the HP 660C printer, a new printer was built with a base that allowed for height adjustment. Consequently, the printed samples could be moved along the x and y planes. That same printer was further modified so that large mammalian cells could be printed. It would be impossible for cells of this size to fit through the nozzle of a regular inkjet printer so modifications had to be made to the print head. The new print head is made of nine individual pumps which can be operated individually, allowing multiple cells types to be printed onto the same sample. The nine pumps can be used simultaneously or a specific pump can be programmed to deposit cells at a given time. New software has been created that allows someone to simply enter the instructions on the computer and then watch the printer carry out those directions (Wilson, Boland, 2003).

In contrast to the inkjet printing method, laser assisted bioprinting has also emerged as a viable bioprinting technique. The Laser Induced Forward Transfer (LIFT) was originally used as a mechanism for transferring metals. It has been applied to bioprinting, resulting in a bioprinter named LaBP, the laser-assisted biological printer. This printer deposits suspended cell material onto a thin metal ribbon which is then hit with a laser pulse. The liquid solution is thereby deposited onto a sample of biopaper. In a recent study, factors such as cell density, viscosity, laser printing speed and laser energy were optimized to result in cell printing with the highest resolution. Rabbit carcinoma cells and human umbilical vein endothelial cells were used as bioink and suspended in liquid form. Different suspensions were prepared and their respective viscosities were measured. A correlation was drawn between high cell viscosity and a small droplet diameter which yields a high printing resolution. Different laser intensities and various laser speeds were tested as well. Decreased laser energy droplets and high laser scanning speeds resulted in high cell printing resolution. This study demonstrated that laser assisted bioprinting could successfully print biological structures, and a high cell-level resolution can be obtained. One advantage that laser-assisted bioprinting holds over inkjet bioprinting is the ability to print a high volume of cells per droplet. This is possible because the LaBP can print cells from a bioink with a concentration as high as 108 cells/ml. Using inkjet printing technology, there is a concern with using high concentration bioink because the printer head can clog. This is not an issue with laser-assisted bioprinting, and as a result high concentrations of cells can be used and cells can be still be printed one by one. The authors suggest further studies that implement a cell recognition scanning technology, which would help ensure that only one cell is being deposited with each laser pulse (Guillotin et. al., 2010).

The Basics of Bioprinting: Biopaper

Cell printing necessitates the use of biopaper so that the cells can be hydrated after printing. The drying process of the ink will have an effect on cell survival, and therefore the materials used as biopaper are imperative to the bioprinting process (Xu et. al., 2006). Therefore, in an innovative study, hydrogels are well suited to act as biopa-

per. The bioprinter that was used in the study was modified so that multiple types of hydrogel with or without cell suspensions can be printed into patterns. A correlation was drawn between certain printer control settings and the width of the printed hydrogel pattern. For example, the nozzle velocity, nozzle diameter and flow rate all have an effect on the hydrogel patterns. The specific hydrogel used in this study was formed by cross-linking hyaluronic acid with polyethylene glycol. This hydrogel (without cell suspensions) was printed multiple times, each time with varied printer settings. These tests yielded pattern widths ranging from 603.218 µm down to 141.38 µm. In all experiments, the temperature and humidity levels were controlled. Printing speed, nozzle diameter and injection speed were all varied, and a narrow hydrogel pattern width (i.e. 141.38 µm) was obtained with a fast printing speed, slow flow rate, and most importantly, small needle diameter (Song et. al., 2010).

In a groundbreaking study, a cell printer successfully printed nine cell types into a 3D construct using thermo-reversible gels. These gels are well-suited to become the biopaper in a printed tissue or organ because of their unique qualities. The gels are biodegradable, nontoxic and thermo-reversible, meaning that they are in a gel state at temperatures above 32° C and in a liquid state at temperatures below 20° C. The authors theorized that by dropping a layer of gel onto a heated substrate, printing cell aggregates onto that biopaper and repeating that process, 3D constructs would be formed as the cell aggregates fused together. In order to successfully perform that experiment, the optimal gel thickness and cell aggregate size had to be determined.

Both thermosensitive gels and collagen gels were prepared and their minimal thicknesses were measured. The reasons for using both types of gels in the 3D construct are to provide stability and strength, as well as forming a 'drug-delivery service.' This allows certain growth factors and bioactive agents to be released throughout the construct in a controlled fashion. The specific advantage of thermosensitive gels is that the time it takes for the gel to form directly affects the distribution of cells within the gel. Therefore, using gels that respond to temperature which gel more quickly than gelation controlled by solvent, pH, or ionic cross linking proves to be beneficial. Cell aggregates were also prepared using bovine aortal endothelial cells. The cells were printed onto series of gel layers. The cells were suspended in a liquid solution and did not spread out once they were printed on the gel layers. In addition, there was little, if any, mingling of the gel layers. But in order for this method to successfully form 3D tissue, fusion needs to occur between the cell aggregates. Although fusion of cell aggregates appeared to be more effective in collagen gels than in thermo-reversible gels, fusion did occur in those gels as well. Furthermore, it was demonstrated that fusion was not limited to the cells in the top layer of gel, but it was equally prevalent within the many layers of gel. In addition, a live/dead assay was performed, and showed that while cells that were not spread throughout the gels underwent cell death, the cells within the gels were remained alive. According to the authors, the adhesiveness of gels for cells is a property that can be modified, so the lower rate of diffusion through thermosensitive gels is not so worrisome. The aspect of the experiment that is slightly problematic is the small amount of apoptosis and necrosis that occurred as a result of cells being printed. The authors suggest further studies using aggregates modified with additional survival factors or genetic antiapoptotic modifications. Though there can be many changes made that will improve the results of this study, it demonstrated that by using thermo-reversible gels as biopaper, 3D organ printing is feasible (Boland et. al., 2003).

The Basics of Bioprinting: Bioink

The physical properties of sodium alginate hydrogel cross-linked with calcium chloride were examined in a study that found it well-suited to behave as bioink. Sodium alginate hydrogel has the unique property of fast gelation at room temperature because it solidifies upon contact with calcium chloride, making it a prime candidate for bioprinting. Because of the ionic cross-link controlled gelation, and because cells and growth factors can easily be suspended within the sodium alginate hydrogel, it makes an effective bioink. A multinozzle printing system was used, allowing the speed of gel injection to be controlled. The bioprinter used in this study was modified with a multinozzle injection syringes, as well as the ability to control stage and syringe motion in the x, y, and z axes. A 3D algi-

nate structure was printed using a four-nozzle system. Each gel layer was composed of a sodium alginate solution followed by a calcium chloride solution printed in the pattern of a lattice structure.

A 'layered pattern accumulation test' was performed to determine whether the sodium alginate-calcium chloride gel could be used to create 3D tissue constructs. The gels were printed using the multinozzle printer into a lattice pattern. The pattern held successfully, although it acquired a sideways slant due to the viscosity of sodium alginate hydrogel. This caused each layer to drag on the layer immediately below it. In future studies that problem should be rectified by using creating a system that will control the gel hardness and solidification time. Despite this setback, the feasibility of using cells suspended in sodium alginate hydrogel to print 3D biological constructs was clearly demonstrated by this study (Song et. al., 2011).

Bioprinting vs. Scaffold-Based Tissue Engineering

Tissue engineering is a field that combines biology, chemistry, physics and engineering in order to create or repair biological tissue. Because of the complexities in the structure and mechanics of biological tissue, there are obviously many challenges in the creation of tissue that performs and functions exactly the way it should. Nonetheless, for years tissue engineering has been incredibly successful. Popular uses of tissue engineering are to repair or replace body tissue including skin, muscle, bone, and blood vessels.

Typically, living cells are used as the primary engineering material in tissue engineering. These cells are placed or 'seeded' into a 3D artificial rigid structure- a scaffold. Scaffolding allows many of the challenges of tissue engineering to be overcome. For example, by using a solid structure like a scaffold, the implanted cells can attach onto its surfaces and eventually are able to form into three-dimensional tissues. One difficulty in creating scaffolds is that they need to be structured in a way that encourages optimal tissue formation to occur. This dictates what material the scaffold will be constructed from (i.e. how porous the material is.) This is important so that the nutrients can diffuse easily through the scaffold and reach the cells as necessary (Chan, Leong, 2008). The scaffolding material should also be biodegradable so that the scaffold will not have to be removed surgically. Instead, the scaffold needs to provide support to the cells while they are still forming their own structures, and then become absorbed by them when the three-dimensional tissue is fully formed. Additional factors that are important in scaffold-based tissue engineering are immunogenicity, the toxicity of the scaffolding material, and inflammatory response by the host (Norotte et. al., 2009).

There are cases when the use of scaffolds in tissue engineering proves detrimental to the newly forming tissue. Scaffolds can reduce the amount of connection between cells and can cause the misalignment of extracellular matrix. Additionally, it is difficult to place the many different types of cells usually found in an organ in specific locations in a solid scaffold. Another major problem with scaffolding is that it is not yet possible for vascular tissue to be formed, resulting in the absence of vascularization in any scaffold-based engineered tissue (Boland et. al. 2003). For these reasons and more, other scaffold- free tissue engineering options have been explored.

Bioprinting uses 3D printing technology to print cells layer-by-layer and create biological materials, and is an example of scaffold-free tissue engineering. One of the largest roadblocks in the success of scaffold-based tissue engineering was the inability to create vascular structures. In 2009, vascular tissue was successfully engineered using scaffold-free bioprinting. In this study, a rapid prototyping technology was developed which instructed a bioprinter to deposit bioink onto biopaper. More specifically, multicellular tissue spheroids of Chinese hamster ovary cells, human skin fibroblast cells, and human umbilical vein smooth muscle cells were used as bioink. In addition, agarose rods were used to build a template for the tubular vascular structure. These materials were placed layer by layer onto a biopaper made of collagen gel using a bioprinter that was designed with two printing heads. This allowed the simultaneous placement of the multicellular spheroids as well as the agarose rods. The use of the agarose rods as a template allowed the diameter of the tubular structure, the wall thickness, and the branching pattern of the vasculature to be accurately controlled. Once the spheroids were all deposited in the correct locations, their fusion was

monitored. It took 5-7 days for fusion to be complete and the tubular vasculature to be fully formed. The fused products were placed in a bioreactor for further maturation. Maturation is important before implantation because there are many cellular mechanical properties that need to be developed.

Once it was determined that the bioprinting was successful, the study was repeated with some variations. In the first, multicellular cylinders are used instead of spheroids. The bioprinter attachments had to be adjusted, but the printing of cylindrical units allowed for computer automation. In another, double-layered vascular tubes were created using both HUVSMC and HCF cylinders in specific patterns. Spheroids of different sizes were tested, and various bioprinter attachments were experimented with.

This study proved the effectiveness of scaffold-free tissue engineering using bioprinting. High cell density was achieved because the engineering materials only consisted of cells. In addition, when multicellular cylinders were used, fusion occurred within 2-4 days and uniform tubes were formed with minimal cell damage. There are some limitations though with methods and materials used in the study. For example, the thickness of the vascular wall prevents all cells from access to the diffused nutrients and oxygen. Therefore, apoptotic cells were observed in no apparent pattern throughout the final construct. In order to avoid this issue, microvasculature is necessary, but even with the advances that have been made, there is no visible solution as of yet. If it were possible to print thinner vascular walls, the cells would avoid apoptosis and cell viability would be increased. But the wall thickness and tube diameter of the vascular tissue is limited by micropipette size and resolution restrictions. Another issue that arose was the removal of the agarose rods. In the current study, the rods are removed manually. But this limits the geometry of the vascular branch necessitating open ends, and becomes more difficult to accomplish with more complex geometric constructs. The authors suggest thermosensitive or photosensitive gels as an alternative to agarose in order to eliminate this problem. This study demonstrated the advantages of scaffold-free tissue engineering over scaffolding, but in the process came up with a host of limitations specific to the methods used (Norotte et. al., 2009).

Successful Bioprinting of Mammalian Cells

One of the major hurdles to overcome in the study of tissue engineering is the complete interaction of the many cell types needed to fabricate complex tissue or organs. These cells need to be placed in very specific locations and fuse together forming a functional biological construct. A study demonstrated that mammalian cells can be successfully printed using a modified HP inkjet thermal printer and retain their functionality. Although bacterial cells had previously been printed successfully, the heat and pressure that are part and parcel of thermal printing had the potential to damage mammalian cells which are more sensitive than their bacterial counterparts. With the use of a modified HP 550C as bioprinter, soy agar and collagen hydrogels as biopaper and Chinese Hamster Ovary cells and embryonic rat motoneurons as bioink, viable mammalian cells were printed.

Suspended cells were printed in circular patterns onto the hydrogel-coated coverslips. Over the next few days the cells were studied under epiflourescent microscopes to determine whether or not the thermal printing process proved lethal. Green fluorescent light was observed, leading to the conclusion that the cells survived the stresses of printing. In addition to monitoring cell growth with advanced microscopy, an assay was performed to measure the percentage of lysed cells. When a cell undergoes lysis, an enzyme called LDH is released. By determining the amount of LDH present, the percentage of cell lysis was measured to be less than 10% in all cases, and 3.3% ±3.7% on average. The reason the cells were not damaged and killed by the temperatures near 300° C is because the heat does not have time to spread through the cells suspended in liquid. The droplets of bioink are printed so quickly that most of the cells do not experience a substantial rise in temperature (Xu et. al., 2005).

Inkjet Printing of Neurons Results in Viable Cell Structures

A lot of research is being devoted to the generation of nervous tissue because most neuronal cells have very low rates of regeneration. In the previously recounted study it was demonstrated that over 90% of cells can go through an inkjet printer and avoid lysis. The physiological

properties of those printed cells were examined in an innovative study. Although cell viability has previously been proven, this study aimed to determine whether cells that have been printed can retain their function. The cells involved in this study were rat primary hippocampal and cortical neurons. Although the temperature and other stresses of bioprinting largely do not affect printed cells, they may affect the electrophysiological properties of neurons. An example of a neural property that might be affected is the ability to fire action potentials.

A modified HP 550 inkjet printer was used to deposit the bioink in a circular pattern onto a collagen gel based biopaper. Axon and dendrite regeneration were demonstrated using immunostaining using MAP-2 as a dendritic marker and NF150 as an axonal filament marker. Immunostaining showed that the hippocampal and cortical neurons had regenerated all axonal and dendritic processes. This had been a concern- that the neurons would lose their neuronal phenotypes through the printing process. That would be very worrisome because the neurons could turn into other cells types like glial cells or cancer cells after losing their own cell phenotypes. After two weeks, the patch-clamp method was used to measure various electrophysiological properties including firing thresholds, repetitive firing, and after-hyperpolarization. This is an electrophysiological technique that studies multiple ion channels in excitable cells such as neurons and records their voltage currents. Results showed that the membranes of the cortical neurons contained mature voltage-gated potassium and sodium channels. In addition, no significant differences in electrophysiological activity were observed between regular hippocampal neurons and those that had been printed. Both cortical and hippocampal neurons were found capable of initiating action potentials. As is the case with mammalian cells, the retention of functionality after printing is due to the incredibly fast timeframe exhibited in thermal inkjet printing. The neurons were also not vulnerable to the shear and pressure of the inkjet printing because they cells had been trypsinized. That meant that the printed cells had no internal architecture and were not damaged by the shear stresses. Had the cells been affected they would have experienced either apoptosis or heat shock. Because the cells retained their function, it can be inferred that neither effect took place. Once these tests were administered on the single-layer neuron structures, 3D structures were printed. Fibrin gel was formed by printing thrombin droplets over layers of fibrinogen. NT2 neurons were printed layer-by-layer with the fibrin gel. High resolution SEM was used to examine the fibrin scaffold, and determined that it was well suited to serve as a scaffold for neurons because of its porous microstructure, allowing nutrients and oxygen to be delivered easily to the neurons within the scaffold. Another advantage that fibrin has over other hydrogel is the strong affinity of neurons for fibrin. Because the neurons attach strongly onto the fibrin scaffold, cell signaling is kept intact and cell functions are carried out. This study examined both 2D and 3D printing of neurons and demonstrated neuron viability and retention of cell phenotype and electrophysiological function (Xu et. al., 2006).

Laser-Assisted Bioprinting of Skin Substitutes

Once a person experiences an extensive burn injury, there are a limited number of options for their rehabilitation. If the wound is large, skin grafts cannot cover the entire area, due to their finite nature. There are a number of clinically approved skin substitutes like Integra and Matriderm which serve as either permanent or temporary wound coverage. These options leave scarring, discoloring, absence of hair follicles and can lead to other damaging side effects as well. Therefore, tissue engineering of skin substitutes is under high demand. Many challenges stand in the way of fabricating skin, due in part to the many cell types which need to be arranged in a very specific pattern. Furthermore, the functions of the engineered skin are greatly affected by the microenvironment of each cell type. A recent study demonstrated that a skin substitute could be created using laser-assisted bioprinting. The different cells types involved in the engineering process included human osteosarcoma cells, mouth endothelial cells, human osteoprogenitor cells, rodent olfactory ensheathing cells, human endothelial cells and human adipose derived mesenchymal stem cells. The cells were mixed with a collagen hydrogel before printing.

Twenty layers each of fibroblasts and keratinocytes were printed on top of a layer of Matriderm using laser printing technology. The Matriderm layer was im-

portant because it helps keep the printed skin constructs more stable during transplantation. The 3D skin constructs were incubated overnight, and then pieces were punched out and transplanted into the skin fold chambers of 12 mice that exhibited full-thickness wounds. In addition to this in vivo approach, the 3D constructs were cultivated in vitro as a control group.

The mice reacted well to the treatment, showing no discomfort or inflammation as a result of the transplantation procedure. In addition, after 11 days, the transplanted skin substitute and the surrounding mouse skin had fused completely together. There were no sharp lines delineating the border between real and substitute skin, and while the substitute skin was shiny at first it became matt as time passed. The keratinocytes and fibroblasts had been labeled before printing and implantation so that extensive tests could be administered even after transplantation. Results of these assays showed that the keratinocytes formed a stratified layer of tissue on top of the fibroblasts and Matriderm much like an epidermis. Although this epidermis was thinner than the natural mouse epidermis, after 11 days the two completely fused together. The thinner epidermis in the substituted skin might pose a problem because it is less stable than the epidermis of natural skin, but the methods of the study can be modified in the future to amend that flaw. In this study it was also shown that fibroblasts formed a multi-layer sheet of tissue. Some remained above the Matriderm where they had been printed and secreted collagen, while others spread through the Matriderm layer.

A skin construct that is incredibly similar to native skin was successfully printed using LaBP. The cells survived the bioprinting process without their phenotype being impacted in any way. One major advantage of the bioprinted skin substitutes is that blood vessel formation was observed in the skin constructs. Fast vascularization is imperative so that the cells can receive oxygen and eliminate cell waste. Complete vascularization was not achieved, but the authors assume that the issue was due to the time constraints of the study and that complete vascularization of the skin substitutes needs more time to be carried out. Despite this setback, this study demonstrated that blood vessels branched from the wound site and spread through the skin substitute very quickly, which is of the highest priority among engineered tissue (Michael et. al., 2013).

Effective Microvasculature Fabrication Using Inkjet Bioprinting

In the previously recounted study, a skin substitute was fabricated using laser-assisted bioprinting technology. Despite the fact that skin is a very complex organ, it is one of the few successfully engineered tissue constructs. Because skin is relatively thin, vascular tissue can either grow from the native skin and migrate through the skin construct, or nutrients and wastes can diffuse through the engineered tissue to and from the vasculature of the host's native tissue. Vasculature is one of the main challenges in tissue engineering because cells cannot survive without pathways for nutrients to be delivered and cellular waste to be eliminated. Another study detailed the use of inkjet bioprinters in the creation of human microvasculature. An HP 500 thermal inkjet printer was modified so that human microvascular endothelial cells and fibrin could be printed simultaneously. HMVEC are the only cells with the ability to form capillaries, and also have the unique property of adjusting their number and structure based on their microenvironments. Fibrin can be used in many ways- fibrin can be produced by the blood and plays a part in natural wound healing, fibrin gels are used as adhesives during surgery, fibrin glue can be used as a skin graft, and fibrin has been utilized extensively in tissue engineering. In this study, fibrin gel was used as a biopaper substrate for the HMVEC to be printed onto, and it was polymerized by combining varying concentrations of fibrinogen, thrombin and calcium. After the printed construct was incubated, a scanning electron microscope was used to facilitate the examination of the microstructure of the fibrin. Its mechanical properties were tested as well using an MTS electromechanical testing system. Results showed that the fibrin gel scaffold underwent only minor deformations as a consequence of the bioprinting process. A Live/Dead Viability/Cytotoxicity Kit was used to stain the HMVEC so that the formation of microvasculature could be observed and analyzed. After only 7 days, proliferation of the cells was detected and a confluent lining of cells was formed after

21 days. The microvasculature exhibited tubular structures which is consistent with the channels and tubes usually formed by endothelial cells. This demonstrates that thermal inkjet printers can be used to successfully fabricate human microvasculature which is fully functional (Cui, Boland, 2009).

Challenges in the Way of Organ Printing

Before any of the technology and methods proposed above can be implemented, one of the first steps to be done in organ printing is creating an organ blueprint. It is a computer-aided design that uses computer software to create a three-dimensional model. The software program then directs the bioprinter to deposit each biocomponent layer-by-layer. The challenge with organ blueprints is that they need to account for the post-printing processing that the 3D printed construct will undergo as a result of tissue fusion and maturation (Mironov et. al., 2008).

Many studies have experimented with various bioprinters, biopapers, and bioink in order to optimize the bioprinting process. In addition to further improvements in these areas, bioreactors are an important component of the bioprinting process. Bioreactors are commonly used in tissue engineering but there are specific properties that bioprinting necessitates. A bioreactor enables the post-processing step, probably bioprinting's most crucial step, to occur. After a tissue construct or organ is printed, the cells need time to fuse together and assemble a functional 3D construct. The bioreactor needs to be integrated closely enough with the bioprinter that the fragile printed constructs can be placed in its sterile conditions without incurring damage. The bioreactor also needs to allow perfusion of the vasculature in the printed construct. It takes time though before the vascular system is developed so the bioreactor also needs to provide a temporary irrigation system. This can be achieved using porous needles with pressure controlled dripper systems that can provide the wet environment that the tissue needs for its development. When the vasculature is sufficiently developed, the irrigation is terminated and perfusion of the vascular tree commences (Mironov et. al., 2011).

The last step in the bioprinting process is post-processing, and as was previously mentioned, it is probably the most crucial one. That is why biomonitoring procedures must be created and applied. It is important to monitor the tissue maturation and the kinetics of tissue self-assembly. In addition, maturogens that aid and accelerate post-processing and tissue maturation are necessary. Maturogens are biological, chemical, or physical factors and procedures that effectively ensure that the printed cell constructs become a fully-functional three-dimensional organ (Mironov et. al., 2008).

The Feasability of Organ Printing

There are three major phases in the organ printing procedure: preprocessing, processing, and post-processing. Preprocessing involves the development of an organ blueprint or alternate CAD. Processing refers to the actual printing of cells onto a substrate, forming a 3D construct. Post-processing concerns the fusion of the cells, the perfusion of the vasculature and tissue maturation. Many studies have been recounted throughout this paper which address every aspect of the organ printing process. Obviously much advancement must be made in every aspect of bioprinting technology before it can be applied to organ printing, but the feasibility of using bioprinting technologies to print an organ is strongly indicated by the groundbreaking scientific research that has inundated this field in the recent years (Mironov et. al., 2003).

Conclusion

After reviewing the scientific data related to bioprinting, it is safe to say that there is currently no way to successfully print a fully functional organ. But that is not to say that bioprinting isn't a viable method for organ regeneration. Bioprinting is a science that is less than two decades old and as a result, the technology and mechanisms are not advanced enough at this stage in time. The research that has been reviewed in this paper demonstrates though that every aspect of the organ printing process is being tackled and is a work-in-progress.

So much success has been achieved in so few years and there is definitely a long way to go. Each study brings forth an important piece of the enormous puzzle that is bioprinting. There are obviously many revisions to the experiments and advancements to the technology that must be undergone before organ printing can make the

leap from the lab to industrial-level production. Nevertheless, the viability of organ printing is affirmed by the enormous amount of progress and success in the bioprinting field.

References

Boland T, Mironov V, Gutowska A, Gutowska A, Roth EA, Markwald RR. Cell and organ printing 2: fusion of cell aggregates in three-dimensional gels. Anatomical Records Part A Discoveries in Molecular Cellular and Evolutionary Biology. 2003;272(2):497–502.

Chan BP, Leong KW. Scaffolding in tissue engineering: general approaches and tissue-specific considerations. European Spine Journal. 2008;7 (4):467-479.

Cui X, Boland T. Human microvasculature fabrication using thermal inkjet printing technology. Biomaterials. 2009;30(31):6221-6227.

Guillotin B, Souquet A, Catros S, Duocastella M, Pippenger B, Bellance S, Bareille R, Remy M, Bordenave L, Amedee J, Guillemot F. Laser assisted bioprinting of engineered tissue with high cell density and microscale organization. Biomaterials. 2010;31(28):7250–7256.

Michael S, Sorg H, Peck C-T, Koch L, Deiwick A, Chichkov B, Vogt PM, Reimers K. Tissue engineered skin substitutes created by laser-assisted bioprinting form skin-like structures in the dorsal skin fold chamber in mice. PLOS ONE. 2013;8(3):1-12.

Mironov V, Boland T, Trusk T, Forgacs G, Markwald RR. Organ printing: computer-aided jet-based 3D tissue engineering. Trends in Biotechnology. 2003;21(4):157-161.

Mironov V, Kasyanov V, Drake C, Markwald RR. Organ printing: promises and challenges. Regenerative Medicine. 2008;3(1):93-1-3.

Michael S, Sorg H, Peck C-T, Koch L, Deiwick A, Chichkov B, Vogt PM, 2008;7(4):467-479.

Mironov V, Kasyanov V, Markwald RR. Organ printing: from bioprinter to organ biofabrication line. Current Opinion in Biotechnology. 2011;22 (5):667-673.

Norotte C, Marga FS, Niklason LE, Forgacs G. Scaffold-free vascular tissue engineering using bioprinting. Biomaterials. 2009;30(30):5910-5917.

Wilson WC, Jr, Boland T. Cell and organ printing 1: Protein and cell printers. The Anatomical Record Part A Discoveries in Molecular Cellular and Evolutionary Biology. 2003;272(2):491–496.

Xu T, Gregory CA, Molnar P, Cui X, Jalota S, Bhaduri SB, Boland T. Viability and electrophysiology of neural cell structures generated by the inkjet printing method. Biomaterials. 2006;27(19):3580-3588.

Neural Plasticity Following Ischemia

Shira Brickman

Abstract

Neural plasticity refers to the ability of one's brain to change its structure and/or function in response to changes in behavior, environment, and neural processes. When a person suffers an ischemic brain injury, it often leads to hemisyndrome with motor and sensory deficits in the arm, leg, and face of one side. This article discusses the various ways that the existing network can be restructured and neuronal connections can be remodeled after the injury to enable partial or complete recovery of motor function. Spontaneous functional recovery after stroke develops through the overlapping sequence of events including a phase of axonal growth, spine remodeling and spine activation, and a phase of establishing and consolidating new neuronal networks.

Introduction

Brain ischemia is a condition where there is insufficient blood flow to the brain to meet the metabolic demand. Focal ischemia occurs when a blood clot blocks a cerebral vessel and it reduces blood flow and causes cell death to a confined region of the brain. Global ischemia, on the other hand occurs when blood flow to the entire brain is drastically reduced and the damage encompasses vast areas of brain tissue. When one suffers from focal ischemia and experiences brain cell death in a certain region, the area surrounding the ischemic core is called the peri-infarct cortex. While this area is compromised, it is potentially salvageable, so researchers study the physiologic changes that take place there. When ischemia occurs, energy hungry neurons stop functioning within seconds due to a lack of oxygen and they quickly show signs of structural damage. As the energy dependent processes fail, neurons cannot maintain their normal transmembrane ionic gradients, leading to cell death, or stroke, and the impairment of sensory and motor functioning.

Stroke, which affects a large percentage of the population, is the cause of a lot of physical and emotional suffering for the victims and their families. This is because it causes significant damage to brain tissue and to external neuronal connections that are involved in cognitive and functional tasks. While there are currently no pharmacological agents that can aid in restoring these functions, by studying and understanding the mechanisms that underlie spontaneous neural plasticity, researchers can identify neurobiological signals which can be critical for treatment and recovery of post-stroke patients (Wieloch T, Nikolich K,2006). This paper details the various mechanisms of brain plasticity and how they occur.

Materials and Methods

The information in this paper was obtained by critical analysis of scientific research articles and reviews. The articles were found in Touro College's online database and from various online medical journals. Most of the information is based on experiments and research done on rats because it is the most feasible way of obtaining vast information about strokes and the brain. While this information may not be completely applicable to the human brain, researchers feel that there is a lot of important information that can be obtained this way.

Discussion

Dendritic Spines:

Pyramidal neurons have a pyramid shaped body and are the most numerous excitatory cell type in the forebrain. Apical dendrites emerge from the apex of the pyramidal cell body and have membranous protrusions, called spines, which are the recipients of most excitatory signals in the brain. They participate in the transmission and integration of these signals and can also help increase the number of possible contacts between neu-

Shira Brickman graduated with a B.S. in Biology on May 2014 and will be attending SUNY Downstate's Physician Assistant Program.

rons. Dendritic spines are very plastic because they can change in shape, volume, and number in a small amount of time. These rapid changes in spine morphology that come as a result of the stroke could potentially influence electrical conduction of postsynaptic potentials (Brown CE, et. al., 2008).

In order to study the rapid, ischemia induced changes in apical dendritic spines, researchers used the photothrombotic method, to induce a stroke in 20 adult male mice, in the part of the sensorimotor cortex that affects the forelimbs. Photothrombosis uses photo activation of injected light sensitive dye. Once it is illuminated, the dye is activated, and it produces singlet oxygen, an electronically excited state of molecular oxygen that causes platelet aggregation and thrombi formation to interrupt local blood flow. This method is used because it produces highly localized and reproducible lesions Results show that focal stroke triggers rapid spine loss and elongation of remaining spines in the peri-infarct cortex (Brown CE, et. al., 2008).

The mouse brain was processed using Golgi-Cox staining. This is a silver staining that is used to view nerve tissues under light microscopy. This method only stains a limited number of entire cells at random, which allows neuroanatomists to track connections between neurons and to make the complex networking structure of many parts of the brain visible. They used this method to view the dendritic spine structures in the cortex at 2, 6 and 24 hours after the stroke. They selected pyramidal neurons from the peri-infarct primary motor cortex/M1and more distant secondary motor cortex/M2 to analyze the spine length and density. Two hours after the stroke, the loss of staining in the infarct core was already evident, while there was still relatively full labeling of neurons in the peri-infarct cortex. At the infarct border, the neurons had an asymmetric appearance because of the fully labeled dendrites on the non-ischemic side of the soma and fragmented dendrites on the other side projecting toward the infarct core. The researchers also measured the spine length of thousands of spines along the primary apical dendrites of neurons in the peri-infarct primary motor, secondary motor, and contralateral barrel cortex (layer 4 of somatosensory cortex). Analysis of this data revealed that spine length varied significantly from the control as a function of time after stroke and distance from the site of infarction. Spines were longer in the peri-infarct M1 but not in the other 2 regions. Additionally, within 24 hours after stroke, spine density levels dropped significantly in the peri -infarct M1. These findings suggest that during the first 24 hours after focal stroke, there is a loss of spines in the peri-infarct cortex. However, the measurements of the spines that remained were longer than that of the controls (Brown CE, et. al., 2008). The data also suggests that the effects of ischemia are limited mainly to neurons close to the infarct border because we do not see significant changes in spine density in the more distant M2 and contralateral barrel cortex. Changes in dendritic spine length or shape can significantly alter the functional properties of neurons by helping to make new connections.

What mechanisms are responsible for enhanced dendritic spine plasticity after stroke? Stroke induces certain gene expressions in the peri-infarct cortex that can regulate neuronal factors including GAP-43. GAP-43 is a growth or plasticity protein that is expressed at high levels during development of neurons in babies. However, the presence of GAP-43 levels in adult presynaptic membranes suggests that it continues to play a role in the functioning of certain synapses throughout life (Stroemer RP, et. al.,1995).

Dendrites and Vasculature

In addition to the plasticity of dendritic spines, the spontaneous recovery of functions after stroke is thought to be brought about through the reorganization and rewiring of surviving brain circuits. The surviving areas of the brain adjacent to the site of stroke reorganize and adopt new roles to compensate for the damage (Brown CE, et. al.,2007). Since dendritic spine turnover is the cause of rewiring during normal development and plasticity, it is likely to take part in bringing about changes that take place during and after stroke.

By using in vivo two-photon imaging, researchers examine changes in dendritic and vascular structure in cortical regions recovering from stroke. In adult control mice, dendritic arbors were relatively stable, however after

stroke, the organization of the dendrites in the peri-infarct cortex was altered. Using two-photon microscopy to monitor real time changes in dendritic structure, results indicate that cerebral infarction causes major changes in the peri-infarct dendrites and vasculature.

For the experiment, adult male mice expressing yellow fluorescent protein were used in order to label/ highlight the specific neurons in layer 5 of the cortex. Once again, the photothrombotic method was used to induce the infarction to the cortical representation of the forelimb. In order to study the organization of the dendrites, the apical dendritic tufts of the highlighted neurons were imaged with two photon imaging every hour for a 6 or 7 hour period. In control mice, the apical dendritic tufts and vasculature were intertwined with one another without any particular spatial relationship. However, these components in the peri-infarct cortex of injured mice displayed a very different pattern of organization; the dendrites and vasculature appear to be parallel with one another and radiate outward from the edge of the infarct border. In addition, researchers took note of the blood flow velocity of the plasma moving through the lumen of the capillaries. While they found that blood flow velocity was similar to that of the control, they observed that the density of the blood vessels in the peri- infarct region increased over time (Brown CE, et. al.,2007). Given the importance of dendrites in neurotransmission, these changes in dendrite structure may very possibly aid in the functional and/or behavioral changes in post stroke victims.

Branching Patterns:

The branching patterns of dendrites are associated with their ability to integrate synaptic inputs from various sources. Therefore, changes in the architecture of the dendrites will determine the size and selectivity of the receptive field of that neuron. Using in vivo time lapse imaging, fully mature pyramidal neurons were seen to be capable of significant structural remodeling when faced with nearby ischemia. They remodel in a manner that conserves the total dendritic length by favoring both growth and retraction equally. Researchers chose to follow specific neurons in 2-3 month old mice. Before inducing stroke, they imaged the apical dendritic tufts for 2-4 weeks and measured the length of the dendritic branch tips. These images indicated that the lengths were relatively stable being that the total length remodeled was only 7% every 2 weeks. After inducing focal stroke in the cortical territory adjacent to these imaged neurons, the dendritic arbors were imaged every 2 weeks for 4 to 6 weeks. The stroke altered the configuration of the apical dendrites in the peri infarct cortex. The total length of the dendrite remodeled was inversely related to the distance of the neuron to the infarct border. Additionally it was observed, that the remodeling mostly occurred through existing branch tips-entire tips were generally not eliminated and there were generally no additions of entirely new branches (Brown CE, et. al., 2009).

These results show that while stroke triggers structural plasticity, it conserves the total length by favoring growth and retraction equally. It was also noted that dendrites facing toward the infarct retracted while those oriented away from the infarct grew. The fact that total dendritic length within the neurons didn't change significantly over time despite the increase in dendritic remodeling suggests that mature cortical neurons may have an innate mechanism that conserves the total amount of space each neuron takes up.

Axonal Outgrowth/ Sprouting of Surviving Neurons

Recovery of function following cortical injury is also associated with enhanced axonal growth and remodeling in the area of the lesion. For example, small ischemic lesions trigger horizontal axonal sprouting between areas that are not normally connected. Neurofilament is a major component of the neuron's cytoskeleton that provides structural support for the axon and regulates the axon's diameter. Phosphorylated heavy neurofilament participates in axonal growth and regulates synaptic functions. Stroke gradually but substantially increases heavy neurofilament axons in the peri-infarct cortex and in homologous areas of the contralateral cortex during the recovery period. At the same time, experimental data revealed that stroke induces a loss in heavy neurofilament axons in the region of the infarct. The expression of neurofilament proteins during axon outgrowth suggests that

they are important for axon development (Ueno Y, et. al., 2012).

The correlation between neurofilaments and axon growth was displayed in Xenopus (African claw frogs used for experiments) embryos. Antibodies against neurofilament were injected into embryos to disrupt neurofilaments on one side of the embryo during axon development. Time lapse video microscopy was used to study neurite outgrowth. Neurites include any projection coming from the cell body, like axons or dendrites. By the second day of axon outgrowth, they produced shorter axons because they spent less time actively elongating (Walker KL, et. al., 2001). This indicates that neurofilaments promote normal rates of axon elongation. This study proves that neurofilaments play an important role in axonal outgrowth.

Cortical Rewiring

After a cortical infarct in the primary motor cortex (or other parts of the brain), other intact, more distant regions of the brain such as premotor areas, remodel. Because the ventral premotor cortex shares extensive connections with the primary motor cortex/M1, after M1 injury, the axons of the ventral premotor cortex that led to M1 degenerate. As a result, these neurons seek new targets and remodel during the post-infarct period. Researchers initially used microelectrode stimulation to observe the neurophysiological mapping between M1 and ventral premotor cortex before the infarct. This information was used to identify the areas sharing connections. By electrically stimulating the hand to move they recorded all the sites at which the stimulation elicited movements. Researchers then induced a cortical infarct destroying the hand representation in the primary motor cortex of 8 adult squirrel monkeys and examined the cortical connections of the ventral premotor cortex several months later by following a tract tracer (biotinylated dextran amine) that was injected into the ventral premotor cortex hand area. Comparing the data with the pattern of connections in uninjured animals, labeling patterns indicated an increase in ventral premotor cortex terminal fields after the injury (Dancause N, et. al., 2005).

Cell Genesis

In addition to repair and regrowth of surviving neurons, cell genesis is stimulated following stroke in certain areas such as the subventricular zone. The subventricular zone is a brain structure situated in the lateral walls of the lateral ventricles that is a known site of neurogenesis. Cell death in the brain triggers a regenerative response in the tissue adjacent to the area of cell death. Stroke induces an increase in the number of immature neurons, or neuroblasts in the subventricular zone. Then, within the first 2 -4 weeks after stroke, these neuroblasts migrate to the tissue adjacent to the stroke site. Eventually they can go on to express markers of mature neurons. To determine the path and signaling systems used in neurogenesis, a focal stroke was induced in the somatosensory barrel field cortex of mice. It was found that new, immature neurons were present in large numbers in the peri-infarct cortex in the first week after stroke. The neuroblasts form close physical associations with peri-infarct blood vessels in a region of active vascular remodeling. Because neurons in the peri-infarct cortex experience abnormal patterns of synaptic transmission due to the lesion, newly born neuroblasts may help to improve behavioral recovery by stabilizing the injured neurons (Ohab JJ, et. al., 2006).

Brain Derived Neurotophic Factor

Brain derived neurotrophic factor (BDNF) is a secreted protein that plays a role in the processes mentioned above-neuronal survival, synaptic plasticity, and neural plasticity. It is one of a family of neurotrophins that influences neuronal proliferation, survival, and differentiation as a result of binding to its tyrosine kinase receptor. BDNF is stored and released from glutamate neurons and aids in recovery from brain injury. Therefore, exogenous treatments with BDNF after stroke, enhances behavioral recovery. In addition, exercise, which increases the amount of brain derived neurotrophic factor, seems to improve behavioral outcome in rodent stroke models. On the other hand, inhibiting BDNF can reduce and slow down the recovery process. These findings support the role of BDNF in motor map reorganization. Thirty two male rats were tested by inducing focal ischemia. On day 4, half of the animals received a BDNF inhibitor (antisense BDNF oligonucle-

otide), while the other half received BDNF. Each group was further divided, half receiving rehab training and half not. Rehab consisted of a running exercise and reach training. These therapies significantly improved skilled reaching in the staircase task after focal ischemia. However, blocking BDNF receptor, prevented behavioral recovery (Ploughman M, et. al., 2009). This study shows that BDNF plays a crucial role in the motor relearning that takes place as a result of poststroke rehabilitation.

fMRI and BOLD

As found in the previous studies, gradual improvement of motor function after stroke, is associated with changes in functional and structural connectivity within bilateral neuronal networks. Brain reorganization leads to normalization of neuronal signal synchronization within the affected sensorimotor cortical network and between the affected and unaffected sensorimotor cortices. Functional imaging (fMRI) studies have provided evidence of cerebral reorganization by displaying task induced activation patterns in the affected/ipsilesional and contralesional brain hemispheres. The resting state fMRIs show a shift toward a more random topology of the motor execution network in recovering stroke patients. Additionally, measurement of blood oxygenation level-dependent (BOLD) fluctuations with fMRI has revealed changes in interhemispheric synchronization of neuronal signaling which correlated with behavioral outcome. It is changes in dendritic morphology, axonal sprouting and synaptogenesis that lead to these critical adjustments of local structural connectivity.

Before making lesions in the rats' brains, researchers identified the baseline functional network that includes the bilateral cortices of all rats using mean BOLD signals from fMRI scans. Then they induced medium and large size lesions in rats while maintaining a group of 10 control rats. The sensorimotor function of the rats was reduced after stroke, especially in the rats with a large stroke. Maps of functional connectivity of the contralesional sensorimotor cortex with the rest of the brain illustrated that the strong baseline interhemispheric functional connectivity between the left and right sensorimotor cortices was lost at three days after the stroke. Shortly after, both groups of rats (medium and large stroke) showed an increase in connectivity, with stroke medium rats returned to baseline levels after day 70 while stroke large rats improved somewhat but remained significantly lower at all time points after stroke. The changes in intercortical functional connectivity after stroke were confirmed by EEG recordings. EEG is an electroencephalogram which measures and records the electrical activity in the brain. Just like the results from the resting state fMRI, a significant loss of intercortical synchronization was shown with EEG signals as compared with baseline. Intraregional signal coherence in the ipsilesional sensorimotor cortex also significantly reduced at days 3 and 7 after stroke. This was restored in stroke medium patients after more than three weeks, but took about ten weeks for stroke large groups. These studies show that the improvement of sensorimotor function is associated with the restoration of interhemispheric connectivity of the bilateral sensorimotor cortex (Van Meer MP, et. al., 2012). n

Conclusion

Despite the large amounts of brain damage that occur after one suffers from a stroke, the above studies prove that there is hope for stroke victims (and their families). While the chances of recovery and the degree of recovery varies from person to person, most people have a chance at recovering some of their lost functions. The degree of recovery depends on various factors such as the size of the stroke and their course of rehabilitation. However, by understanding all the neuronal mechanisms that underlie post-stroke recovery, like axonal growth, spine outgrowth, spinal remodeling, and cortical rewiring, researchers can hopefully develop strategies to enhance these processes.

References

Brown CE, Boyd JD, Murphy TH. Longitudinal *in vivo imaging reveals balanced and branch-specific remodeling of mature cortical pyramidal dendritic arbors after stroke. J Cereb Blood Flow Metab. 2010; 30(4): 783–791.*

Brown CE, Li P, Boyd JD, Delaney KR, Murphy TH**.** Extensive turnover of dendritic spines and vascular remodeling in cortical tissues recovering from stroke. *The Journal of Neuroscience. 2007;27(15):4101– 4109*

Brown CE, Murphy TH. Livin' on the edge: imaging dendritic spine turnover in the peri-infarct zone during ischemic stroke and recovery. *Neuroscientist. 2008;14(2): 139-146*

Brown CE, Wong C, Murphy TH. Rapid morphologic plasticity of peri-infarct dendritic spines after focal ischemic stroke. *Stroke. 2008;39:1286 -1291.*

Dancause N, Barbay S, Frost SB, Plautz EJ, Chen D, Zoubina EV, Stowe AM, Nudo RJ. Extensive cortical rewiring after brain injury. *The Journal of Neuroscience. 2005;25 (44):10167-10179*

Ohab JJ, Fleming S, Blesch A, Carmichael ST. A neurovascular niche for neurogenesis after stroke. *The Journal of Neuroscience. 2006;26 (50):13007-13016*

Ploughman M, Windle V, MacLellan CL, White N, Dore JJ, Corbett D. Brain-derived neurotrophic factor contributes to recovery of skilled reaching after focal ischemia in rats. *Stroke. 2009;40:1490-1495*

Stroemer RP, Kent TA, Hulsebosch CE. Neocortical neural sprouting, synaptogenesis,and behavioral recovery after neocortical infarction in rats. *Stroke.1995;26:2135-2144*

Ueno Y, Chopp M, Zhang L, Buller B, Liu Z, Lehman NL, Liu XS, Zhang Y, Roberts C, Zhang ZG. Axonal outgrowth and dendritic plasticity in the cortical peri-infarct area after experimental stroke. *Stroke. 2012;43:2221 -2228*

van Meer MP, Otte WM, van der Marel K, Nijboer CH, Kavelaars A, van der Sprenkel JW, Viergever MA, Dijkhuizen RM. Extent of neuronal network reorganization and functional recovery in relation to stroke severity. *The Journal of Neuroscience.2012;32(13)4495-4507*

Walker KL, Yoo HK, Undamatla J, Szaro BG. Loss of neurofilaments alters axonal growth dynamics. *The Journal of Neuroscience. 2001;21(24):9655 -9666*

Wieloch T, Nikolich K. Mechanisms of neural plasticity following brain injury. *Current Opinion in Neurobiology.2006;16:258-264*

Stem Cells as a Cure For Amyotrophic Lateral Sclerosis

Chaya K. Hirsch

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a fatal motor neuron disease which affects approximately 30,000 Americans at any given time (alsa.org, 2010). The etiology of this terminal disease unfortunately remains an unsolved mystery and has therefore severely limited the ability to find a cure. The use of stem cells to regenerate neurons has been vastly studied and have produced very promising results. However, its practicality as a cure or treatment for neurodegenerative diseases, such as ALS, is greatly compromised. Three different therapies involving stem cells were examined, Embryonic Stem Cells (ESC), induced pluripotent stem cells, (iPSC) and direct reprogramming of adult stem cells into motor neural cells, and their advantages and limitations discussed. While ESC, iPSC and induced motor neural cells (iMNC) may have astonishing potential as a treatment they also have severe limitations ethically, clinically and effectively.

Introduction

Amyotrophic Lateral Sclerosis (ALS), commonly known as "Lou Gehrig's Disease," is a fatal neurodegenerative disorder characterized by the slow degeneration of the motor neurons (MNs) of the entire human body, including, but not limited to, the MNs of the cerebral cortex, spinal cord and limb, axial and respiratory muscles. ALS is an adult-onset, rapidly progressive disease, killing 50% of patients within 2 and a half years of symptom onset. There are two types of disease onset, Limb-onset, which is characterized by degeneration of upper and lower motor neurons (UMN and LMN) identified by weakness, fasciculations and spasticity of the limbs, and Bulbar-onset, degeneration of tongue muscles causing weakness and fasciculations in the tongue as well as spastic dysarthria and dysphagia. Eventually, no matter the type of onset, the motor neuron degeneration spreads throughout the body resulting in paralysis of the limbs and eventually the diaphragm, causing respiratory distress and ultimately death (Kiernan, et al. 2011).

ALS has been categorized into two classes, familial ALS, which is genetic and presented through a Mendelian pattern of inheritance, accounts for 5-10% of people with ALS, and Sporadic ALS which accounts for the remaining 90% (Kiernan, et al. 2011).

At present, the etiology remains complex and unresolved and has confounded scientists over the century. As a result, finding a treatment for ALS has proved to be extremely challenging. The etiology seems to be multifaceted with many genetic and molecular factors contributing directly and indirectly to the degeneration of motor neurons, including gene mutations, neurotoxins, glutamate-induced excitotoxicity, and structural abnormalities of the mitochondrion, sodium potassium pump and axonal structures. Mutations in SOD1 gene generates a toxic gain of function of SOD1 enzyme, formation of free radicals and formation of aggregates due to improper folding of the SOD1 protein, resulting in impaired motor neuron function and neuron death (Kiernan, et al. 2011). The SOD1 gene, which accounts for 20% of familial ALS, is of significant importance because it can transduce mice into effective ALS models for stem cell therapy and other research (Gurney, et al. 1994).

To date, only one drug, Riluzole, remains effective in treating ALS symptoms, by inhibiting glutamate receptors and slowing the disease progression by 3-6 months. This unfortunate lack of a significant cure or treatment may be a result of the inadequacy of diagnostic tests. By the time a patient is diagnosed with ALS, many of their motor neurons have allready died, and their muscles atrophied. Since ALS can only be diagnosed once clinical symptoms appear, there is no early detection and therefor, no early prevention (Kiernan, et al. 2011).

Stem cells, however, provide an astonishing and

Chaya K. Hirsch graduated in May 2014 with a B.S. in Biology.

effective alternative. They can accomplish what Riluzole cannot, providing hope for a future regenerative cure for neurodegenerative diseases. Stem cells are cells capable of giving rise to new cells of the same type or to different cell types through the process of differentiation. Modern research has discovered that stem cells can be induced into neurons, which can regenerate neurons, reestablish neural connections and protect the remaining neurons from further degeneration when transplanted into mouse or human ALS models and patients (Miles, et al. 2004; Nizzardo, et al. 2013; Peljto, et al. 2010; Son, et al. 2011; Takahashi, Yamanka, 2006; Takahashi, et al. 2007; Umbach, et al. 2012; Vierbuchen, et al. 2010; Wichterle, et al. 2002; Xu, et al. 2009; Yao, et al. 2013) If the safety and efficiency of stem cells can be determined, the progression of ALS can be halted and reversed, rather than just slowed down.

Stem cells derived from embryos, called Embryonic Stem cells (ESC) can differentiate in to the three germ layers, ectoderm, mesoderm and endoderm and then be forced through specialized transcription factors or signals to differentiate into functioning neurons in vitro and in vivo (Miles, et al. 2004; Peljto, et al. 2010; Umbach, et al. 2012; Wichterle, et al. 2002). However, ESCs provide an ethical issue because the embryo is sacrificed in exchange for stem cells. Therefor a modern approach was created by inducing differentiated stem cells, including post-natal and adult stem cells, into a pluripotent state, which can then be induced into neurons and other cells through transcription factors with ESC-like efficiency (Nizzardo, et al. 2013; Takahashi, Yamanka, 2006; Takahashi, et al. 2007; Yao, et al. 2013). However, the induction of a stem cell into a pluripotent state requires the use of oncogenes and tumorigenic factors which can be preserved in the induced neuron state. These factors can produce malignant tumors in vivo, a challenging clinical hurdle to overcome (Lee, et al. 2013). The most recent research has been devoted to directly converting stem cells into neurons, skipping the pluripotent state and avoiding the formation of potentially malignant neoplasms (Son, et al. 2011; Vierbuchen, et al. 2010). These three approaches each have advantages and limitations, which will be discussed in further detail, and provide a promising hope for a future cure for ALS.

Embryonic Stem Cells

To begin the process of generating motor neurons from ESC we must understand the developmental stages and the signaling characteristics of motor neurons from the ectoderm. Ectodermal cells develop into a neural progenitor state through the regulating signals, bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wnt. These signals play a vital role during development of neural progenitor cells by regulating cell-to-cell interactions and controlling the activation or deactivation of specific genes (Wilson, Edlund, 2001). These neural progenitor cells, which are characterized by the expression of neural biomarkers, Neuronal Nuclei (NeuN), Sex determining region Y- box 1(Sox1), and Neuronal Class III β –Tubulin (TuJ1), can then differentiate into all classifications of neurons through the help of specific transcription factors and signals. Through the action of Sonic hedgehog (Shh) signaling these neural progenitor cells are transformed to a progenitor motor neuron (pMN) domain, which are destined to the fate of spinal motor neurons. These cells in the pMN domain are characterized by the expression of Pax6, Nkx6.1 and Olig2 proteins which each have a role in the differentiation of various spinal motor neurons (Wichterle, et al. 2002). Additionally, these factors direct the expression of Hb9, a transcription factor found in MNs.

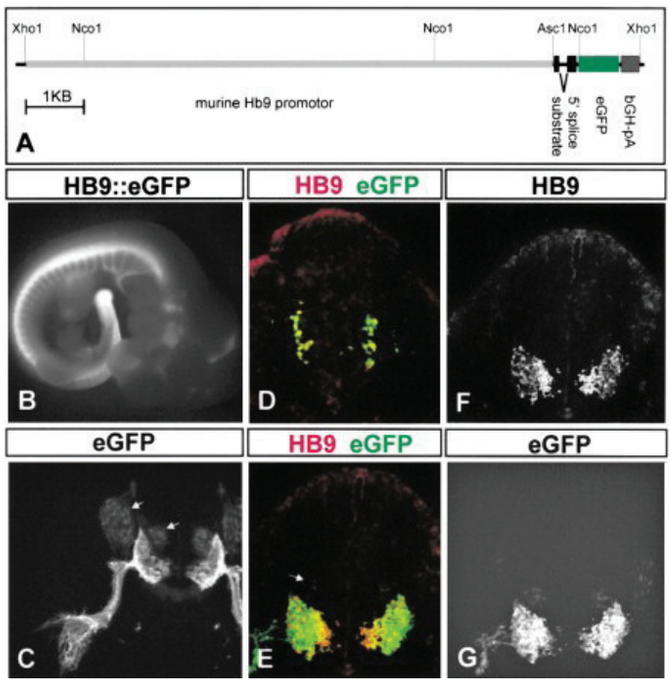


Figure 1: A: Insertion of green fluorescent protein (eGFP) in the Hb9 promoter. **B-G:** eGFP expression in an embryonic mouse,

indicative of Hb9 transcription factor and presence of spinal motor neurons (source Wichterle, et al. 2002).

Hb9 is commonly used to identify motor neuron presence in vivo and in vitro research, through the genetic insertion of Green Fluorescent Protein (GFP) in the Hb9 promoter site (Figure 1). The GFP will be expressed and visually viewed if Hb9 is expressed, a factor indicative of motor neuron presence (Wichterle, et al. 2002).

Wichterle et al. began the transformation of ESC into motor neurons by isolating ESCs from a mouse embryo and growing them in culture to produce embryoid bodies (EBs). These EBs produced very few of the neural progenitor state factors, Sox1, NeuN and TuJ1. In contrast, when retinoic acid (RA) was added to the culture, many neurons were detected, evident by Sox1, NeuN and TuJ1 expression. To determine whether RA exposed EBs can be generated into motor neurons specifically, the expression of pMN domain proteins, Pax6, Nkx6.1 and Olig2, was monitored. Few of these proteins were detected and these cells continued to differentiate mostly into interneurons, instead of motor neurons, demonstrating that RA alone cannot sufficiently induce motor neurons. Therefore, Shh was added to RA-exposed EBs, to generate pMN domain cells. After the addition of Shh, expression of Pax6, Nkx6.1 and Olig2 proteins and Hb9 transcription factor was observed, indicating the development of motor neurons. To further prove the involvement and necessity of Shh, a function blocking anti-Hh antibody, which impairs the function of Shh, was added to the RA-exposed EBs. No Hb9 was observed under these conditions. However, when increased levels of Shh were added, the amount of Hb9 increased significantly, in a "concentration dependent manor (Wichterle, et al. 2002)," indicating a RA and Shh dependent motor neuronal pathway (Figure 2).

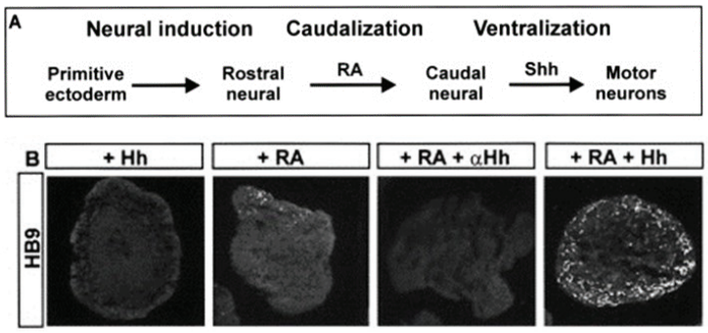


Figure 2: A: generation of motor neurons from the ectoderm of an embryo, requiring the involvement of RA and Shh **B:** expression of Hb9 in ESC exposed to: Shh alone, RA alone, RA with function blocking Hh antibody and RA with Shh. Motor neurons, indicated by white dots, are found in minor amount in RA only culture and in abundance in RA and Shh culture. Absence of motor neurons occur in the absence of RA and in the presence of function blocking Hh antibody. **C:** quantity of GFP/Hb9 cells grown in RA and different quantities of Shh. -1000 refers to cells grown without RA. (Source: Wichterle, et al. 2002).

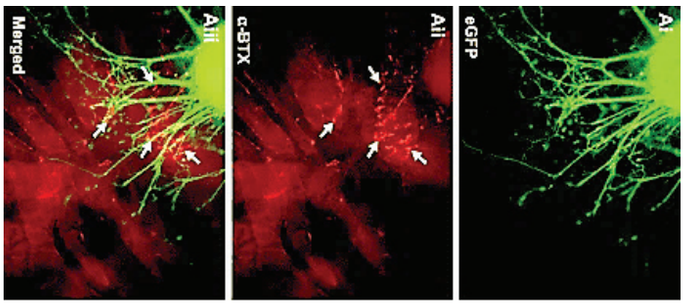
Survival and differentiation of these induced motor neurons was tested in the spinal cord of a mouse in vivo. ESC-derived motor neurons were grafted onto a mouse spinal cord. After 3 days, GFP was detected in the ventral regions of the spinal cord, indicating the ability of these induced motor neurons to survive and differentiate in a living model (Wichterle, et al. 2002). The positioning, trajectory and extension of ESC-derived motor neurons in vivo was then compared with normal motor neuron behavior in embryos. Normal extension along the ventral roots of the spinal cord was detected by use of GFP. Additionally, the motor neurons extended to appropriate muscles, depending on where it was grafted, cervical, thoracic and lumbar segments of the cord (Wichterle, et al. 2002). In another study, two types of ESC-derived MNs, median columnar motor neurons (MMC), which are normally found medially in the spinal cord and innervate axial muscles, and lateral columnar motor neurons (LMC), which are positioned laterally and innervate limb muscles, were mixed together and transplanted in the spinal cord of a mouse. MMC and LMC were found to aggregate in their appropriate medial and lateral regions, next to other motor neurons of their type. In addition to settling in their appropriate regions, the MNs projected along the appropriate pathways, the LMC extending to the limb musculature and the MMC extending to axial musculature (Figure 3) (Peljto, et al. 2010). This implies that ESC-derived MNs can populate all levels of the spinal cord and project along the proper trajectories to specified muscles in vivo.

These studies demonstrate the possibility of generating motor neurons from ESCs and their ability to posi-

tion and project correctly in a host spinal cord. The question still remains if these motor neurons can function as normal motor neurons. In order to use stem cell therapy to treat neurodegenerative disorders, ESC-derived MNs must be able to establish connections, release neurotransmitters, respond to neurotransmitters, and generate action potentials.

MNs containing GFP were prepared through ESC exposure to RA and Shh. Several tests were then conducted to assess MN properties. To determine whether the MNs can connect to muscles, the ESC-derived MNs were placed with myotubes, muscle progenitor cells found in embryo. After 1 day GFP-labeled MNs extended across the myotubes. Additionally, greater than 95% of the GFP labeled MNs that migrated onto the myotubes, expressed ChAT, an enzyme involved in the formation of the neurotransmitter, acetylcholine, indicating the possible ability of the MNs to release cholinergic neurotransmitters (Wichterle, et al. 2002). To establish whether the MNs can send functional neurotransmitters and thereby form a functional connection with muscles, the MNs were cultured together with myotubes and analyzed. After one day, acetylcholine (ACh) receptors, which take up ACh to stimulate muscle contraction, were found on myotubes adjacent to the axons of the ESC-derived MNs (figure 4). These ACh receptors were not observed in the absence of these motor neurons, indicating the ability of ESC-derived MNs to form functional connections with muscles (Miles, et al. 2004).

Figure 4: Ai: axons of GFP-ESC-derived MN. Aii: ACh receptors on myotubes (arrows). Aiii: merged picture of axon and ACh receptor. (Source Miles, et al. 2004).



The effect of neurotransmitters on the ESC-derived MNs was then tested to demonstrate whether GABA, glutamatergic and glycine receptors were present on the MNs and whether their corresponding neurotransmitters can generate a post synaptic action potential on the motor neuron. GABA and glycine, are characteristically inhibitory neurotransmitters in the mature state, however in the embryonic and early postnatal stages they act as excitatory neurotransmitters. Glutamate is an excitatory neurotransmitter which normally generates action potentials in motor neurons. Voltage clamp recording was used to read the membrane potential as the experiment was conducted. In all 3 cases action potentials in the MN membranes were generated when bathed in the neurotransmitters. GABA, glycine and glutamate generated an excitatory post synaptic potential typical of embryonic MNs, demonstrating the ability of these ESC-derived MNs to respond appropriately to neurotransmitters (Miles, et al. 2004).

Next, the possibility of generating multiple trains of action potentials was determined. It seems that the action potential development of ESC-derived MNs develop similarly to MNs in embryo. In early embryonic stages only single trains of action potentials can be fired. In later stages, however, repetitive trains of action potentials can be generated. Similar results were found in ESC-derived MNs, using a current-clamp technique to insert pulses of current. During the first 3 days in culture, only single action potentials could be induced. By the third day, however, repetitive trains of action potentials were detected in 52 out of 61 ESC-derived MNs (See Figure 6) (Miles, et al. 2004).

These findings indicate the possibility of generating motor neurons from embryonic stem cells and the ability of these ESC-derived MNs to function as normal motor neurons. Although these findings are remarkable, there is a strong controversy regarding the source of the stem cells, since human embryos have to be sacrificed to apply this therapy to humans. Studies were conducted to determine whether a less controversial method of inducing motor neurons could be employed. The primary alternative method discovered was the induction of pluripotency of a differentiated, non-embryonic stem cell.

Induced Pluripotent Stem Cells:

Pluripotency is an undifferentiated cell state. A cell

that maintains pluripotency can differentiate into any cell type with the aid of specified factors and signals. By means of specialized transcription factors, differentiated stem cells destined to a certain cell fate can be reprogrammed into a pluripotent stem cell state. These induced pluripotent stem cells (iPSCs) can then differentiate into any cell, including motor neurons, through a similar process to the induction of ESC-derived MNs. Fibroblasts, differentiated stem cells destined to produce connective tissue components, such as collagen and other materials, are most commonly used. Fibroblasts can be isolated from the adult human body as well as embryos, enabling the generation of patient-specific cells from a noncontroversial source. With this research, a patient's own cells can be generated into a pluripotent state and eventually into motor neurons that can be transplanted more safely and efficiently in to a host since they will not be rejected by the patient's immune system (Lee, et al. 2013). The patient will not have to take Immunosuppressant drugs, because the body will recognize the cells as its own, reducing the risk of infections after transplantation.

To reprogram a cell into a pluripotent state, genes found in the regulation and maintenance of pluripotency in embryos were added to a culture of mouse embryonic fibroblast cells. A combination of 24 possible genes were found to induce colonies similar in morphology to ESCs (Figure 6A). To narrow down the candidates, each gene was individually removed to determine whether or not each gene had a positive effect on the induction of pluripotency. The withdrawal of four genes, Oct3/4, klf4, Sox2 and c-Myc, resulted in the absence of any colony formation. The addition of these four genes alone to fibroblast culture generated colonies more similar in morphology to ESCs than the combination of 24. The absence of any one of these genes produced colonies of different morphology than ESCs (Figure 7B), indicating the individual necessity of each of these 4 genes in the renovation of fibroblasts into an ESC-like pluripotent state (Takahashi, Yamanka, 2006).AB

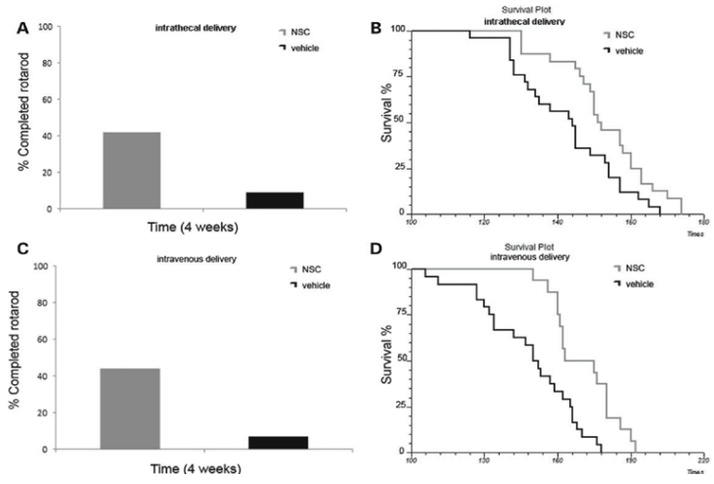
To determine the pluripotency of these iPSCs reprogrammed from fibroblasts by 4 factors, they were injected into mice and monitored. Teratomas consisting all three germ layers, which further differentiated into neural tissues, muscle tissue, cartilage, epithelium and other materials, were observed (Takahashi, Yamanka, 2006). The development of these tumors denotes the ability to create pluripotent stem cells, from differentiated cells, that are identical to ESCs in morphology and pluripotency. These studies were similarly applied to adult human fibroblasts which were successfully induced into a pluripotent state with comparable efficiency to mouse embryonic fibroblasts (Takahashi, et al. 2007).

To examine the possibility of reprogramming ALS-patient specific cells, an ALS mouse model was prepared by inducing a mouse with the mutant SOD1 gene, initiating motor neuron degeneration in the mouse (Gurney, et al. 1994; Yao, et al. 2013). Fibroblast cells were then isolated from the tail of the SOD1-mutated mouse and introduced to the four transcription factors, Oct3/4, klf4, Sox2 and c-Myc. Expression of ESC markers, such as specialized transcription factors, were observed in the iPSCs, indicating a successful reprogramming of fibroblasts into an undifferentiated state. To confirm whether the iPSCs could be generated into motor neurons with a similar efficiency to ESC-derived motor neurons, RA and Shh were added to the iPSCs. After the addition of RA, ~95% of the iPSC expressed the neural marker Tuj1. Shh was then added to induce a motor neuron fate. ~85% of these MNs expressed ChAT, the enzymatic precursor for ACh neurotransmitter and ~24% expressed Hb9. Whole-cell patch clamp technique was used to determine the electrical potential of these induced MNs. Multiple trains of action potentials were generated suggesting the ability of these induced MN to function normally (Yao, et al. 2013).

To assess the therapeutic potential of using iPSC to treat ALS, neural stem cells (NSCs), generated from iPSCs from human somatic fibroblasts, were delivered to SOD1-mutated mice. The NSCs were treated with RA and Shh to induce motor neurons and injected into the cerebral spinal fluid. They were injected intrathecally or intravenously, rather than direct intraspinal transplantation, to minimize the invasiveness of the therapy and maximize safety. GFP was inserted in the NSCs to trace their trajectory. Analysis of the spine and GFP suggested that the NSCs integrated in appropriate regions of the spinal cord, especially in great quantities in regions of active neuron degeneration. In both cases of delivery, survival of mice were significantly

extended. Intrathecally administered NSCs extended survival by ~10 days in all 24 mice in comparison to vehicle treated control SOD1-mutated mice and all 25 intravenously treated mice survived ~23 days longer than vehicle treated control mice (Figure 7B and D). Although the survival extension seems very limited, it is statistically significant and importantly, the extension remained steady in all the mice. Additionally, in a rotarod test, which evaluates neuromuscular function, NSC treated mice had a much higher rotarod functional outcome, indicating improved neuromuscular function (Figure 7A and C) (Nizzardo, et al.)

Figure 5: A: comparison of Rotarod function in control mice and after intrathecal delivery. **B:** survival comparison between control and NSC intrathecally treated mice. **C:** comparison of Rotarod function in control mice and after intravenous delivery. **D:** survival comparison between control and NSC intravenously treated mice. (Source: Nizzardo, et al. 2013) Further studies were conducted to determine whether the induced motor neurons transplanted can prevent further MN degeneration. The number of motor neurons was counted after 140 days in both control and stem cell treated mice. Mice treated with stem cells contained 40% more motor neurons than control mice, a very significant difference. Additionally, axonal density was preserved by 50% in stem cell treated mice while significant reduction was observed in control mice (Nizzardo, et al. 2013). These differences indicate the ability of induced MNs generated from iPSCs to protect against further motor neuron degeneration, thereby indicating the possibility of stem cell therapy to halt the disease progression of ALS.



Clinical Hurdle for iPSCs:

While inducing pluripotency offers a noncontroversial and comparable alternative to ESCs, there is one chief clinical hurdle crucial to overcome. The process of inducing pluripotecy in differentiated stem cells requires the use of oncogenes. Genes such as Klf4, c-Myc and others, which are central to inducing pluripotency, are fundamentally interconnected with the networks of benign and malignant tumor formation. The oncogenic network includes proliferation, differentiation and other properties that are major factors in pluripotency (Lee, et al. 2013). In fact, the way researchers detect pluripotency is by injecting these iPSCs into mice where they form teratomas (Lee, et al. 2013; Takashi, Yamanka, 2006; Takashi, et al. 2007; Yao, et al. 2013). The induction of pluripotency can cause malignant tumors by inducing genetic aberrations associated with tumorigenesis. Deletions in tumor-suppressor genes and activation of oncogenes by integration of transcription factors, such as Myc, are some examples of how the induction of pluripotency causes potentially malignant neoplasms (Figure 8).

Besides for Myc, transcription factors such as Oct4, Klf4 and other core pluripotency factors are associated with tumorgenicity. These factors contain the possibility of maintaining their oncogenic tendency and causing malignant neoplasms when transplanted in humans (Lee, et al. 2013). To ensure patient protection, the clinical expression of these oncogenes will have to be researched further. Other options have to be explored to guarantee the safest and most efficient process of introducing stem cells into a human patient.

Direct Reprogramming of Stem Cells into Motor neurons:

In the face of obstacles such as ethical controversy and tu morigenic potential of ESCs and iPSCs respectively, researchers have searched for other methods to apply stem cell therapy to treat ALS. Direct reprogramming of somatic stem cells into motor neurons, skipping the pluripotent state, seems to be an ideal alternative and considerable research has been devoted to this plight.

Vierbuchen et al. describes the process of directly reprogramming fibroblasts into neurons. Both mouse embryonic fibroblasts and mouse post natal fibroblasts were used to

ensure that both somatic and embryonic stem cells express similar efficiency. Five factors, prepared from an original pool of 19 were isolated based on their positive role in inducing neural development, morphology and activity in fibroblasts. Patch-clamp recording was used to identify whether action potentials could be generated in these induced neurons (iNs). A significant quantity of iNs, 85.1% , could elicit action potentials when induced with current. Additionally, iNs were responsive to GABA, glutamine and other excitatory neurotransmitters, indicating properly functioning receptor sites. iNs were placed into an existing neural network in vitro to determine whether the cells could integrate into complex neural networks. After 7 days, normal neuronal behavior was observed (Vierbuchen, et al. 2010). This suggests the capability of iNs to integrate into and cooperate with the complex human neural network, a critical factor in human stem cell therapy.

To directly induce a more specified subtype of neurons, such as a motor neurons, transcription factors that induce motor neuron identity in normal embryonic development were applied first to mouse embryonic fibroblasts, and later to human-derived fibroblasts. Eight candidate factors, responsible for various developmental stages of MNs, in addition to 3 of the 5 general neural transcription factors, described previously, were inserted into mouse embryonic fibroblasts. Hb9-GFP was used to indicate motor neuron presence. Seven factors were deemed necessary to generate induced motor neurons (iMNs). Using a patch-clamp technique, the resting membrane potential was found to be -49.5mV, very similar to ESC-derived MNs, which is -50.5mV. Single and multiple trains of action potentials were elicited using a current-clamp technique in 90% of iMNs. The ability of iMNs to respond to typical inhibitory and excitatory neurotransmitters was then tested by exposure to GABA, glycine and glutamate. The iMNs elicited normal functioning inhibitory and excitatory action potentials upon contact with these neurotransmitters, indicating functional response of iMNs to neurotransmitters (Son, et al. 2011).

To determine the capability of iMNs to form functional synapses with muscles, called neuromuscular junctions, and to stimulate muscle contraction by release of ACh, iMNs and myotubes were co-cultured. Remarkably, the iMNs extended along the myotubes and after several days began inducing rhythmic contractions in the myotubes. To determine whether the contractions were caused by ACh, curare was added to the culture. Curare is an antagonist of ACh and will inhibit ACh from binding to the ACh receptor site and stimulating muscle contraction. After the curare was added the contractions stopped, indicating the ability of iMNs to develop and dispatch functional ACh neurotransmitters capable of stimulating muscle contraction. iMNs were then transplanted into the spinal cord of chick embryos to determine whether these cells can migrate to appropriate regions, survive and maintain function in vivo. Using Hb9-GFP, proper migration was observed in the spinal cord of the embryo. Additionally, 80% of iMNs extended their axons out of the spine toward the musculature. The same methods were applied to human fibroblasts with similar results (Son, et al. 2011). These findings indicate the similarity of iMNs to ESC-derived MNs and the capability of MNs to function normally both in vitro and in vivo.

These studies determined the ability of differentiated cells to be directly converted into specific subclasses of neurons, including motor neurons, skipping the induced pluripotent state. The use of oncogenes and tumorigenic factors are avoided, because the fibroblasts are not required to pass through an undifferentiated, ESC-like state.

Results

In this study, 3 different stem cell therapies were presented and discussed, embryonic stem cells, induced pluripotent stem cells and direct reprogramming of differentiated cells into neurons. All three therapies demonstrated the ability to produce functional motor neurons from stem cells in vitro and some in vivo (Miles, et al. 2004; Nizzardo, et al. 2013; Peljto, et al. 2010; Son, et al. 2011; Takahashi, Yamanka, 2006; Takahashi, et al. 2007; Umbach, et al. 2012; Vierbuchen, et al. 2010; Wichterle, et al. 2002; Xu, et al. 2009; Yao, et al. 2013). Embryonic stem cells can successfully generate functional motor neurons using factors and signals found in normal embryonic development (Miles, et al. 2004; Peljto, et al. 2010; Umbach, et al. 2012; Wichtelle, et al. 2002). Because of its ethical controversy alternative methods are being researched. However, ESC-derived motor neurons remains a model for oth-

er research, such as induced motor neurons from pluripotency and induced MNs from differentiated stem cells, because of its success in generating normal embryonic-like motor neurons. The induction of pluripotency from differentiated stem cells demonstrated ESC-like character and could generate into functional neurons (Nizzardo, et al. 2013; Takahashi, Yamanka, 2006; Takahashi, et al. 2007; Yao, et al. 2013). The significance of this method lies in its capability of using adult cells, as well as embryonic cells, to generate motor neurons (Takahashi, et al. 2007). Pluripotency, however, is intricately linked to the oncogenic network (Lee, et al. 2013). Many of the factors used to induce pluripotency are found in the regulation and maintenance of tumor formation (Lee, et al. 2013; Takashi, Yamanka, 2006). These tumorigenic factors can potentially be preserved through the process of generating MNs and cause malignant teratomas when transplanted into humans (Lee, et al. 2013). Direct reprogramming of differentiated stem cells into motor neurons skips the pluripotent state and reduces the risk of tumorigenesis. These directly induced motor neurons can be generated from both embryonic and adult differentiated stem cells, with similar efficiency to ESC-derived MNs (Son, et al. 2011; Vierbuchen, et al. 2010). This method can provide an ideal alternative to the controversial ESC-derived MNs and the tumorigenic iPSCs.

Conclusion

Although the success of inducing motor neurons has been established preclinically, the safety and clinical efficiency of these stem cell therapies in human patients with ALS still have to be determined. Several clinical trials, conducted by “Neuralstem inc.,” “BrainStorm Cell Therapeutics,” and others, are currently underway to evaluate the safety of these methods. If safety can be determined, trials can begin to assess efficiency. While preclinical trials have been successful in generating motor neurons with proper function, clinical success may prove to be more difficult in ALS human patients. Since the etiology remains unknown, the degeneration initiating factors cannot be controlled and the new motor neurons may be subject to the same factors that caused the motor neuron degeneration in the first place. To continue survival, frequent MN transplantations into ALS patients may have to take place each time degeneration reoccurs. While there are many clinical hurdles to overcome, with further research stem cell therapy can become the cure ALS patients have been waiting for.

References:

ALS Association. About ALS. ALS Association. 2010. Available at: http://www.alsa.org/about-als/. Accessed January 8, 2014.

Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hetati A, Kwon YW, Deng HX, Chen W, Zhai P, Sufit RL, Siddique T. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science. 1994;264:1772-1775.

Kiernan MC, Vucic S, Cheah BC, Turner MR, Eisen A, Hardiman O, Burell JR, Zoing MC. Amyotrophic Lateral Sclerosis. Lancet. 2011;377:942-955.

Lee AS, Tang C, Rao MS, Weissman IL, Wu JC. Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies. Nature Medicine. 2013;19:998-1004.

Miles GB, Yohn DC, Wichterle H, Jessell TM, Rafuse VF, Brownstone RM. Functional properties of motoneurons derived from mouse embryonic stem cells. The Journal of Neuroscience. 2004;24:7848-7858.

Nizzardo M, Simone C, Rizzo F, Ruggieri M, Salani S, Riboldi G, Faravelli I, Zanetta C, Bresolin N, Comi GP, Corti S. Minimally invasive transplantation of iPSC-derived ALDHhiSSCloVLA4+ neural stem cells effectively improves th phenotype of an amyotrophic lateral sclerosis model. Human Molecular Genetics. 2013;23:342-354.

Peljto M, Dasen JS, Mazzoni EO, Jessell TM, Wichterle H. Functional diversity of ESC-derived motor neuron subtypes revealed through intraspinal transplantation. Cell Press. 2010;7:355-366.

Son EY, Ichida JK, Wainger BJ, Toma JS, Rafuse VF, Woolf CJ, Eggan K. Conversion of mouse and human firoblasts into functional spinal motor neurons. 2011;9:205-218.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka Tomoda K, Yamanka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell Press. 2007;131:861-872.

Takahashi K, Yamanka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663-676.

Umbach JA, Adams KL, Gundersen CB, Novitch BG. Functional neuromuscular junctions formed by embryonic stem cell-derived motor neurons. PLoS ONE. 2012;7:1-6.

Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. Direct conversion of fibroblasts to functional neurons by defined factors. Nature. 2010;463:1035-1042.

Wichterle H, Lieberam I, Porter JA, Jessell TM. Directed differentiation of embryonic stem cells into motor neurons. Cell Press. 2002;110:385-397.

Wilson SI, Edlund T. Neural induction: toward a unifying mechanism. Nature Neuroscience Supplement. 001;4:1161-1168.

Xu L,Ryugo DK, Pongstaporn T, Johe K, Koliatsos VE. Human neural stem cell grafts in the spinal cord of SOD1 transgenic rats: differentiation and structural integration into the segmental motor circuitry. National Institutions of Health. 2009;4:297-309.

Yan J, Xu L, Welsh AM, Hatfield G, Hazel T, Johe K, Koliatsos VE. Exten-

sive neuronal differentiation of human neural stem cell grafts in adult rat spinal cord. PLoS MEDICINE. 2007;4:318-332.

Yao XL, Ye QH, Liu Q, Wan J, Zhen J, Xiang AP, Li WQ, Wang Y, Su H, Lu XL. Motroneuron Differentiation of induced pluripotent stem cells from SOD1G93A mice. PLoS ONE. 2013;8:1-the help of specific transcription factors and signals. Through the action of Sonic hedgehog (Shh) signaling these neural progenitor cells are transformed to a progenitor motor neuron (pMN) domain, which are destined to the fate of spinal motor neurons. These cells in the pMN domain are characterized by the expression of Pax6, Nkx6.1 and Olig2 proteins which each have a role in the differentiation of various spinal motor neurons (Wichterle, et al. 2002). Additionally, these factors direct the expression of Hb9, a transcription factor found in MNs. Hb9 is commonly used to identify motor neuron presence in vivo and in vitro research, through the genetic insertion of Green Fluorescent Protein (GFP) in the Hb9 promoter site (Figure 1). The GFP will be expressed and visually viewed if Hb9 is expressed, a factor indicative of motor neuron presence (Wichterle, et al. 2002).

Similarities Between Corticosteroids

M. Einhorn

Abstract

Corticosteroids are a class of potent drugs with important physiological effects on the body. Regular use is linked with common and serious side effects. This paper uses studies to analyze the similarities between various corticosteroids. All drugs in this class are molecules that contain the same steroid backbone and are therefore associated with the same cellular receptor. This results in a comparable mechanism and parallel overall effect in the body. Side effects of corticosteroids are analogous as well. Using the knowledge of physiological changes in the body due to corticosteroids will allow healthcare providers to determine the most effective corticosteroid least likely to cause adverse effects, and treat and monitor the patient accordingly.

Introduction

Corticosteroids are a class of commonly prescribed drugs containing a backbone of four fused cycloalkanes. These drugs are prescribed to treat a wide variety of ailments including: autoimmune disorders, arthritis, asthma, dermatitis, cancer treatment, pain management and others. Corticosteroid drug therapy is currently used in the treatment of over 175 diseases. Additionally, these drugs are commonly used by patients, as seen by the fact that Advair diskus, a corticosteroid inhaler, was sixth in retail sales during the year 2013. The drug can be administered and absorbed by the body in a variety of different forms: topically applied creams, inhaled sprays, injected systemically, drops for the eyes or ears, and orally. What are the common physiological effects of the different corticosteroids? The medications in this class share a common molecular backbone and cause analogous effects on a cellular level. Much research has been done trying to determine an exact mechanism of steroidal function. Mechanisms have been proposed, but as of today, they are hypotheses that explain some, but not all, effects. Though corticosteroid drugs are used for seemingly disparate pathologies, there are many similarities in physiological effects of corticosteroids, both medicinal as well as detrimental.

Methods

The similarities between the corticosteroid class of drugs, as well as the differences between specific corticosteroids, were analyzed using original studies and critiques obtained by typing corticosteroid along with other associated keywords, such as inflammation, into databases, such as Ebsco, Medline, ProQuest, and other similar databases.

Similarity in Structure

Steroids are molecules with a backbone consisting of three bonded cyclohexane rings fused with a cyclopentane. The backbone can vary in oxidation state as well as in functional groups, producing numerous different steroid molecules. The body produces cholesterol and steroid hormones as part of normal function, most importantly: aldosterone, and the sex hormones. Synthetic steroids can be introduced into the body.

Corticosteroids are a class of drugs similar to the hormone cortisol, and they contain this steroid backbone. Different corticosteroids vary somewhat in shape due to the differing functional groups and oxidation state. Though all steroid molecules are quite similar in structure, slight changes between each molecule's backbone results in differing affinity between the molecule and the corresponding glucocorticoid receptor . Structural changes also affect the bioavailability of a corticosteroid. Inhaled corticosteroids with a hydroxyl group on carbon 21 have been shown both in vitro and in vivo to undergo esterification inside lung cells and therefore take a longer period of time to eliminate from the body (Kelly, 2009).

Mechanism of Action

The physiological effects of corticosteroids are well

M. Einhorn is pursuing a B.S. in Biology.

documented, and medical practitioners administer these drugs because the effects are beneficial to the patients. Corticosteroids are sometimes referred to as a "miracle drug" because of the diverse pathologies that are alleviated or managed by these drugs. The precise mechanism of action, nevertheless, is as of yet unknown. Much research has been done to discover this mechanism, and varying hypotheses have been suggested. There is a strong possibility that corticosteroids have numerous effects on a cellular level, resulting in what seems to be several valid mechanisms.

Corticosteroids function by diffusing into a cell and binding to glucocorticoid receptors in the cytoplasm (Barnes, 2005). The receptor corticosteroid complex stimulates changes in the cell by influencing transcription. Research testing cardiovascular effects of the corticosteroid dexamethasone, found that treatment of cells with dexamethasone and the glucocorticoid antagonist mifepristone (RU 486) blocked the known effects of the corticosteroid (Hafezi-Moghdam et al., 2002). When cells are treated with mifepristone, the glucocorticoid receptor is inactivated. When the cell with the inactivated receptor is treated with corticosteroid, no corticosteroid effect takes place. This demonstrated that a corticosteroid must bind to the receptor for an effect to occur in the cell.

This theory was replicated in a study showing that dexamethasone inhibits vasculogenesis in a tumor through suppressing vascular endothelial growth factor A. Treatment of steroid along with mifepristone prevented this suppressive effect (Greenberger et al., 2010). A limit of the conclusion, however, is that both studies were done using the same corticosteroid dexamethasone, and therefore, does not prove that other corticosteroids need to bind to the glucocorticoid receptor to have an effect.

Further proof of this theory lies in the clinically seen observation that different topical corticosteroids have different potencies. Corticosteroids are therefore separated into classes. In the United States, corticosteroids are divided in classes from one, the extremely potent, to seven, the least potent. In Europe, classes are numbered differently with class one, the mild corticosteroids, to class four, the strongest of the corticosteroids. Percentage of active ingredient in corticosteroid creams, lotions, or ointments fluctuates depending on the strength of the corticosteroid.

In a double blind study testing the bioavailability of different corticosteroids, desoximetasone 0.25%, clobetasol-17-propionate .05%, hydrocortisone 1.0%, and betamethasone dipropionate .064% were applied on the volar forearm of 30 healthy volunteers. After six hours, skin color changes were noted. The degree of skin blanching is associated with the efficacy of the corticosteroid. Though the corticosteroid concentration in each cream was different, the vasoconstrictive effect of the corticosteroid, as seen by blanching of the skin was quite similar between three of the four corticosteroids tested. Hydrocortisone caused milder skin blanching and in fewer individuals, but is known to be a weak corticosteroid so the results were not atypical (Borelli et al., 2008). The clinical observation that three of the four creams caused similar blanching despite differing concentrations, clearly demonstrated that corticosteroids differ in potency.

Research has shown that each corticosteroid has a different binding affinity to the glucocorticoid receptor because of structural differences (Kelly, 2009). Clinical trials show that the change in binding affinity from one corticosteroid to another is associated with the potency of the corticosteroid (Kelly, 2009). This correlation that greater the affinity of the corticosteroid to the receptor the more powerful its effects, bolsters the theory that corticosteroids need to bind to the glucocorticoid receptor to have physiological effects. The corticosteroid receptor complex causes numerous changes in the cell, both in the short term as well as long term.

After binding to the corticosteroid, the activated glucocorticoid receptor complex is transported from the cytoplasm into the nucleus and binds to the promoter of specific genes containing the glucocorticoid response elements. This induces transcription of glucocorticoid response elements mRNA (Barnes, 2005). In endothelial cells, dexamethasone induced promoter activity for the glucocorticoid response elements and caused transcription of these genes beginning four hours after treatment of cells. Actinomycin D, which inhibits transcription, and 5, 6-dichlorobenzimidazole riboside, which inhibits RNA polymerase, given in conjunction with the corticosteroid

blocked the promoter activity (Hafezi-Moghdam et al., 2002). These results indicate the influence of corticosteroids on transcription, which will subsequently affect proteins produced by the cell.

Some of the genes contained within the glucocorticoid response elements code for proteins with anti-inflammatory effects, such as interleukin 10. Leucine zipper protein and I kappa beta alpha (IkBα) are proteins synthesized in a cell exposed to corticosteroid. They suppress an important proinflammatory transcription factor called nuclear factor kappa B (Barnes, 2006). Some genes are indirectly inhibited by corticosteroids through the suppression of nuclear factor kappa B (Maneechotesuwan et al., 2009). There are also genes directly inhibited by corticosteroids, and these genes are considered to be negative glucocorticoid response elements. Examples of these directly suppressed genes include genes that regulate osteocalcin and keratin (Barnes, 2006). The suppression of these proteins results in common side effects of corticosteroids, such as epidermal, and to a lesser extent, dermal thinning and increased risk of fractures.

Corticosteroids inhibit proteins that have deleterious effects on specific pathologies. As a result corticosteroid therapies are beneficial for these diseases. A study seeking to explain the function of corticosteroid therapy in the treatment of infantile hemangioma found that the medicinal property was due to a negative glucocorticoid response elements and a decrease in amount of a corresponding protein. Corticosteroids suppressed the expression of vascular endothelial growth factor A mRNA and protein in hemangioma stem cells in a dose dependent manner, suggesting that corticosteroids may slow the growth of cancer cells. . Untreated infantile hemangioma cells express larger quantities of vascular growth factor than normal endothelial cells, but use of corticosteroids inhibited growth factor mRNA and protein. This effect was replicated using different corticosteroids including dexamethasone, prednisone, prednisolone, methylprednisolone, and hydrocortisone (Greenberger et al., 2010) leading to a conclusion that this effect is common to all corticosteroids. Other proangiogenic factors were suppressed in the hemangioma stem cells, in addition to vascular endothelial growth factor A. These factors included matrix metalloproteinase 1, interleukin-6, and monocyte chemoattractant protein 1. The latter two molecules are both involved with the immune system (Greenberger et al., 2010). Inhibition of metalloproteinase is also seen in corticosteroid treatment of arthritic diseases (Fubini et al., 2001). Steroidal medications induce changes in gene expression. The expressed genes, as well as the repressed genes, have physiological effects on the cell, which are attributed to the corticosteroid.

Numerous studies demonstrate that corticosteroids affect transcription, but this does not explain all of the physiological effects of corticosteroids. Corticosteroids inhibit cytokine production in T helper 2 cells, specifically interleukin 4, 5, and 13. The genes coding these interleukins are not fully regulated by nuclear factor kappa B and are not known to be part of the glucocorticoid response element (Maneechotesuwan et al., 2009). Additionally, effects of dexamethasone were seen in cells after only ten minutes , quite a short time for the transcription of glucocorticoid response element genes and translation of corresponding proteins. Furthermore, although the inhibitor of transcription actinomycin D blocked the promoter of the glucocorticoid response elements, overall the molecule did not inhibit the physiological changes of a cell exposed to dexamethasone (Hafezi-Moghdam et al., 2002). Based on these observations, the theory that corticosteroids binding to a receptor cause a cell to express certain genes and repress others is insufficient.

T cells exposed to anti CD3 antibodies and anti CD 28 antibodies cause GATA-3, a transcription factor, to be imported into the nucleus and stimulate the expression of interleukin 4 and 5. T lymphocytes that were exposed to the corticosteroid, known as fluticasone propionate, were found to have inhibited GATA-3, which affected interleukin gene expression. Interleukin 4 expression, which is regulated by GATA-3, was inhibited, and GATA-3 did not bind to the promoter of interleukin 5. Research on this phenomena found that GATA-3 uses importin alpha for protein to enter the nuclear membrane. Activated glucocorticoid receptors use importin alpha for nuclear translocation as well, leading to competition between GATA-3 and the glucocorticoid receptor for importin alpha. This decreased quantity of importin alpha available to GATA-3 results in

less nuclear translocation, less transcription, and less translation of interleukins 4,5, and 13 (Maneechotesuwan et al., 2009).

Corticosteroids were found to have non-transcriptional effects as well. Treating endothelial cells with dexamethasone stimulated endothelial nitric oxide synthase, an enzyme that has physiological effects on the cells. Activating the enzyme begins with the corticosteroid binding to the glucocorticoid receptor. The complex stimulates protein kinase B (also known as Akt) either directly or indirectly through the phosphoinositide 3-kinase/Akt pathway. Protein kinase B phosphorylates endothelial nitric oxide synthase thereby activating this enzyme (Hafezi-Moghdam et al., 2002). Researchers carried out this experiment with only one corticosteroid, dexamethasone, and the cells were evaluated for the function of only one enzyme. This raises an important question whether a corresponding mechanism applies to other corticosteroids and the phosphorylation and activation of other enzymes.

General Effects of Corticosteroid Drugs

Based on proteins produced or inhibited, as well as the enzymes activated in a cell exposed to corticosteroids, these drugs have predictable physiological effects. One of the most important effects of corticosteroid drugs is the suppression of inflammation. This effect is so significant that other drugs that also have anti-inflammatory properties are separated into a class titled nonsteroidal anti-inflammatory drugs (NSAIDs). Healthcare practitioners therefore often prescribe corticosteroids in the treatment of pathologies involving an inflammatory reaction. Inflammation is associated with the immune system, and corticosteroids influence cytokine production, increasing some cytokines while decreasing others, resulting in an immunosuppressant effect. Repressing the immune system is beneficial for the treatment of pathologies caused by an overactive immune system, but detrimental to a patient on long-term corticosteroid therapy. Immune system suppression increases patient susceptibility to infections, which can become quite serious. This effect is especially deleterious to patients with compromised immune system due to other medical issues. Corticosteroids affect mRNA transcription and proteins produced in the cell. Researchers are studying the effects of proteins produced or repressed by cells exposed to corticosteroids and the physiological effects these proteins have on the cell.

Therapeutic Usage of Corticosteroids

The properties listed above are what cause corticosteroids to have a therapeutic influence on a wide variety of diseases. One of the most common uses of corticosteroids is in the treatment and management of asthma (Eurich et. al, 2013) and other diseases characterized by an obstructed airway (Sibila et al, 2013). Corticosteroids are used to treat inflammation in other areas of the body as well. Therefore, corticosteroid drug therapy is commonly used to treat inflammatory skin disease,s such as eczema and psoriasis. Injections of corticosteroids are used in the controlling of inflammation and the suppression of matrix metalloproteinase activity in arthritic diseases (Fubini et al.,2001). Corticosteroids are beneficial for autoimmune disorders because of the immunosuppressive effect. Some pathologies, such as allergies, are aided by both the anti-inflammatory and immunosuppressive effects of corticosteroids.

The proteins produced in cells exposed to corticosteroids are beneficial to some diseases. Cardiovascular disease can be treated using corticosteroids, though a high dose is needed to achieve results (Hafezi-Moghdam et al., 2002). Dexamethasone increased the activity of the enzyme endothelial nitric oxide synthase (eNOS) and the production of nitric oxide. This radical protects muscle microvessels in mice after ischemia. Under normal conditions, leukocytes are present in the blood and do not adhere to venule walls. Following ischemia and reperfusion, leukocytes' velocity in the bloodstream decreases and they begin to stick to the venule walls. Mice given dexamethasone, directly following the ischemia and reperfusion, did not have the decrease in leukocyte velocity and the increase in adhesion normally seen after ischemia and reperfusion (Hafezi-Moghdam et al., 2002). This effect was studied in the cremaster muscle, but corticosteroids were found to have beneficial effects on myocardium as well. Dexamethasone treated mice were found to have myocardial infarctions affecting a smaller percentage of their region at risk for infarction, while mice in the control group

had a larger percentage of their region at risk affected by a myocardial infarction. Other studies have demonstrated that corticosteroids given to cardiovascular patients improved short-term survival following a myocardial infarction. In the experiments using mice, only very high doses caused physiological effects, and an established clinical dose offered no cardiovascular protection (Hafezi-Moghdam et al., 2002).

Corticosteroids are effective in reducing pain, as evidenced by a study comparing the effectiveness of intravenous hydrocortisone plus conventional treatment versus a placebo and conventional methods at reducing a severe postdural puncture headache. The results clearly showed the effectiveness of corticosteroids at reducing intense pain. Dural puncture is thought to cause a headache by causing cerebral spinal fluid leakage and a lessening of the fluid cushioning around the brain. The mechanism for the resolution of the headache is unknown, but possibilities include the anti-inflammatory properties of corticosteroids at the puncture site, the suppression of interleukins and prostaglandins, and causing the reabsorption of cerebrospinal fluid (Alam et al., 2012). Similarly, corticosteroids, in addition to antibiotics, relieved the pain and symptoms of patients suffering strong discomfort from pharyngitis sooner than than antibiotics and a placebo did (Bergeson et. al., 2013).

Topical corticosteroids have the benefit of maintaining a localized concentration of corticosteroids. Patients with bullous pemphigoid, a potentially fatal autoimmune disease of the skin, were divided into one group receiving treatment of 40 grams of the topical corticosteroid 0.5% clobetasol propionate twice daily, and the other group was treated with one milligram per kilogram of oral prednisone. The topical corticosteroid was found to be more effective at controlling the disease and caused a lower incidence of side effects than the oral corticosteroid (Joly et al., 2002). Similarly, topical steroids were found to be effective at reducing inflammation and ear discharge in otitis media. Clinicians commonly prescribe ear drops, containing a combination of an antibiotic, such as Augmentin®, and a corticosteroid, to treat otitis media with discharge (Florea et al., 2006).

Corticosteroids are used in oncology treatment of infantile hemangioma. They suppress vascular endothelial growth factor A, thereby inhibiting tumor vasculogenesis and limiting tumor growth (Greenberger et al., 2010). Additional research is needed to determine whether this effect extends to other forms of cancer and can be used accordingly, as a part of treatment. Furthermore, the safety of corticosteroid therapy in cancer patients must be evaluated as well.

Side Effects Common to Corticosteroids

The change in proteins produced by a cell exposed to corticosteroids has numerous effects on the cell, both positive and negative. Side effects of a corticosteroid depend on the binding affinity, which is the potency of the given corticosteroid; the length of time for which it is taken; and the method of introduction in the body. Side effects of topically applied corticosteroids generally affect the location of application.

Skin atrophy is a common effect of corticosteroids (Cobman and Wezel, 2006). In a double blind study, twenty healthy volunteers applied four different topical corticosteroids with differing potencies to their volar arm for four weeks. Researchers quantified skin atrophy, using optical coherence tomography to measure epidermal thickness, and using high frequency ultrasound to measure dermal thickness. Additionally, they used a profilometer, an instrument that measures the profile of a given surface, to quantify epidermal roughness. All of the volunteers experienced epidermal thinning, demonstrating the frequency of this side effect. The epidermis thickened considerably after treatment was completed, but three weeks after finishing treatment, the epidermis was still measurably thinner than it had been before treatment. The subjects were followed for only three weeks following steroid use, so the length of time for which this effect persisted and the time needed for the epidermis to return to its original thickness was not determined in the study. The dermis of volunteers thinned as well. The dermal thinning, however, was less than that of the epidermal thinning. Additionally, three weeks post-treatment the dermis had almost returned to its original thickness. Using the profilometer, areas of corticosteroid use had a decreased roughness. The effect of the corticosteroid on the skin corresponded with its known potency; the more potent corticosteroids caused more significant

thinning. Hydrocortisone, a mild corticosteroid, did not cause significant atrophy.

It is evident from this study that in many cases, the skin, particularly the epidermis, is affected by corticosteroid use (Cobman and Wezel, 2006). This effect is likely because corticosteroids directly inhibit genes coding the protein keratin; these genes are part of the negative GRE. Healthcare providers should treat a patient using the least potent corticosteroid found to be effective and should monitor skin changes in the patient (Cobman and Wezel, 2006). Similar results were obtained in a study testing the effects of corticosteroids on different skin phototypes. An additional side effect of corticosteroids was seen in the study: changed skin pigmentation (Shlivko et al., 2013).

The method used to introduce corticosteroids into the body influences the side effects experienced by the patient. The effects of topical corticosteroids are concentrated and limited to the location of application. Therefore, topical corticosteroids may be more effective for treatment of the skin and are known to have a lower toxicity as compared to oral corticosteroids (Joly et al., 2002).

Based on numerous studies, adverse effects of oral corticosteroids are not detected at low and infrequent doses. Patients treated with oral corticosteroids were evaluated for risk of bone fractures. Those on medium to high doses of prednisone or prednisolone were found to have increased risk of fracture. Significantly, the fracture risk remained elevated for up to a year after the last dose, indicating that effects of corticosteroids linger even after discontinuing drug therapy . Budesonide has a low systemic availability, as it is designed for release in the intestine. Due to the low systemic concentration, budesonide was not associated with increased risk of fracture. Methylprednisolone is generally not used regularly, and as a result, did not cause an increased risk of fractures. Likewise, low doses of corticosteroids and doses taken intermittently were not associated with increased risk (Vestergaard et al., 2008). Similarly, short-term use of corticosteroids in treating the intense pain of a postdural headache did not cause short-term negative effects (Alam et al., 2012). Additionally, in the study using corticosteroids to treat severe sore throat, the corticosteroids did not exacerbate illness. Likewise, the use of corticosteroids in croup and mononucleosis did not worsen the infection (Bergeson et al., 2013). These studies did not follow up with the patients to see if any long-term effect or non-immediate reaction to the corticosteroid occurred. Additionally, it is possible that corticosteroids cause subtle effects that are not detected by laboratory means currently available.

A low to medium dose of inhaled corticosteroid is not likely to cause adverse effects (Kelly, 2009). As an immunosuppressant, regular corticosteroid use may negatively impact the immune system's ability to fight infection. Eighty nine patients, with a diagnosis of community-acquired pneumonia and had previously taken inhaled corticosteroids, were compared with a control group of 575 patients, with the same diagnosis who had not taken any inhaled corticosteroids. Inhaled corticosteroids are used in the control of disorders involving lung inflammation, such as asthma and COPD. The corticosteroid inhaler is generally used daily or on a regular basis. The patients, who had previously used inhaled corticosteroids, were more likely to have an illness caused by an antibacterial resistant microbe and presented a more critical case of pneumonia upon admission. Clinicians measure pneumonia by using the Pneumonia Severity Index and Curb-65 scores, which measures the likelihood of the pneumonia patient to die from their disease within 30 days (Sibila et al., 2013). Similarly, a study of 6874 patients, who had community-acquired pneumonia, found that inhaled corticosteroid use in high-risk patients increased the risk of a repeat incident of pneumonia by ninety percent (Eurich et al., 2013). Healthcare providers should relay this risk to asthmatic patients and instruct the patient, or the patient's caregiver, to seek medical care as soon as they feel symptoms of pneumonia. Earlier diagnosis will lead to treatment and a better prognosis.

Treatment of arthritis using corticosteroids has been proven effective. There are negative effects, however, and a healthcare provider should perform a benefit versus risk assessment and treat the patient accordingly. Corticosteroid injections into joints negatively affected chondrocytes, in addition to the cartilage damaged by the arthritis. A single dose of methylprednisolone succinate suppressed the expression of type 2 procollagen mRNA and decreased the relative percentage of the fibronectin form

unique to cartilage. Researchers found that injecting the recommended dose of methylprednisolone acetate into synovial fluid resulted in a high corticosteroid concentration, that was maintained for a longer period of time, because the half-life of corticosteroid in synovial fluid is found to be 10.3 hours (Fubini et al., 2001).

Conclusion

Corticosteroids are a class of drugs with similarities in structure, and they have immunosuppressant and anti-inflammatory properties with comparable mechanisms. Gene expression is affected by corticosteroids, with genes coding the glucocorticoid response elements expressed more than normal, while other genes coding interleukins are inhibited. Various corticosteroids are used in a wide variety of disparate pathologies, because of their immunosuppressant and anti-inflammatory properties. These properties are responsible for the side effects of corticosteroids, including a decreased immune function and changes in cells exposed to high concentrations of corticosteroids. Differences in corticosteroid structure influence bioavailability and affinity to glucocorticoid receptor; both of which are directly related to potency. Additionally, the method of introduction of corticosteroid into the body, as well as the place of introduction, are major determinants of corticosteroid effects.

Overall, corticosteroids are an important method of treatment for illnesses involving an overactive immune system and/or inflammation. However, their use should be limited, as much as possible, due to their side effects. Before prescribing corticosteroids, physicians and other healthcare providers should evaluate the patient's overall immune function and assess the need for corticosteroid therapy. Healthcare providers should experiment with the patient to determine the least potent, lowest dose of corticosteroid found to be effective, particularly in patients requiring long-term use. Patients or the primary caregiver, in the case of children or the elderly, need to be informed of the possible side effects such as increased risk of infection and thinning of the skin. Awareness of corticosteroid risk, along with monitoring by medical professionals, will likely diminish many of the adverse side effects accompanying corticosteroid use.

References

Alam M, Rahman M, Ershad R. Role of very short-term intravenous

hydrocortisone in reducing postdural puncture headache. Journal Of Anaesthesiology, Clinical Pharmacology [serial online]. April 2012;28 (2):190-193. Available from: Medline, Ipswich, MA. Accessed January 8, 2014.

Barnes P. How corticosteroids control inflammation: Quintiles Prize

Lecture 2005. British Journal Of Pharmacology [serial online]. June 2006;148(3):245-254. Available from: Medline, Ipswich, MA. Accessed January 8, 2014.

Bergeson K, Rogers N, Prasad S. PURLs: corticosteroids for a sore

throat?. The Journal of Family Practice [serial online]. July 2013;62 (7):372-374. Available from: Medline, Ipswich, MA. Accessed January 8, 2014.

Borelli C, Gassmueller J, Fluhr J W, et al. Activity of different

desoximetasone preparations compared to other topical corticosteroids in the vasoconstriction assay. Skin Pharmacology and Physiology [serial online]. June 2008;21(3):181-187. Available from Google Scholar. Accessed January 12, 2014.

Cobmann M, Welzel J. Evaluation of the atrophogenic potential of different

glucocorticoids using optical coherence tomography, 20-MHz ultrasound and profilometry; a double blind, placebo-controlled trial. British Journal Of Dermatology [serial online]. October 2006;155(4):700-706. Available from: Academic Search Complete, Ipswich, MA. Accessed January 8, 2014.

Eurich DT, Colin L, Thomas JM, Sumit RM. Inhaled Corticosteroids and

risk of recurrent pneumonia: a population-based, nested case-control study. Clinical of Infectious Diseases [serial online]. 2013;57(8):1138-1144. Available from Oxford Journals. Accessed December 2, 2013.

Florea A, Zwart J, Jung T, et al. Effect of topical dexamethasone versus

rimexolone on middle ear inflammation in experimental otitis media with effusion. Acta Oto-Laryngologica [serial online]. September 2006;126(9):910-915. Available from: Academic Search Complete, Ipswich, MA. Accessed January 8, 2014. Accessed January 8, 2014.

Fubini SL, Todhunter RJ, Burton-Wurster N, et al. Corticosteroids alter the

Differentiated phenotype of articular chondrocytes. Journal of Orthopaedic Research [serial online]. July 2001;19(4):688-695. Available from: ProQuest. Accessed December 29, 2013.

Greenberger S, Boscolo E, Adini I, Mulliken J, Bischoff J. Corticosteroid

suppression of VEGF-A in infantile hemangioma derived stem cells. The New England Journal of Medicine [serial online]. March 18, 2010;362 (11):1005-1013. Available from: Medline, Ipswich, MA. Accessed January 8, 2014.

Hafezi-Moghdam A, Simoncini T, Liao J, et al. Acute Cardiovascular

protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. Nature Medicine [serial online]. May 2002;8(5):473. Available from: Academic Search Complete, Ipswich, MA. Accessed January 8, 2014.

Joly P, Roujeau J, Benichou J, et al. A comparison of oral and topical

corticosteroids in patients with bullous pemphigoid. New England Journal of Medicine [serial online]. 2002;346(5):321-327. Available from: ProQuest. Accessed January 8, 2014.

Kelly HW. Comparison of inhaled corticosteroids: an update. The Annals

Of Pharmacotherapy [serial online]. March 2009;43:519-527. Available from: Medline, Ipswich, MA. Accessed December 12, 2013.

Maneechotesuwan K, Yao X, Barnes P, et al. Suppression of GATA-3

nuclear import and phosphorylation: a novel mechanism of corticosteroid action in allergic disease. Plos Medicine [serial online]. May 12, 2009;6(5):e1000076. Available from: Medline, Ipswich, MA. Accessed January 8, 2014.

Sibila O, Laserna E, Mortensen E, Anzueto A, Restrepo M. Effects of

inhaled corticosteroids on pneumonia severity and antimicrobial resistance. Respiratory Care [serial online]. September 2013;58(9):1489-1494. Available from: Medline, Ipswich, MA. Accessed January 8, 2014.

Shlivko IL, Kanmensky VA, Donchenko EV, Agrba P. Morphological

changes in skin of different phototypes under the action of topical corticosteroid therapy and tacrolimus. Skin Research and Technology [serial online]. May 2013;0:1-5. Available from:Penn State Hershey ILL lending. Accessed December 17, 2013.

Vestergaard P, Rejnmark L, Mosekilde L. Fracture risk associated with

different types of oral corticosteroids and effect of termination of corticosteroids on the risk of fractures. Calcified Tissue International [serial online]. March 2008;82(4):249-257. Available from: Google Scholar. Accessed January 12, 2014.

The Grapefruit Juice Effect

Rebbeca Leitner

Abstract

A drug interaction is the effect that a substance can have when taken together with a drug. Grapefruit juice has proven to be a source of interaction with many drugs, causing increased bioavailability, leading to possible toxicity and increased instances of side effects. This paper discusses the mechanisms of the interaction and the components of grapefruit juice responsible for the interaction, as well as two classes of drugs that are affected, calcium channel antagonists and HMG-CoA reductase inhibitors, and possible ways to avoid the interaction. The mechanisms are inhibition of the enzyme cytochrome P-450 3A4 (CYP3A4) and the transport molecule P-glycoprotein. Furancoumarins are responsible for the interaction. In general, drugs that are affected have a low oral bioavailability and are substrates of CYP3A4. The interaction is best avoided by not drinking grapefruit juice, or choosing a drug that doesn't interact. Other possibilities include juice low in furanocoumarins due to harvest factors, and furanocoumarin free juice. In conclusion, one should exercise caution unless it has been proven that the drugs being taken do not interact with grapefruit juice.

Introduction

In 1997, twenty one percent of the households in America chose to buy grapefruit juice to drink as their breakfast beverage. In recent years, the public have realized the importance of including antioxidants in their diets, and the choice of grapefruit juice, which contains those compounds, reflects that awareness. Several chronic diseases, most importantly cardiovascular disease and some cancers, could possibly be prevented by flavenoids and other components of grapefruit juice. Additionally, there was a study that was done that showed that grapefruit and grapefruit juice might promote weight loss (Mertens-Talcott, et. al. 2006). Due to its possible health benefits, it's hard for many people to believe that grapefruit juice can have an adverse effect when taken together with many common medications.

A drug interaction is a name given to the effect that any substance can have when ingested together with a medication. The possible effects fall into two categories: pharmacokinetics and pharmacodynamics. Pharmacokinetics includes changes in absorption, distribution, metabolism, and excretion. Pharmacodynamics is the description of the relationship between the concentration of the drug and its effect (Mertens-Talcott, et. al. 2006). Grapefruit juice causes pharmacokinetic interactions. When taken together with grapefruit juice, many medications display an increased bioavailability (Fuhr, 1998). This leads to undesired effects, such as decreased efficiacy or toxicity. Increased side effects can occur as well (Mertens-Talcott, et. al. 2006). The unpleasantness of toxicity can cause patients to stop taking the drugs, and in extreme cases, toxicity can even cause death (Bailey, Dresser, 2004).

The phenomenon of the grapefruit juice- drug interaction was discovered by chance in 1989. An experiment was being done to determine if there was an interaction between felodipine, a calcium channel antagonist, and ethanol. Grapefruit juice was used to mask the taste of the ethanol. When the results were analyzed, it was discovered that the concentrations of felodipine were significantly higher than those recorded in other experiments, and the subjects had lower blood pressure and higher instances of unwanted side effects. Further testing was performed to try to discover the cause, and it was found that the increased concentration was a result of the grapefruit juice. Since then, numerous drugs have been tested, and many have been found to interact with grapefruit juice (Dahan, Altman, 2004). This research paper will discuss the possible mechanisms behind this phenomenon and the components of grapefruit juice thought to cause the interaction. The effect and clinical relevance of grapefruit juice on two classes of cardiovascular medications, calcium channel antagonists and HMG-CoA reductase inhibitors, will be analyzed, and several ways of avoiding the interac-

Rebecca Leitner will be attending Long Island University in September, pursuing a degree in pharmacy.

tions will be explored as well.

Methods

Research was done using Touro College's search engines such as ProQuest, and MEDLINE, and most frequently, EBSCO. The method of research included evaluating original studies and peer reviewed articles. Keywords such as "grapefruit juice", "drug interaction", and specific names of drugs were used.

Mechanisms of Action

There are several possible mechanisms that have been suggested to explain the interaction. Since the interaction only takes place with drugs that are ingested orally, the mechanism must occur at some point during the digestion process (Mertens-Talcott, et. al. 2006).

Cytochrome P-450 3A4

Cytochrome P450 is a family of enzymes that is responsible for the breakdown and detoxification of, among other substances, steroids, environmental carcinogens, and drugs (Girennavar, et. al. 2007). Cytochrome P-450 3A4 (CYP3A4) specifically is the isoenzyme highly prevalent in the human digestive system, found in the small intestine and the liver. Drugs that are metabolized by CYP3A4 have low oral bioavailability, so only a small amount of the total that is ingested enters circulation. Because of this, larger doses are administered so that enough of the drug will be available to cause the desired effect. If CYP3A4 is inhibited, greater oral bioavailability results, and there is a possibility for undesired effects to occur (Bailey, Dresser, 2004).

Grapefruit juice has been shown to inhibit CYP3A4 in in vitro studies. In one study, the enzyme was tested with 1%, 10%, and 25% concentrations of different species of grapefruit and pummel juices. Significant inhibition was observed, especially at concentrations of 10% and 25%. At those concentrations, inhibition ranged from 96.69% to 99.88% (Girennavar, et. al. 2007). In another study that used human liver microsomes, inhibition was observed as well. (Seden, et. al. 2010)

Grapefruit juice has been proven to be an inhibitor of CYP3A4 in in vivo studies as well. Diminished CYP3A4 enzyme presence, but not mRNA, has been observed after drinking just one 300 mL glass of grapefruit juice. The decreased levels indicate that the inhibition is not competitive in nature. (Bailey, Dresser, 2004). Additionally, an investigation that measured CYP3A4 levels after six days of drinking grapefruit juice recorded greatly decreased amounts of the enzyme but not of the CYP3A4 mRNA (Seden, et. al. 2010). Due to these observations, it can be assumed that grapefruit juice most likely does not inhibit production of the enzyme. Instead, the juice causes destruction of the enzyme through suicide or mechanism-based inhibition. A component of grapefruit juice is thought to be converted to a reactive metabolite that bonds to CYP3A4 and causes inactivation (Bailey, Dresser, 2004).

If this were the case, synthesis of new enzymes would be required for restoration of CYP3A4 activity, which explains the prolonged effect that grapefruit juice can have. Studies performed with felodipine to determine the length of time of the effect grapefruit juice support the theory. It was shown that the maximum effect can still be observed when the juice is ingested 4 hours before the drug is administered, and increased levels of felodipine were still recorded when there was a 24 hour interval between juice and drug intake (Bailey, Dresser, 2004). Recovery times of 72 hours for nisoldipine (Bailey, Dresser, 2004) and 74 hours for midazolam (Mertens-Talcott, et. al. 2006) have been observed as well.

It is interesting to note that the effect of the interaction varies widely among individuals. Those with initial high CYP3A4 activity have been shown to have greater inhibition than those with naturally lower activity of the enzyme. Therefore, ingestion of grapefruit juice together with an affected drug by these individuals will cause a greater increase in drug concentration than would result in subjects with low CYP3A4 activity (Mertens-Talcott, et. al. 2006).

P-glycoprotein

P-glycoprotein is an ATP-dependent drug transporter. It is an efflux pump located in the small intestine, liver, kidneys, and at the blood brain barrier. P-glycoprotein decreases the bioavailability of many drugs by limiting the amount absorbed from the intestines (Bailey,

Dresser, 2004). P-glycoprotein has many substrates, and a majority of them overlap with the substrates of CYP3A4. (Seden, et. al. 2010)

The inhibition of P-glycoprotein by grapefruit juice was first demonstrated by the increased uptake of vinblastine into Caco-2 cells. Since vinblastine is a substrate of both CYP3A4 and P-glycoprotein, no definite conclusion could be drawn regarding the inhibition of P-glycoprotein (Mertens-Talcott, et. al. 2006). A study done using human renal proximal cells proved that grapefruit juice does in fact inhibit P-glycoprotein expression and activity (Romiti, et. al. 2004) Another study investigated P-glycoprotein inhibition in Caco-2 cells as well, using colchicine as the probe. Grapefruit juice caused increased transport of colchicine into the cells. As is the case with vinblastine, colchicine is both a CYP3A4 and a P-glycoprotein substrate, so the cause of the increased uptake can't be credited solely to P-glycoprotein inhibition. The same study investigated colchicine permeability in the small intestine of rats in situ. It was found that grapefruit juice significantly increased the permeability of the drug (Dahan, Amidon, 2008). In an additional investigation, rats were given talinolol, a substrate of P-glycoprotein that does undergo metabolization. Because of that, there is no potential for the inhibition of CYP3A4 to interfere with the results. When given together with grapefruit juice, concentrations of talinolol were increased (Bailey, Dresser, 2004).

Although the in vitro studies demonstrate that there is a possible inhibition of P-glycoprotein by grapefruit juice, there have been difficulties in reproducing the effects in human trials (Seden, et. al. 2010). Digoxin is another drug that is known to be an unmetabolized substrate of P-glycoprotein. The drug did not display a great change in bioavailability when administered with grapefruit juice as compared to water. But since digoxin has a high bioavailability of 70-80%, it is not expected that inhibition of P-glycoprotein would drastically change its concentration (Bailey, Dresser, 2004).

Although research has yet to produce clear proof, the above studies demonstrate that there is an interaction between grapefruit juice and P-glycoprotein. Further studies must be done to determine the extent of the inhibition and how significantly it affects grapefruit juice-drug interactions. According to the current information, it seems to be that the interaction is mainly caused by inhibition of CYP3A4, and that inhibition of P-glycoprotein plays a small role as well.

Components of Grapefruit juice Possibly Responsible

There are hundreds of chemical components present in grapefruit juice. The amounts present in a specific juice depend on a variety of factors, including the genetic background of the plant, and the conditions during the growth, maturity, harvesting and processing of the fruit. Most of the constituents are present in other citrus fruits as well, but the amounts of the specific ones differ among the species. Since juices from numerous sources have displayed interactions with drugs, it seems that the component or components that cause the interaction are always present. Two of the typically present substances, flavonoids and furanocoumarins have been presented as the most probable sources of the grapefruit juice effect (Fuhr, 1998).

Flavonoids

The flavonoid naringin is present in much higher levels in grapefruit juice than in other citrus fruit juices. Naringenin, the active metabolite of naringin, is known to be a strong inhibitor of CYP3A4. Several in vitro studies have shown that both naringin and naringenin are able to inhibit the metabolic process of the drugs tested (Fuhr, 1998). Additionally, it has been demonstrated that naringenin has the ability to inhibit P-glycoprotein in human renal proximal cells (Romiti, et. al. 2004).

Although these trials seem promising, human trials have not gotten conclusive results. Studies that gave subjects naringin in the form of capsules or in solution did not observe any significant increase in concentrations of the drugs that were tested. Furthermore, in a study in which naringin was removed from grapefruit juice, CYP3A4 activity was still inhibited (Fuhr, 1998).

Furanocoumarins

Furanocoumarins are mainly found in the peels of grapefruit, but there are significant amounts present in the juice as well (Fuhr, 1998). The most abundant, bergamottin and its metabolite 6'7'-dihydroxybergamottin (DHB), have

been identified as CYP3A4 inhibitors. Several in vitro studies have proven this.

The individual furanocoumarins have been isolated and tested in several studies. In one study, DHB, bergamottin, paradisin A, bergaptol and geranylcuomarin were tested for the extent of the inhibition that they caused. It was found that paradisin A was the strongest, followed by DHB, then bergamottin, then bergaptol, and finally geranlycoumarin (Girennavar, et. al. 2007). Although DHB is not the strongest, it is the most abundant which is most probably why the inhibitory effect is generally attributed to its presence. In another study, the furanocoumarins were isolated and tested as a whole to determine their overall inhibitory capacity. When all were combined, the inhibition was similar to that observed with whole grapefruit juice. Removing any one of the furanocoumarins from the mixture decreased the inhibition, which presents the possibility that all of furanocoumarins play a role in the grapefruit juice effect (Dahan, Altman, 2004).

Further reinforcement of the role of furanocoumarins can be seen in studies that evaluated the effect of furanocoumarin-free grapefruit juice. In one study, most of the furanocoumarins were removed using Aspergillus niger, a strain of fungus. The altered juice was tested in vitro and found to have a reduced inhibitory effect (Myung, et. al. 2008). In another study, approximately 99% of the furanocoumarins were removed using food grade solvents and absorption resins. In vitro, the processed juice did cause some inhibition, but to a markedly lesser extent than did whole grapefruit juice. The same study performed a human trial, using felodipine as the probe. It was found that when compared to orange juice, the control, furanocoumarin-free grapefruit juice had no great effect on the pharmacokinetics of the drug. This was the first study to show that the grapefruit juice effect can be credited completely to furanocoumarins (Paine, et. al. 2006).

Drugs that Exhibit an Interaction

The drugs that are affected by grapefruit juice follow a general trend. First of all, because the interaction occurs in the gastrointestinal tract, the medication must be ingested orally. Second, the drug in question must be either metabolized by CYP3A4 or transported by P-glycoprotein. Finally, since grapefruit juice increases bioavailability, the drug must have a normally low bioavailability (Bailey, Dresser, 2004).

Dihydropyridine Calcium Channel Antagonists

Calcium channel antagonists are vasodilators that are used to treat hypertension and other cardiovascular disorders. Excessive vasodilation can result in headaches, swelling of the ankles, and facial flushing. These are not serious side effects, but they are unpleasant enough to cause patients to stop taking the medication, thus leaving their conditions untreated. The more serious side effects include severely low blood and pressure myocardial infarction (Bailey, Dresser, 2004).

Felodipine, the drug with which the grapefruit juice interaction was first observed, has been studied extensively. It has an absolute bioavailability of 15% (Bailey, Dresser, 2004). Over the course of these studies it has been determined that depending on the amount and timing of the ingestion of grapefruit juice, the concentrations of felodipine can increase to between double and triple the usual levels. Greater instances of adverse side effects were observed as well (Fuhr, 1998).

Nifedipine, another calcium channel antagonist with low oral bioavailability, has been shown to be affected by grapefruit juice as well. In a single case study on a 50 year old man, 500 ml of grapefruit juice caused nifedipine levels to more than double (Nakagawa, Gotu, 2010). Although this was a single case study, and therefore no definite conclusions can be drawn, the results match those of larger studies. In another study, sixteen subjects drank 250 ml of grapefruit juice. The observed result was much less dramatic than that of other studies, but there was an increase in bioavailability. This can possibly be explained by the smaller amount of grapefruit juice administered. In most other studies, the amount ingested was between 400 and 500 ml (Odou, et. al. 2005). Other drugs in this class that have displayed an interaction are nicardipine, nisodipine, and nitrendipine. The average changes in concentration for the above range from 1.5 to four times the normal amount (Bailey, Dresser, 2004).

The final calcium channel antagonist, amlodipine, has a high oral bioavailability of between 64% and 80%

(Seden, et. al. 2010). Because of this, it is not expected to interact with grapefruit juice, and studies support this hypothesis. In the same single case study discussed previously, grapefruit juice did not affect the plasma concentration of the drug (Nakagawa, Gotu 2010). In a study performed using twenty volunteers, 240 ml of grapefruit juice was ingested together with the amlodipine, and an additional 200 ml was taken each day for 8 days following drug administration. Bioavailability was not altered significantly (Vincent, et. al. 2000).

HMG-CoA Reductase Inhibitors

HMG-CoA reductase inhibitors, commonly known as statins, are used to lower cholesterol. They have the potential to cause serious toxicity. Side effects range from diffuse myalgia, or muscle pain, to rhabdomyolysis, which is severe skeletal muscle degeneration. Acute renal failure is a possibility as well (Bailey, Dresser, 2004).

Simvastatin, lovastatin, and atorvastatin are all metabolized by CYP3A4 and therefore have low bioavailability. Simvastatin and lovastatin are inactive forms that are converted to their active acid forms by esterases. Before the conversion, they are extensively metabolized by CYP3A4, resulting in a bioavailability of approximately 5% (Bailey, Dresser, 2004). In one study, ten volunteers ingested 200 ml of grapefruit juice for two days before and then together with a dose of simvastatin. The average increase in concentration was 3.6 times greater than that observed with water (Lilja, et. al. 2004). In other studies that tested simvastatin and lovastatin, the average increase was 15-fold the normal (Bailey, Dresser, 2004).

Atorvastatin has a bioavailability of 12%. In a study using eight subjects, grapefruit juice was ingested for three days before and concomitantly with atorvastatin. Juice was also administered 4 and 12 hours after drug intake. Concentrations increased between 1.5 and 1.7-fold (Ando, et. al. 2005). In another study that surveyed twenty subjects, grapefruit juice was consumed at regular intervals before, during and after administration of atorvastatin. The average increase that was observed was 83% (Fukazawa, et. al. 2003). Finally, a much larger study was performed using 130 subjects. Patients drank grapefruit daily for ninety days together with their medication, and there was a small but still statistically significant increase in concentrations (Reddy, et. al. 2011).

Pitavastatin, a synthetic statin, has a bioavailability of 60% (Ando, et. al. 2005). It is metabolized not by CYP3A4, but by CYP2C9, and to some extent by CYP2C8 (Seden, et. al. 2010). In the study that surveyed the effects of grapefruit juice on atorvastatin in eight subjects, pitavastatin was tested as well. It was found that the juice only slightly increased concentrations of pitavastatin in the blood (Ando, et. al. 2005).

Like pitavastain, pravastatin is not metabolized by CYP3A4. Pravastatin was surveyed in the second study described for atorvastatin that tested twenty subjects. No significant effects were recorded (Fukazawa, et. al. 2003). Two other statins, fluvastatin and rosuvastatin, have not yet been tested (Seden, et. al. 2010). But fluvastatin is a CYP2C9 substrate, and rosuvastatin appears not to be a substrate of either CYP3A4 or P-glycoprotein, so no interactions are predicted (Bailey, Dresser, 2004).

How to Avoid the Interaction

The simplest way to avoid the interaction would be to abstain from drinking grapefruit juice while undergoing treatment with a drug that is known to, or has been predicted to, interact with grapefruit juice. If the patient has no interest in giving up the juice, the next best solution would be to select one of the drugs that have been proven to have no interaction with the juice. However, if that specific drug is required, and changing to a non-interacting medication is impossible, there are several methods that might limit the possibility of an interaction occurring.

As it has been proven that furanocoumarins are the cause of the grapefruit juice-drug interaction, the ideal way to minimize the possibility of an interaction occurring is to limit exposure to furanocoumarins. Below are several possible ways in which the levels of furanocoumarins can be reduced.

Harvest Factors and Different Species of Grapefruit

It has been proven that the maturity, location, processing, and storage of grapefruits and grapefruit juice affects the levels of furanocoumarins present in the final ingested product. Furanocoumarins are molecules that are

produced in times of stress so there are many factors that affect their levels. It was found that furanocoumarin levels are highest at the beginning of season, and that they decrease throughout the season. In evaluating location, it was discovered that in general, grapefruits grown in Florida and California had higher levels than grapefruits grown in Texas. Hand squeezing versus commercial processing tests revealed that levels were higher in hand squeezed juice. Post-harvest, furanocoumarin levels were lowered when stored at both 24 and 9°C. Finally, levels were evaluated in processed juice stored in three different types of containers: cans, cardboard bottles, and cartons. All displayed a decrease in levels over time, but the cartons contained the highest levels of furanocoumarins (Girennavar, et. al. 2008). Based on these findings, those who would like to drink grapefruit juice and minimize the possibility for an interaction should choose commercially processed juice stored in cans or bottles, and made from organic grapefruits grown in Texas that were harvested late in the season.

Furanocoumarin-Free Grapefruit Juice

Furanocoumarins have been extracted from grapefruit juice for studies, but commercial furanocoumarin-free juice has not been developed. Based on the studies, there are two possible ways to extract them. In one study, the fungi Aspergillus niger was incubated with grapefruit juice. The fungus was able to absorb most of the non-polar furanocoumarins, but not the polar ones, and not the flavonoids. The juice was then tested on CYP3A4, and it was found that inhibition of the enzyme was significantly reduced. This method still needs further development though, because the strain of fungus that was used could produce toxins. For the removal of furanocoumarins from grapefruit juice by fungus to become possible commercially, a method employing food-grade, edible fungi must be developed (Myung, et. al. 2008). In another study, furanocoumarin-free grapefruit juice was created using food-grade solvents and absorption resins. 99% of the furanocoumarins were able to be removed. When tested in a human trial, the juice had no effect on the concentrations of felodipine (Paine, et. al. 2006).

Conclusion

As seen in the above studies, grapefruit juice has the potential to interact with many drugs. Any medication that is either a substrate of CYP3A4 or transported by P-glycoprotein can possibly be affected when taken together with grapefruit juice. The components in the juice that are responsible for the interaction are the furanocoumarins, which bind to and inactivate CYP3A4 and P-glycoprotein. Avoidance of the interaction is best accomplished by not drinking grapefruit juice, or selecting a drug that is known not to interact. If such actions are not possible, juice that was processed so as to minimize furanocoumarins might be a possibility, and there is a chance that furanocoumarin-free grapefruit juice will be developed sometime in the future.

Since there are so many drugs that have the possibility of interaction, it is important to simply be aware of this phenomenon. Patients must know to let their doctors know if they drink grapefruit juice habitually and to ask if any medications that they are on can interact. Unless it has been proved that there is no possibility of a reaction, any drug that might interact based on the method in which it's metabolized should be treated as if it is privy to the grapefruit juice-drug interaction.

References

Ando H, Tsuruoka S, Yanagihara H, Sugimoto K, Miyata M, Yamazoe Y, Takamura T, Kaneko S, Fujimura A. 2005. Effects of grapefruit juice on the

pharmacokinetics of pitavastatin and atorvastatin. Br J Clin Pharmacol. 60(5):494-497.

Bailey DG, Dresser GK. 2004. Interactions between grapefruit juice and

cardiovascular drugs. Am J Cardiovasc Drugs. 4:281-297.

Dahan A, Altman H. 2004. Food-drug interaction: grapefruit juice augments drug

bioavailability-mechanism, extent and relevance. European Journal of Clinical Nutrition. 58:1-9.

Dahan A, Amidon GL. 2008. Grapefruit juice and its constituents augment colchicines intestinal absorption: potential hazardous interaction and the role of P-glycoprotein. 2009. Pharmaceutical Research. 26 (4):883-892.

Fuhr U. 1998. Drug interactions with grapefruit juice. Drug Safety. 18 (4):251-272

Fukazawa I, Uchida N, Uchida E, Yasuhara H. 2003. Effects of grapefruit juice on

the pharmacokinetics of atorvastatin and pravastatin in Japanese. Br J Clin Pharmacol. 57(4):448-455.

Girennavar B, Jayaprakasha GK, Patil, BS. 2007. Potent inhibition of human

cytochrome P450 3A4, 2D6 and 2C9 isoenzymes by grapefruit juice and its furocoumarins. Journal of Food Science. 72(8):C417-C421.

Girennavar B, Jayaprakasha GK, Patil BS. 2008. Influence of pre- and post-

harvest factors and processing on levels of furocoumarins in grapefruits (Citrus paradise Macfed.). 2008. Food Chemistry. 111:387-392.

Lilja JJ, Neuvonen M, Neuvonen PJ. 2004. Effects of regular consumption of

grapefruit juice on the pharmacokinetics of simvastatin. Br J Clin Pharmacol. 58(1):56-60.

Mertens-Talcott SU, Zadesensky I, De Castro WV, Derendorf H, Butterweck V.

2006. Grapefruit-drug interactions: can interactions be avoided?. J Clin Pharmacol. 46:1390-1416.

Myung K, Manthey JA, Narciso JA. 2008. Binding of furanocoumarins in

grapefruit juice to Aspergillus niger hyphae. Appl Mirobiol Biotechnol. 78:401-407.

Nakagawa K, Gotu T. 2010. Effects of ingestions of grapefruit juice or grapefruit

on the hypotensive effect and plasma concentrations of dihydropyridine calcium antagonists (amlodipine and nifedipine): a case study. Clinical and Experimental Hypertension. 32:71-75.

Odou P, Ferrari N, Barthelemy C, et al. 2005. Grapefruit juice-nifedipine

interaction: possible involvement of several mechanisms. Journal of Clinical Pharmacy and Therapeutics. 30:153-158.

Paine MF, Widmer WW, Hart HL, Pusek SN, Beavers KL, Criss AB, Brown SS, Thomas BF, Watkins PB. 2006. A furanocoumarin-free grapefruit

juice establishes furanocoumarins as the mediators of the grapefruit juice-felodipine interaction. Am J Clin Nutr. 83:1097-1105.

Reddy P, Ellington D, Zhu Y, et al. 2011. Serum concentrations and clinical

effects of atorvastatin in patients taking grapefruit juice daily. Br J Clin Pharmacol. 72(3):434-441.

Romiti N, Tramonti G, Donati A, Chieli E. 2004. Effects of grapefruit juice on the

multidrug transporter P-glycoprotein in the human proximal tubular cell line HK-2. Life Sciences. 76:293-302.

Seden K, Dickinson L, Khoo S, Black D. 2010. Grapefruit-drug interactions.

drugs. 70(18):2373-2407.

Vincent J, Harris SI, Foulds G, Dogolo LC, Willavize S, Friedman HL. 2000. Lack

of effect of grapefruit juice on the pharmacokinetics and pharmacodynamics of amlodipine. J Clin Pharmacol. 50:455-463.

Is Fluoridating Water Beneficial to Our Children: A Dental Perspective

Daniel Weidberg

Abstract

The addition of fluoride to our water system may not be as beneficial to children as previously thought. While fluoride is effective at preventing dental caries formation, it would be more beneficial if it was administered only as an oral topical treatment and not introduced into the water. This paper will examine research that explains dental caries formation and the caoriostatic mechanism of fluoride. Additionally, the adverse effects of fluoride by causing fluorosis will be reviewed. By analyzing studies that compare the prevalence of dental caries and fluorosis in both fluoridated areas and non-fluoridated areas, it can be concluded that while dental caries decrease in both areas, a drastic increase in fluorosis is noticed only in those fluoridated areas.

Introduction

The Center for Disease Control claims that fluoridating the water is one of the greatest achievements in the twentieth century in terms of preventing disease (CDC, 1999). In fact, it is estimated that by 2005 60% of the country already had access to fluoridated water (Kauffman, 2005). The supposed benefit is that fluoride help reduce dental caries. While the exact mechanism is not fully understood there is a prevailing theory as to why it does work. Indeed, it has been noticed that since the induction of fluoride to water supplies worldwide there has been a downward trend in cavities (Jones, et al. 2005). However, fluoride use is synonymous with fluorosis, the discoloration of teeth. While this isn't a significant health risk, it is unsightly and can make the child feel uncomfortable. Currently, there is limited treatment available for fluorotic teeth ranging from bleaching to crown restoration (Sherwood, 2010). It remains to be seen if fluoridation of the water is the optimal method for delivering fluoride to our children. In fact, given the widespread dissemination of fluoridated products there are other methods to ensure that children have adequate fluoride intake. By examining the data and studying trends associated with fluoride use both in water and as a supplement, scientists can use the resulting information to suggest necessary changes in the child's fluoride intake and modify their diet to achieve the maximized benefit from fluoride intake.

Method

In conducting this research Touro's online library, and the publications it is affiliated with, specifically Pubmed were searched. In addition, Google Scholar and online search engines as well as the resources available at the public library and its computer access to various online journals were utilized.

Discussion

Dental Caries

Dental caries, is the most common infectious disease affecting children, both in developed and third world countries (Colak, et. Al. 2013). They are formed primarily in pits and fissure where bacteria cling and metabolize sugars . The tooth is composed of two sections , the crown which is the exposed section, and the root which goes down and is embedded in the bone. The outermost layer of the crown is enamel which is comprised primarily of hard tightly packed rods of hydroxyapatite ($Ca_{10}(OH)_2PO_4)_6$) . The saliva in the mouth greatly aids in biofilm formation and allows various bacteria to colonize the tooth (Talaro, 2009). In fact, there is a diverse population of bacteria that inhabit the mouth estimated to be up to 300 different species (Loesche, 1986). When carbohydrates are consumed and broken down in the mouth by salivary enzymes into sugar components, oral bacteria will metabolize them and produce acid as a by-product. Current research suggests

Daniel Weidberg is graduated in May 2014 with a B.S. in Biology.

that *S. mutans* are the primary bacteria responsible for dental caries formation (Loesche, 1986) . As sugars are broken down in the mouth and acids are produced, the pH gets lowered and shifts the optimal conditions for bacterial growth in favor of these bacteria. Consequently, their metabolic activity will increase and produce even more acid while stunting other bacterial growth which don't produce as much acid and prefer more basic conditions. This will lead to significant acid buildup in the mouth. Caries are then formed over time by the acid in the mouth tunneling into the enamel layer through demineralization. If it is left untreated it can tunnel into the dentin layer and cause serious disease (Talaro, 2009).While it is known that teeth undergo a continuous process of mineralization and demineralization, it is the disturbance of this equilibrium in favor of increased demineralization that causes dental caries (Marsh, 1994)

History of Flouride

The cariostatic of fluoride was discovered by in 1901 by Dr. Frederick McKay (NIH, 2014) in Colorado Springs, CO when he moved there to open a dental practice. As soon as he got there he saw that ninety percent of the children had dark spots on their teeth. The locals told him that they suspected that there was something in their diet that contributed to this interesting phenomenon. Although this claim sounded preposterous to him he was intrigued and started doing research to uncover what was causing what was dubbed as the Colorado Brown Stain, which until then was unreported in medical literature. In 1909 dental researcher Dr. G.V. Black went to Colorado to help investigate the cause of this discoloration. He then discovered two interesting facts. First of all this discoloration was only an issue in children, meaning that those adults whose teeth calcified without discoloration weren't at any risk at all and secondly, that there was little prevalence of dental caries within the population of Colorado Springs (NIH, 2014).

In 1923 Dr. Mckay went to Oakley, Idaho where he heard that the children there suffered as well from tooth discoloration. He suspected that the water was causing this discoloration . Amazingly, when the water supply was rerouted the discoloration stopped. This convinced Dr. Mckay that somehow, the water is responsible for the discoloration of teeth. A similar phenomenon was noted in Bauxite, Arkansas where discolored teeth were prevalent in children, yet in other towns a mere few miles away their teeth were normal.

This was of great scientific significance as many companies had toxic fluoride waste that they had to get rid of and used this substantiation to dump their fluoride, albeit treated , into public water. Furthermore, this initial validation of the benefits of fluoride is still used today. However, there is much worldwide opposition to fluoridating water from a dental perspective and general health as well (Bryson, 2004).

Cariostatic Mechanism of Fluoride

How does fluoride work to prevent cavities from forming? We must consider this question form two angles, namely, does it work in pre or post eruptive teeth? Furthermore, what is the pharmacological effect of fluoride on developing teeth.

Rosin-Grget ,et. al. concluded that the mechanism of fluoride is more preventative in nature in mature teeth than pre eruptive ones where fluoride changes the crystalline structure and reduces the formation of dental caries (Rosin-Grget, et. al. 2013). They quote a study from LeGeros investigating enamel structure in deciduous teeth where prenatal fluoride supplements were administered They found that there is less acid etching, higher mineral, and more organized crystalline structures with smaller prisms in the teeth. He concluded that this makes the enamel more perfect and as a result less acid soluble . However Rosin-Grget et al. (2013) refuted this claim because in vitro studies show the effect of fluoride on enamel solubility is minor and it is unlikely that the pre eruptive effect of incorporating fluoride will have any significant contribution, if any, in the reduction of caries.

An interesting study was done to determine the in situ effect of bacteria on shark teeth. Shark teeth ,unlike human teeth, are naturally high in fluoride and composed almost exclusively of fluorapaptite ($Ca_5(PO_4)_6F_2$) . They placed orthodontic bands with 0.2% NaF solution on both sets of teeth which will allow biofilm growth and found the plaque accumulation to be almost identical in both samples. They concluded that the effect of fluoride is influ-

enced primarily by topical, post eruptive effects. However, when treating both sets of teeth with fluoride rinses plaque growth was inhibited (Ogaard, et. al. 1991). We clearly see that the effects of fluoride is topical.

Rosin- Grget et al. proposed that the mechanism of action for fluoride is twofold. When fluoride is introduced into the mouth topically it forms a covering over the teeth in the form of Calcium Fluoride. This will inhibit acid demineralization by secreting fluoride ions which neutralize the acid. Since teeth also undergo a constant mineralization/ demineralization process, the fluoride will be incorporated into the crystalline structures. Normally, the main component of teeth is hydroxyapatite crystals (Ca5(PO4) 6OH2), however fluoride will be exchanged in place of some of the hydroxyl groups and form fluorapatite (Ca5 (PO4)6F2) which is more resistant to acid breakdown. This is because when acid breaks down the fluorapatite crystalline structures it will release the free fluoride ions which then work similarly to topical fluoride. So while fluoride is important from a remineralization point of view, its efficacy is based on its ability to form crystals which won't dissolve quickly, thereby inhibiting enamel demineralization. Therefore , a constant low level of fluoride ions in saliva reduces the rates of enamel demineralisation during the caries process and enhances the remineralisation of enamel.

Some have suggested that fluoride in the form of HF will affect the bacteria at the metabolic level but further research is necessary to substantiate this claim (Rosin-Grget, Peros, Sutej, & Basic, 2013). However , if this is true that fluoride disrupts bacterial metabolic activity, there is cause for concern that it may alter the body's normal flora equilibrium (Bryson, 2004)

Fluorosis

Fluorosis is defined as the discoloration of teeth due excess fluoride. The initial discovery of the efficacy of fluoride was through the discoloration of children's teeth. After McKay's initial work, The National Institute of Health dental department, headed by Dr. H.Trendely Dean, began investigating acceptable levels of fluoride in water supply systems that won't cause fluorosis (NIH, 2014). Interestingly enough, Dean was originally a strong opponent of fluoridating water for this very reason but later after being promoted to head the NIH he changed his mind. This led critics to question whether there was any reason to believe in the safety of fluoride or if Dean changed his mind for unknown reasons (Kauffman, 2005). After performing various tests he discovered that fluoride levels up to 1.0 ppm won't causes severe fluorosis in most people and only mild fluorosis in many (Science, 1993). Furthermore he compiled an index, famously known as Dean's index which measures fluorosis severity levels and is still used today (Table 1). These guidelines are defined by taking the two most discolored teeth and then classifying the tooth in its entirety , not just the discolored spots (Science, 1993)

Table 1: Fluorosis classifications. Based on information obtained from the National Institute of Science (Science, Health Effects of Ingested Fluoride, 1993)

Classification	Fluorosis Symptoms
Normal	Clear white surface
Questionable	Small white flecks where fluorosis isn't clear.
Very Mild	Small white flecks that occupy up to 25% of the tooth
Mild	White opaque spots are clearly noticeable but don't occupy 50% of the tooth
Moderate	All surface of the tooth are affected and brown spots are sometimes apparent
Severe	The general structure of the tooth is affected and the tooth can corrode. It is marked by brown splotches

Flourosis, while the exact molecular mechanism is still unknown , is known to be caused by the incorporation of fluoride during the mineralization of the enamel during tooth development (Lyaruu, et al., 2014). It arises as result of long term uptake of fluoride ions and the hypermineralization of fluoride in enamel during tooth development. Consequently, it is a concern for children during development and it won't affect them after their teeth are already formed. In fact, after the teeth erupted they will be cariosatstic as previously discussed.

Ireland Water Fluoridation

Dr. Máiréad Antoinette Harding and Dr. Denis Martin O'Mullane reviewed the results obtained from this study (Harding & O'mullane, 2013). In an effort to combat the widespread dental caries plaguing the general population, the government passed a law in 1964 requiring Dublin to fluoridate its water. BY 1970 most communities in Ireland had fluoridated their water supply system. The government also mandated that a baseline survey be taken before the fluoridation of the water and compare it with results that would be obtained at a later date. To ensure the study's integrity, the RoI also stipulated that regular surveys of the water fluoride levels be conducted as often as necessary. Furthermore, the fluoridation was carried out by the Department of Sanitation while the Department of Environment is responsible for ensuring optimal fluoride levels are maintained throughout the study. The fluoride levels were kept between 0.8 -1.0 ppm with a target range of 0.9 ppm. Additionally, since this was a government project it is safe to assume that there was a large study pool. Also, because fluoride is introduced directly into the water, patient compliance is a non-issue. Furthermore, because the water wasn't previously fluoridated we know that the data was gathered accurately and reflects the nature of the study.

The method of the study was to measure the decayed, missing, filled teeth(DMF) of children age 5 from both communities that have access to fluoridated water and comparing it to communities that drink unfluoridated water. Results were then recorded using the DMF index. Measurements were taken in 1965 when fluoridation was first introduced and then again in 1983-1984 and 2002.

Another study was conducted as well in Northern Ireland (NI) in 2000 where water fluoridation hadn't been introduced. The same criteria as the 1984 study were used here as well. The purpose of the study was to study the effect of fluoridating water on DMF teeth as well in 5 year olds.

Another component of the study was to measure the prevalence of fluorosis in fluoridated areas versus those areas that weren't fluoridated. They were measured and ranked using Dean's Index and results were recorded with percentages from 0 -100 of those taking part in the study who exhibited fluorosis . It is important to note that only fluorosis data from normal, questionable, and very mild were recorded in the survey. Thus we can't quantify the data with respect to mild- severe with resulting from this survey.

Results

The following results were obtained in respect with dental caries measured in DMF indices.

Table 2: Data showing the prevelance of decayed, missing, filled teeth.(DMF) in areas of fluoridated water (Ful FL) and non-fluoridated water(Non FL) in the Republic of Ireland(RoI). (Harding & O'mullane, 2013).

RoI 5 yr olds	RoI	NI	
Year	Full FL	Non FL	Non FL
1960	-----	5.6	4.8
1983-84	1.8	3.0	4.5
2002	1.3	1.0	1.8

As we can see from this data, in the year 1960 there is a no data between fluoridated and non-fluoridated areas as there was no fluoridation done at this point, but we did see that the DMF is higher than the following years. However, in the year 1983 we see a clear distinction between full FL and non-FL areas which follows the expected results. In the year 2002 we little difference if any between FL and non-FL areas. Furthermore, when analyzing data in

non – FL areas alone we see a step decline in the DMF index.

This can be seen as well when analyzing the data from NI. In the year 1960 the DMF is 4.8 and in 1983-84 it is 4.5. Again, we see that although there wasn't any change in the fluoride levels in the water, nevertheless there is a drop in the DMF index. This correlates with data found worldwide that dental caries were on the decline with the advent of fluoridated products especially toothpaste which was introduced in the 1970's (Jones, et. al. 2005).

Perhaps the most intriguing data is from 2002 where all the data is within the same range, both from the FL and non-FL area. Furthermore, even the NI data from 1985 which was slightly higher when compared to non-FL (4.5 vs 3.0) is within 0.1 in 2002. This further solidifies the observation that given the widespread availability and incorporation of fluoridated products in our lifestyle we see a dramatic decline in dental caries worldwide. This is evident where we see no distinguishable difference in the data between fluoridated and non-fluoridated areas. It is important to notice that this study doesn't account for the participants' dietary intake and so it is impossible to make any substantial claim with respect to the effect of fluoridated products alone without drinking fluoridated water.

Table 3: Percentages of fluorosis in Republic of Ireland (RoI) and Northern Ireland (NI) in both fluoridated areas and non - fluoridated areas. Degrees of fluorosis follow Dean's Index. (Harding & O'mullane, 2013)

Fluorosis	RoI Fluoridated	RoI non-Fluoridated	NI (non FL)		
	1984	2002	1984	2002	2002*
Normal	94	76	98	90	90
Questionable	5	11	2	7	6
Very Mild	1	8	0	2	3
Mild	0	4	0	0	0
Moderate	0	0	0	0	0
Severe	0	0	0	0	0

It is logical to assume that there will be a similar trend in the data with respect to fluorosis. However, this is not so. In fact, the number of normal teeth in nonflouridated areas vs. fluoridated areas in 1984 is 98% and 94% respectively, while in 2002 the numbers for non-FL and FL is 90% and 76%. There is no documented data available for 1984 in NI (table 3).

This means that although there is a decrease in the prevalence of dental caries both in fluoridated and non-fluoridated areas, we only see a marked increase in fluorosis in fluoridated areas.

Another interesting observation is when comparing the data within non fluoridated areas. We find that although the DMF index has decreased in 2002 from the year 1984, we don't see a significant increase in fluorosis during that time period. However we do see some increase in fluorosis. This data correlates with data found worldwide that the prevalence of fluorosis increased during this time.

While this study clearly shows the disparity between fluoridated and non fluoridated areas, it alone is inconclusive because we don't know if the people from non fluoridated areas drank water from other sources. Furthermore, we don't know if there was any moderate or severe cases associated with those that lived any of these areas. Additionally, perhaps the reason we find such a drastic increase of fluorosis in 2002 in fluoridated areas is because they too used products containing fluoride and drastically increased their fluoride intake levels. Perhaps if they would not have used these products their fluorosis levels would mirror those from 1984.

Perhaps a possible explanation for the decrease in dental caries in non fluoridated areas is given the availability of dental products that specifically target dental caries. However, since these products are used as directed we won't find a high level of fluorosis accompanying it.

An explanation is available the lower levels of fluorosis in non fluoridated areas versus fluoridated. While they too use fluoridated product, as evident from the decrease in dental caries, it doesn't contribute to fluorosis

with the same intensity as fluoridated water. This can clearly be seen from another study that was conducted in 1995 in Kingston and Newburgh N.Y. to measure the development of fluorosis and dental caries in children (Kumar, et al., 1998). These two cities were chosen to partake in the study because of their similar demographics and dental lifestyles which can be easily compared. The city of Newburgh was fluoridated in 1945 during Dean's initial project to fluoridate the public water system (Kauffman, 2005) Newburgh has maintained a fluoride level of 1.0 ± 0.2 ppm except for a slight fluctuation between 1978-1981. In contrast, the city of Kingston has a fluoride content of less than 0.3 ppm. After an initial study was conducted in 1986, another study was done in 1995 to compare the results and note any changes.

The criteria for the study in 1995 were the same as the guidelines set forth in the 1985 study to allow for comparison. These included fluorosis measurements in accordance with Dean's fluorosis index. However unlike the RoI study fluorosis levels were recorded for severe and moderate cases as well which yields a complete set of data. Children between the ages of 7-14 representing various demographics were examined in this study and data of 1496 children were analyzed which represented a significant percent of the respective populations.

Although an increase in fluorosis was noticed in both communities since 1985 , a trend which was noticed worldwide, there was nevertheless a marked increase in the fluoridated Newburgh (table 4).

Table 4: Fluorosis levels in percentiles obtained from a study done in 1995 in Newburgh N.Y. and Kingston N.Y. (Kumar et.al., 2005)

Dean's index levels	Newburgh	Kingston
Questionable	19.4	6.9
Very Mild	13.2	8.3
Mild	5.7	3.1
Moderate/ Severe	0.7	0.3

Perhaps most noticeable was the following data obtained when examining the results closely. The prevalence of fluorosis was compared when fluoridation alone was used and when only supplements were used and the following was found. When fluoride was obtained through fluoridation alone: 17.9%, but when fluoride supplements alone were used fluorosis prevalence was merely 8.8%.

Conclusion

We can conclude that while fluoridating water has its benefits by reducing dental caries, it also is solely responsible for the rapid increase of dental fluorosis in children. While many would say that the risk is well worth it, this can avoided by stopping to fluoridation the water and use of fluoride products instead. As we see from our data, the increase of fluorosis that comes as a side effect from fluoridation isn't noticed with the same intensity when using fluoridated products alone. While we still do see a slight rise in fluorosis as evident from both studies this can be attributed to our inability to monitor our fluoride intake at optimal levels especially in children (ADA, 2005). This can be due the ubiquity of fluoride in many things, including fruit juice (Kumar, et al., 1998). However, with further modifications we should be able to spare our children from -developing fluorosis at all. Furthermore, since fluorosis occurs at the pre-eruptive stage while the efficacy of fluoridation is primarily post eruptive we are exposing our children's growing teeth to the harms of water fluoridation while the benefits aren't yet fully effective.

References

ADA, A. D. (2005, Dec). Fluoride- Natures Cavity Fighter. Journal of the American Dental Association, 136(12) p. 1783.

Bryson, C. (2004). The Fluoride Deception. New York, NY: Seven Stories Press. p.16

CDC. (1999, Oct 22). Recommendation for Using Fluoride to Prevent Dental Caries in the U.S. Retrieved Dec 4, 2013, from CDC.gov: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4841a1.htm

Colak, H., Dülgergil, Ç. T., Dalli, M., & Hamidi, M. M. (2013). Early childhood caries update: A review of causes, diagnoses, and treatments. Journal of Natural Science, Biology and Medicine, 4(1),29-31.

Harding, M. A., & O'mullane, D. M. (2013). Water fluoridation and oral health. Acta Medica Acedemia, 42(2),131-139.

Jones, S., Burt, B. A., Petersen, P. E., & Lennon, M. A. (2005, Sep). The effective use of fluoride in public health. Bulletin of the World Health

Organization, 83(9), 670-679. Retrieved Jan 9, 2014, from World Health Organization.

Kauffman, J. M. (2005). Water Fluoridation: a Review of Recent Research and Actions. Journal of American Physicians and Surgeons, 10(2), 38-44.

Kumar, J. V., Swango, P. A., Lininger, L. L., Leske, G. S., Green, E. L., & Haley, V. B. (1998). Changes in dental fluorosis and dental caries in Kingston and Newburgh NY. American Journal of Public Health, 88(12),1866-1870.

Loesche, W. (1986). Role of Streptococcus Mutants in Tooth Decay. Microbiology Reviews, 50 (4), 354.

Lyaruu, D., Medina, J., Sarvide, S., Bervoets, T., Everts, V., DenBesten, P., Bronckers, A. (2014). Barrier formation- potential molecular mechanism of enamel fluorosis. Journal Dental Research, 93(1), 96-102.

Marsh, P. (1994). Microbial Ecology of Dental Plaque and its Significance in Health and Disease. Advanced Dental Research, 8(2),263-271.

NIH. (2014, Jan 6). Story of fluoridation. Retrieved Dec 25, 2013, from National Institute of Dental and Craniofacial Research: http://nidcr.nih.gov/OralHealth/Topics/Fluoride/TheStoryofFluoridation.htm

Ogaard, B., Rolla, G., Dijkman, T., Ruben, J., & Arens, J. (1991). Effect of fluoride mouthrinsing on caries legion development in shark enamel; an in situ study. European Journal of Oral Sciences, 99(5), 372-377.

Rosin-Grget, K., Peros, K., Sutej, I., & Basic, K. (2013). The cariostatic mechanism of fluoride. Acta Medica Acedimia, 42(2),179-188.

Science, N. A. (1993). Health effects of ingested fluoride. Washington D.C.: National Academy Press.

Sherwood, A. (2010, Jun 23). Fluorosis Varied Treatment Options. Journal of Conservative Dentistry, 13(1), 52. Retrieved from National Institute of Health.

Talaro, K. P. (2009). Foundations in Microbiology 7th Ed. New York,NY: Mcgraw-Hill.

Hutchinson-Gilford Progeria Syndrome: Pathophysiology and Possible Treatments

Pearl Hersh

Abstract

Named after the two scientist who independently described the condition, Hutchinson-Gilford Progeria Syndrome (HGPS) occurs due to a mutation in the LMNA gene that codes for Lamin A, a filament protein that acts to form the nuclear lamina in the cell nucleus. This mutation is a single C-to-T substitution at nucleotide 1824 of the LMNA gene. As a result of this mutation, an abnormal protein named 'progerin' is synthesized instead of Lamin A, causing the nuclear membrane to be malformed. Since protein farnesylation is needed to target progerin to the nuclear rim, farnesyltransferase inhibitor has been proposed as a form of treatment that could reduce the occurrence of misshapen nuclei and alleviate HGPS symptoms.

Introduction

Aging is a process that's inevitable. Over a typical lifespan, the body loses its ability to maintain homeostasis and fight off disease. The elderly also experience atherosclerosis that can lead to strokes and myocardial infarctions. Arthritic joint pain and stiffness are also common, as is thinning of the skin. Researches have speculated about the precise physical changes in the body that manifest themselves as the process of aging (Burtner and Kennedy 2010).

The answer may lie in a very rare segment of the population who suffer from progeriod syndromes that cause accelerated aging. The most severe of this group is Progeria, which causes rapid aging in children and death by adolescence. The calculated mean lifespan in Progeria patients is only 13 years of age (Kudlow et al. 2007).

HGPS Pathophysiology

Hutchinson-Gilford Progeria Syndrome (HGPS) is caused by a de novo heterozygous point mutation, changing a GGC sequence to GGT in exon 11 of the LMNA gene. This mutation causes a 50 amino acid sequence deletion at the carboxyl terminus of prelamin A, producing a truncated progerin in the place of Lamin A (Neelam et al. 2012). Lamins are intermediate filament proteins that polymerize to form nuclear lamina, a meshwork forming the inner nuclear membrane. Lamin A belongs to the A-type lamins that includes Lamin A and Lamin C. Lamin C differs very slightly from Lamin A by its C-terminal extension, which has 90 less residues than Lamin A. Both Lamin A and C contribute to the shaping of the nuclear membrane. When progerin is present in place of Lamin A, the nuclear membrane takes on an abnormal morphology (Rakha, et al. 2011).

The Proteins of the nuclear lamina were once thought to play merely a structural role. Through studying diseases that affect these proteins, it has become apparent that they also play an important role in various cellular functions such as signal transduction and gene expression (Scaffidi and Misteli, 2006). Therefore, mutations affecting these proteins can have devastating effects on multiple organ systems in the body, as in the case of Hutchinson-Gilford Progeria Syndrome (HGPS).

HGPS is part of a group of rare diseases known as laminopathies, in which the proteins of the nuclear lamina are affected. These diseases all result from LMNA gene mutations and include lipodystrophy, muscular dystrophy, peripheral neuropathy and Progeria (Liu, et al. 2010). In lipodystrophy, symptoms begin at the onset of puberty and patients experience loss of subcutaneous fat, high cholesterol and type-II diabetes. Muscular dystrophy is similar in that symptoms appear close to puberty and cause progressive muscle wasting. Peripheral Neuropathy is a laminopathy that causes nerve dysfunction. Progeria is perhaps the most devastating of them all since symptom onset begins at a few months of age and death from cardiovascular

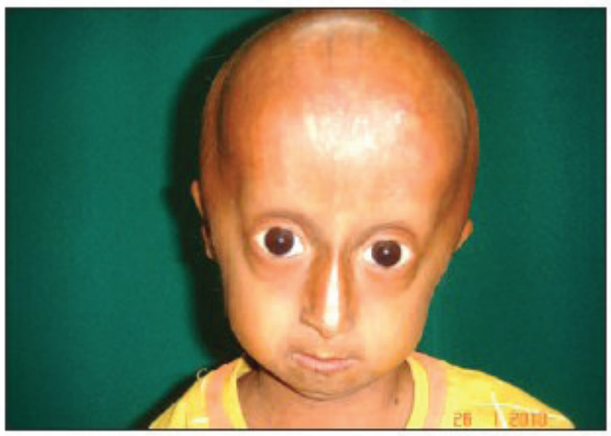
Perl Hersh graduated with a B.S. in Biology and will be attending New York Medical College .

illness is likely by age 13.

HGPS Phenotypes

HGPS disease symptoms include; slow growth, scleroderma-tous changes of the skin, alopecia, osteoporosis and atherosclerotic vascular disease. (Yang, et al. 2006) These symptoms contribute to a very distinctive look common to all Progeria patients. They are sometimes described as appearing like 'little aliens' due to their diminutive stature, lack of hair, and small heads with prominent noses.

Figure 1: Face of a Progeria patient showing alopecia, loss of eyebrow and eyelashes and prominent eyes with a tapered nasal tip. (Agarwal, et al. 2010)



In one particular Chinese girl with Progeria, HGPS diagnosis was made at 2 months of age. The child was the second-born of healthy parents with no remarkable family medical history. The first symptom that raised concern was a loss of subcutaneous fat in her abdomen and buttocks, where a baby would normally exhibit plumpness. Her hair was also unusually sparse and her veins were noticeably prominent for 2 months of age (Chen, et al. 2012).

At 2 years of age, the child had already lost all of her hair and was beginning to develop a characteristic Progeria face. Her forehead jutted out, her eyes were prominent and her jaw was undersized. Imaging studies taken over ages 4 and 5, showed patterns of bone resorption in her clavicles and distal phalanges along with hip displacement and dental crowding (Ibid).

The symptoms progressed rapidly and by age 6 the deformities caused speech and swallowing difficulties and hearing loss. When measured at age 8, she fell below the 3rd percentile in both height and weight compared to other her age and she remained very thin and small in stature (Ibid).

Diagnoses came later for an Indian boy who showed up at a Dermatology clinic at age 4. He had loss of hair, including eyelashes and eyebrows since age 1, and stunted growth. Doctors noticed his distinct prominent veins and eyes, beaked nose and high-pitched voice. His problems were far from merely dermatological, and he was diagnosed with Progeria after confirmatory imaging studies (Agarwal, et al. 2010)

Vertebrate Progeriod Models

Researchers attempting to better understand and treat Progeria, need models to work with. The incidence of Progeria is so infrequent in the population due to the rarity of the specific LMNA mutation that causes it. Nearly all Progeria patients in the world today are well known and have participated in studies by contributing medical data and samples. In the century or so since the discovery of the syndrome, only about 100 cases have been documented (Mohamed and Jayachandran 2009) and today, with only 48 known patients whose lifespans are short, these samples are severely limited.

Human Progeria research subjects are not only limited in number, it often isn't safe for them to participate in early stages of drug trials. Before a drugs potency can be determined, use in humans is unethical. Thus, creating animal models to test Progeria drugs is an important key to ultimately finding a cure.

After much trial and error, mice have been successfully genetically modified to be ZMPSTE24 deficient, causing the expression of Progeria-like symptoms (Osorio, et al. 2009). ZMPSTE24 is an enzyme that completes the post-translational processing of Pre-lamin A by cleaving it into its mature form of lamin A (Liu, et al. 2010). Without the ZMPSTE24 enzyme, Pre-lamin A processing is halted and this results in a truncated Lamin A protein similar to the progerin protein found in patients with HGPS.

Zebrafish models expressing truncated Lamin A

proteins have also been genetically engineered and studied. Using a splice-block method to delete the 8 amino-acid site of pre-lamin A cleavage in zebrafish embryos, researchers created progeriod zebrafish models. These zebrafish then exhibited accelerated aging and shortened lifespans along with other symptoms such as cell-cycle arrest and cartilage defects that appeared as craniofacial abnormalities (Koshimizu, et al. 2011).

Both the ZMPSTE24 deficient mice and the progeriod zebrafish models were genetically altered in a way where Lamin A was not able to be processed into its mature form. Another mouse model however, was modified so that it would not produce the Lamin A protein at all. This lamin A deficient mouse would only produce Lamin C, a similar Lamin protein normally produced alongside Lamin A. The Lamin C-only mice had no tissue abnormalities and appeared non-diseased (Fong et. al 2006).

In a state of complete lack of Lamin A, Lamin C seems to compensate and therefore, no pathologies result. It seems that the truncated, pre-lamin or progerin form of the Lamin A that accumulates when the protein's processing is halted, is the cause of the diseased cell (Fong et. al 2006). This realization can help researchers treat laminopathic diseases by developing drugs that can prevent immature lamin proteins from doing harm and possibly trigger the non-affected Lamin types (unaffected Lamin C in Progeria patients) to compensate for the missing protein.

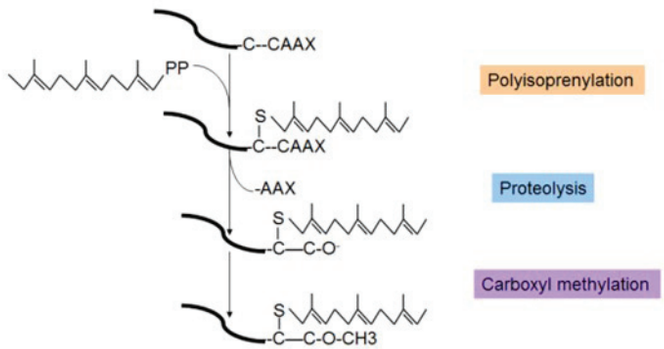
FTI Treatment

The advantage of farnesyltransferase inhibitor (FTI) is that it can mask HGPS symptoms even while the abnormal Progerin protein is still being expressed. By preventing progerin from attaching to the nuclear rim, FTI can increase the amount of normal-shaped nuclei (Capell, et al. 2005).

Prelamin A requires a 3-step prenylation processing to be converted into mature Lamin A. The prenylation occurs rapidly and unprocessed Prelamin A is barely detectable (Gao, et al. 2009). A similar form of post-translational modification occurs in all CaaX proteins. This category of proteins contains a specific amino acid sequence at the c-terminal consisting of a cysteine residue, 2 alipathic residues and another c-terminal acid 'x' that varies, hence the acronym 'CaaX Protein'. In Prelamin A, the CaaX terminal is made of cysteine, alipathic serine and isoleucine, and methionine (CSIM) (Dominici, et al. 2009).

In the first processing step of polyisoprenylation, farynesyltransferase recognizes methionine and adds a 15 carbon farnesyl to the CSIM sequence. Proteolysis is next, in which endoprotease ZMPSTE24 removes the 'SIM' portion of the CSIM sequence (Dominici, et al. 2009). In the final prenylation step, carboxyl methylation of the farnesylated prelamin A occurs via methyltransferase to produce carboxymethylated-farnesylated prelamin A (preLA-farnesyl-C-H_3).

Figure 2: Prenylation process of the CAAX proteins. The protein prenylation process includes 3 steps: polyisoprenylation, proteolysis, and carboxyl methylation. Polyisoprenylation is the attachment of an isoprenoid lipid by protein farnesyltransferase (FTase) or geranylgeranytransferase type I (GGTase-I) to CAAX box. In the second step, the CAAX residues are proteolysed by prenyl protein peptidase RCE1 family to release -AAX. This is followed by subsequent endoproteolytic trimming and carboxyl methylation significantly increases the hydropho-



Progerin, a truncated form of pre-lamin A, is missing the ZMPSTE24 cleavage site and will therefore retain the cysteine C-terminal that is farnesylated and carboxymethylated (Liu, et al. 2010). The retention of the toxic farnesyl group will cause progerin to incorporate itself into the nuclear envelope in an abnormal fashion. Once in the nuclear envelope, progerin will produce an abnormal heterochromatin assembly and lead to an increase in DNA

damage (Zaremba-Czogalla et al. 2011). Structurally, the nuclear membrane will also become misshapen and stiff. This characteristic is known as nuclear blebbing and is a distinct phenotype of progeria.

Lonafarnib, a farnesyltransferase inhibitor drug initially used for cancer treatment, has recently been tested on patients with HGPS in a clinical trial. In 2007, 25 progeria patients from 16 countries around the world came to Boston, Massachusetts over a period of 2 years to receive Lonafarnib. They were measured for changes in weight, skeletal rigidity, hearing and cardiovascular health. The study showed that the children improved in at least one or more of the measured areas over the course of the trial. Most importantly, in the essential area of cardiovascular health, which is the cause of death in progeria patients, all but one participant showed improvement (Gordon, et al. 2012)

Other Treatments

In the presence of farnesyltranferase inhibitors, the cell will often adapt and turn to other processing pathways such as grenygrenylation (Gordon et al. 2008). In place of polyisoprenylation (addition of a 15-carbon farnesyl) via farnesyltransferase, a similar enzyme, geranylgeranyltransferase I (GGTase I) can add a 20-carbon geranylgeranyl group to the Pre-lamin A (Sousa et al. 2008). This adaptation mechanism can lessen the effectiveness of FTI treatment. A combination of statins and aminobisphosphonates, combined with FTI treatment can inhibit both farnesylation and grenygrenylation pathways.

Although not originally developed for progeria, the statin Pravastatin and the aminobisphosphonate Zoledronic acid have been implemented in a 2012 clinical trial, along with FTI Lanofarnib, in progeria patients. Pravastatin, an HMG-CoA inhibitor, is often prescribed to lower cholesterol levels and keep atherosclerosis from worsening. Zoledronic acid is used in bone cancers and multiple myeloma, can help prevent hyepercalcaemia that occurs in Progeria patients. These two drugs work principally by preventing farnesyl group formation, a process vital to progerin in the course of the disease (Neelam et al. 2012).

Lastly, stem Cell use has been researched as a form of treatment. Since Lamin A expression is developmentally regulated, it isn't present in embryonic cells and stem cells (Mounkes et al. 2003). This can be seen in the normal appearance of Progeria-modeling mouse embryos and progeria patients up until a few months of age, when progerin starts to proliferate in cells in place of Lamin A. Stem cell use has been explored as a means of initiating tissue regeneration and offsetting the accelerated rate of cell aptosis that causes rapid aging in Progeria patients (Halaschek-Wiener and Brooks-Wilson 2007).

Conclusion

Progeria is a rare, devastating and terminal disease caused by a single gene mutation. Since the mutation is located on the LMNA gene, the Nuclear Lamina Proteins are affected. A single nucleotide substitution causes a 50 base pair deletion of pre-lamin A (Lain A pre-cursor). Lamin A is then prevented from completing its processing and remains in an immature form called progerin. The accumulation of progerin has a multi-systemic effect leading to rapid aging and early death from complications of atherosclerosis. Lonafarnib, a FTI inhibitor, has been proposed as a form of treatment and recent studies have shown it is effective in alleviating some of the symptoms of Progeria.

References

Agarwal U, Sitaraman S, Mehta S, Panse G. Hutchinson-gilford progeria syndrome. Indian Journal of Dermatology, Venereology and Leprology. 2010;76(5):591.

Burtner CR, Kennedy BK. Progeria syndromes and ageing: What is the connection? Nature Reviews.Molecular Cell Biology. 2010;11(8):567-78.

Capell B, Erdos M, Collins F, et al. Inhibiting farnesylation of progerin prevents the characteristic nuclear blebbing of Hutchinson-Gilford progeria syndrome. Proceedings Of The National Academy Of Sciences Of The USA. September 6, 2005;102(36):12879-12884.

Chen C, Lin S, Lin D, Liu Y, Hsu LJ, Wang W. Clinical Imaging Findings In A Girl With Hutchinson-Gilford Progeria Syndrome. Genetic Counseling. 2012;23(1):1-7.

Dominici S, Fiori V, Magnani M, et al. Different prelamin A forms accumulate in human fibroblasts: A study in experimental models and progeria. European Journal of Histochemistry : EJH. 2009;53(1):43-52.

Fong LG, Ng JK, Lammerding J, Vickers TA, et al. Prelamin A and lamin A appear to be dispensable in the nuclear lamina. J Clin Invest. 2006;116 (3):743-52.

Gao, Juehua, Liao, Jie, Yang, Guang-Yu. CAAX-box protein, prenylation

process and carcinogenesis. American Journal of Translational Research. [serial online]. 2009. 1(3): 312–325.

Gordon, Leslie B., Kleinman, Monica E. et al. Clinical trial of a farnesyltransferase inhibitor in children with Hutchinson–Gilford progeria syndrome. Proceedings of the National Academy of Sciences.2012;109(41).

Gordon LB, Harling-Berg C, Rothman FG. Highlights of the 2007 progeria research foundation scientific workshop: Progress in translational science. The Journal of Gerontology. 2008;63A(8):777-87.

Halaschek-Wiener J, Brooks-Wilson A. Progeria of stem cells: Stem cell exhaustion in hutchinson-gilford progeria syndrome. The Journal of Gerontology. 2007;62A(1):3-8.

Koshimizu E, Imamura S, Qi J, et al. Embryonic senescence and laminopathies in a progeroid zebrafish model. PLoS One. 2011;6(3).

Kudlow BA, Kennedy BK, Monnat,Raymond J.,,Jr. Werner and hutchinson-gilford progeria syndromes: Mechanistic basis of human progeroid diseases. Nature Reviews.Molecular Cell Biology. 2007;8 (5):394-404.

Liu Q, Kim DI, Syme J, LuValle P, Burke B, Roux KJ. Dynamics of lamin-A processing following precursor accumulation. PLoS One. 2010;5(5).

Mohamed Riyaz S, Jayachandran S. Progeria. Indian Journal of Dental Research. 2009;20(4):508-10.

Mounkes L, Kozlov S, Burke B, Stewart CL. The Laminopathies: nuclear structure meets disease. Curr Opin Genet Dev. 2003;13:223-230.

Neelam S, Krishna M, Semwal BC, Shravan P, Kuldeep S, Deepak S. Progeria: A Review. International Journal of Pharmaceutical Sciences Review and Research. 2012;14(1):4449.

Osorio FG, Obaya ÁJ, López-otín C, Freije JM, P. Accelerated ageing: From mechanism to therapy through animal models.Transgenic Res. 2009;18(1):7-15.

Rakha P, Gupta A, Dhingra G, Nagpal M. Hutchinson-- Gilford Progeria Syndrome: A Review. Der Pharmacia Sinica [serial online]. February 2011;2(1):110-117.

Scaffidi P, Misteli T. Good news in the nuclear envelope: Loss of lamin A might be a gain. J Clin Invest. 2006;116(3):632-4.

Sousa SF, Fernandes PA, Ramos MJ. Farnesyltransferase inhibitors: A detailed chemical view on an elusive biological problem. Curr Med Chem. 2008;15(15):1478-92.

Yang SH, Meta M, Qiao X, Frost D, al e. A farnesyltransferase inhibitor improves disease phenotypes in mice with a hutchinson-gilford progeria syndrome mutation. J Clin Invest. 2006;116(8):2115-21.

Zaremba-czogalla M, Dubiska-magiera M, Rzepecki R. Laminopathies: The molecular background of the disease and the prospects for its treatment. Cell Mol Biol Lett. 2011;16(1):114-48.

Apoptotic Neurodegeneration in the Developing Human Brain: Possible Role of General Anesthetics in its Genesis, and of L-Carnitine in its Reversal

Yisroel Yitzchok Rosenfeld

Abstract

Recent studies suggest that general anesthetic (GA) agents administered to developing rats, through its mechanism as an NMDA antagonist or a GABAa mimetic, may damage developing neural cells by inducing a higher rate of programmed cell death (apoptosis). Similar heightened degeneration was also apparent in higher primates such as the monkey. This warrants strong concern, as every year thousands of pregnant women and children below 1 year of age undergo a surgical procedure in which GAs are used. A spike in neuroapoptosis may lead to long term cognitive deficiencies lingering into adulthood. Are humans vulnerable to these affects? Different pathways are under investigation as to the cause of the damage in animals, but humans have different metabolic pathways than even higher primates, and the basic mechanism by which GAs take affect is not well understood. Scientists continue to unravel the underlying mechanism, seeking to stop the apoptotic cascade, all the while maintaining the benefits of the sedative effect. Promising new hope comes

Introduction

Anesthetic Pathway / Mechanism

For conformation to occur between an anesthetic agent and its target, it would be expected that the agent would be highly specific. But the broad arrays of such agents, with their varied structural and chemical compositions seem to belie such a nature. Nevertheless, all general anesthetics work through either of two primary transmission mechanisms: NMDA (N-methyl-D-aspartate) receptor antagonists or GABA mimetic receptors (gamma-aminobutyric acid).

GABA receptors, a type of ligand-gated ion channel, are the main inhibitory neurotransmitter in the central nervous system (Colquhoun et al. 2004). The sedative effect of the GABAa agonists is caused by the inhibition of firing new action potentials. When molecules bind extracellularly, a selective Cl ion pore is opened. This increased accessibility of the Cl ion drives it towards -65mV, a membrane potential at which there is no overall change in the ion concentration on either side of the membrane, a situation called reversal potential.

NMDA receptor antagonists block the glutamate and glycine from binding to the receptor, blocking signal transmission between the spinal cord and the brain (Olney et al. 1991). NMDA receptors are important in the function of neuronal migration and differentiation, synaptic plasticity, and in promoting learning and memory. Glutamate receptors are the most common excitatory neurotransmitter found in mammals. NMDA glutamate receptor antagonists include ketamine and nitrous oxide, both widely used in pediatric anesthesia. The collective effect of GABAa agonists and NMDA antagonists is to block synaptic transmissions from occurring between neurons and the spinal cord.

Methods

Data presented in this paper was mined from case studies, review articles, online sources, and scientific journals using search engines of the Touro College Library database, EBSCO, Pubmed, and Google Scholar.

Discussion

Effects on lower mammals

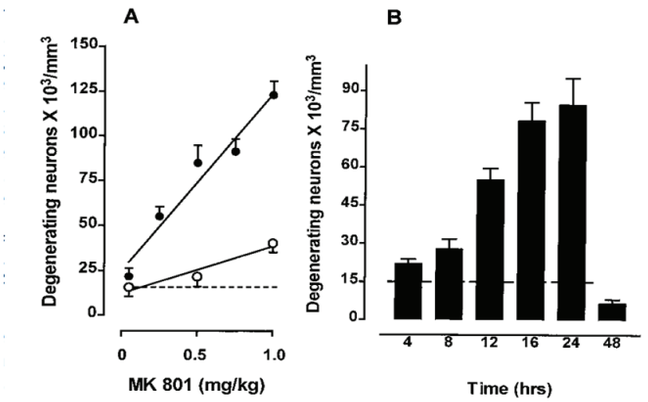
Deleterious effects of anesthetics on developing neural cells was first observed in extensive exposure of rat pups on postnatal days 0,3,7, and 21 to Dizocilpine [(+)MK 801], an NMDA receptor antagonist (Ikonomidou et al. 1999). NMDA receptor activation promotes survival of their re-

Yisroel Yitzchok Rosenfeld graduated in May 2014 with a B.S. in Biology.

spective cells. Even brief periods of inhibition were shown to cause brain-wide apoptosis. Rats were injected variously with .5mg, .75, and 1mg per kg of body weight and apoptosis was measured after 24 hours using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). TUNEL is a common technique for identifying DNA fragmentation representative of apoptotic signaling cascades by marking the terminal end of nucleic acids.

Pervasive apoptosis was perceived throughout the brain including within the hippocampus, dentate gyrus, thalamus, hypothalamus, frontal cortex, parietal cortex, cingulate cortex, and retrosplenial cortex, as evidenced by decreased cell density. The apoptotic effect was most pronounced in 7 day old rats when expression of NMDA receptors peak. The neural deletion was commensurate with the influence of both dosage and time factors (Figure 1A). The enantiomer, (-)MK 801, known to be less effective in its ability to inhibit NMDA receptor signal transmission, was also tested. The effects of (-)MK 801 remained negligible relative to the vehicle treated rats (the control; rats just given the solvent without the active ingredient, MK801) up to a dose of 1.0 mg/kg. Electron microscopy demonstrated that these degenerate cells showed no physiological or ultrastructural distinction from corresponding apoptotic cells.

Figure 1: Part (A) shows the detrimental effect of MK 801 is proportional to the dosage. Solid circles denote (+)MK 801; Empty circles represent (-)MK 801. The dashed line represents vehicle treated rats. Part (B) demonstrates a relationship between the degeneration and the time of exposure. Only (+)MK 801 was used. The dashed line shows the level of spontaneous apoptosis. (Source: Ikonomidou et al.1999)



Embryonic rats in utero were also given 0.5mg (+)MK 801 at embryonic days 17, 19, and 21 for 8 or 16 hours, and were examined after 24 hours. Significant apoptosis occurred in the embryonic 21 day old rats in the dentate gyrus, hippocampus and hypothalamus. Embryonic day 19 rats showed some modest apoptosis while embryonic day 17 rats showed no sign of apoptosis.

At certain points in the postnatal period rats were more vulnerable to neural degeneration than during the fetal period. Increased vulnerability in the postnatal period may result from the subunit composition of the NMDA receptor which is modified in the transition from the embryonic to the postnatal stage. Higher expression of NMDA receptors at certain periods would make the cells more vulnerable to the NMDA antagonist.

Most concerning was that ketamine, a common pediatric anesthetic, was also shown to cause neuroapoptosis in the developing rat brain. Humans are more developmentally advanced than rats and these results are difficult to extrapolate, but these findings suggest a period of susceptibility in humans during the corresponding period. This time frame is controversial, and an early postnatal study in rats may compare to late gestation or the newborn stage. Perhaps the danger exists throughout the corresponding brain growth spurt period (synaptogenesis) which lasts from mid-gestation to several years after birth.

GAs were also shown to have a time dependent effect with apoptosis shown to continually accentuate when rats were given 0.5mg/kg and were killed variously at 4,8,12,16,24, and 48 hours after being subjected. With the passage of just 4 hours apoptotic effects were apparent. A peak effect was reached after 24 hours while after 48 hours the effects mostly subsided (Figure 1B).

Apoptosis - Essential for Life

During normal development of the central nervous system apoptosis plays an integral part in the development. Signals that proliferate or restrain the apoptotic program using a precise biochemically guided system controls the number of cells extant and are important in shaping controlling and establishing the development of cells. When apoptotic caspases were eliminated to block the apoptotic mechanism in rats, abnormalities such as

increased subpopulations in the brain occurred and in some cases resulted in lethality. Apoptosis further aids in sexual differentiation, development of olfactory and thermoregulatory systems, and processing of pheromones (Broad et al. 2009). While a moderate amount of neural cells undergo apoptosis to support healthy development, the number of cells undergoing apoptosis in regions throughout the brain upon administration of general anesthetics was excessive.

Do disturbances in other major excitatory transmitter systems of the brain also cause widespread neuroapoptosis? No, it is not apparent in antagonists of excitatory pathways such as the muscarinic or non-NMDA glutamate antagonists. Nor does it occur in agonists of the inhibitory dopaminergic system. Only with the use of NMDA antagonists and GABAa agonists is widespread apoptosis apparent (Ikonomidou et al. 1999). Because of these discoveries, researchers set out to discover the effects of GABAa mimetics, NMDA antagonists, and the combined neurotoxic effects of the two.

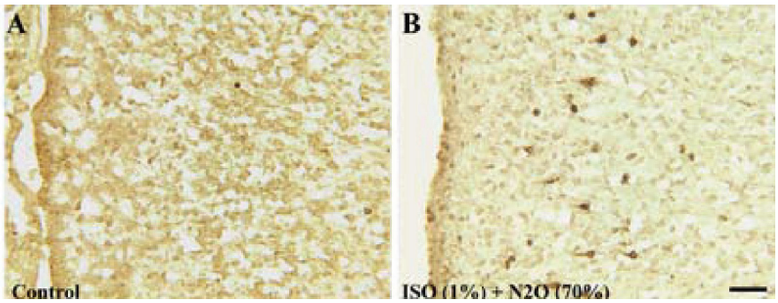
Permanent Effects on Intelligence

Even presuming robust apoptotic effects upon subjection to anesthetics, what practical significance would it have? Jevtovic-Todorovic et al. (2003) showed that exposure of developing rodents to anesthetics results in a decline in brain density leading to a long-term decline in cognitive function. Seven day old rats were anesthetized for 6 hours with N2O, isoflurane (ISO), and midazolam (triple anesthetic cocktail). They were put in the Morris Water maze and trained to swim to an observable stand. Their performance concerning path length and vigor in the Morris Water maze was on a par with that of the control. When the stand was submerged in the same location, the triple anesthetic cocktail rats showed substantial learning deficits in the middle trials, although they performed at the same level as the controls by the test's conclusion. When retested as adults, their lower memory capacity was evident when they spent less time searching for the submerged stands in the area of the pool in which it was had formerly been raised. Control rats spent more time searching the appropriate pool area for the stand's location. Spatial memory was shown to be impaired in the anesthetic cocktail rats by use of a radial arm maze, with a significant learning deficit as compared to the performance of the control.

Ikonomidou et al. (1999) learned that NMDA receptor antagonists can cause apoptosis. Wang et al. (2012) however contends that rats subjected to a dosage commonly used in clinical settings [NMDA receptor antagonist N2O (70%), or to the GABAa agonists ISO (1%)] showed no significant measure of apoptosis upon harvest and TUNEL examination after 6 hours. Rather, they needed to be subjected to prolonged and substantial doses of the NMDA receptor antagonist N20 or ISO for any measurable damage (Figure 2).

Separate administration did not cause significant apoptosis, but when administered concurrently, apoptotic levels were accentuated. While gene expression was altered when N2O and ISO was delivered independently the pathways of these genes are not closely associated with neurons, suggesting that cascades are inducing apoptosis rather than the anesthetic doing so directly. This provides hope to researchers. If the anesthetic itself does not directly cause the damage but instead generates a cascade to do so, scientists may be able to determine the point at which the damage occurs. A drug can then be developed to act in concert with the anesthetic and inhibit propagation of the damaging pathway. Accordingly, pediatric surgeons can continue to benefit from the sedative effects without concern for the health of the developing brain.

Figure 2. The apoptotic damage in the ISO + N2O group relative to the control is apparent (Source: Wang et al. 2012)



Widespread Effects Observed

How serious are the effects generated by neuroapotosis? Huang et al. (2012) proposed that ketamine causes permanent learning and memory impairment. Postnatal day 7 rats were given doses of 25, 50, and 75 mg/kg of ketamine for 3 days. At two months old, the group subjected to 75 mg/kg had a diminished capacity for memory and learning, as measured by the Morris water maze. Behavioral issues were observed as well. These effects were not apparent in the groups exposed to the smaller dosages.

Groups of rats were given the same treatment as described previously but dispatched after 3 days. TUNEL-stained hippocampal neurons were compared between the hippocampal sub-regions cornu ammonis 1 (CA1), CA2, and CA3, and the dentate gyrus in rat pups. Groups subject to lower doses of 25or 50 mg/kg did not have higher rates of apoptosis while a group subjected to 75 mg/kg showed significant apoptosis in these regions. Protein expression of PKCγ, ERK1/2 and Bcl-2 in the hippocampus was measured by western blot. The greater dose of ketamine inhibited p-PKC, p-ERK1/2 and Bcl-2 manifestation but not that of t-PKC or t-ERK. These findings corroborate the position that the malign effects of ketamine are associated with p-PKC, p -ERK1/2 and Bcl-2 expression, which in turn is related to persistent destructive cognitive effects.

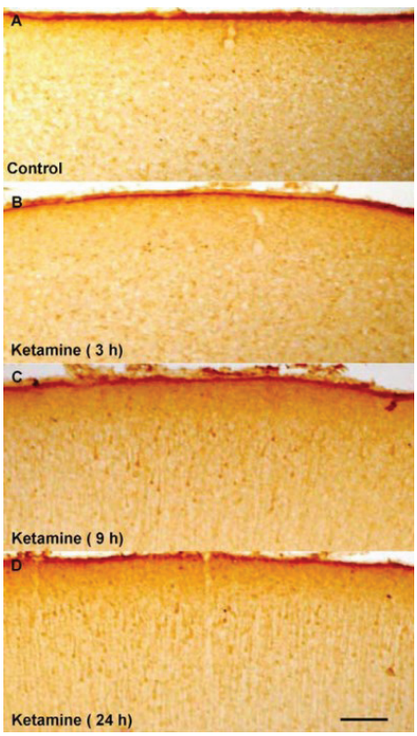
Dikranian et al. (2001) suggested perhaps scientists were mistaken in comparing neuronal cells believed to be undergoing apoptosis to non-neuronal cells. Maybe the detailed biological structure of a neuronal cell undergoing apoptosis would have a different ultrastructure than a corresponding cell in the rest of body. NMDA antagonists, and GABAa agonists were used to effect neuroapoptosis and the cellular ultrastructure of apoptotic cells was compared to that of a physiologically dying cell as it occurs naturally in the brain. It was verified to have similar properties to that set forth by the Kerr/ Wyllie team, the scientists who originally discovered and formulated the word apoptosis and classified the structure and formation of such a cell.

Apoptosis Seen in Higher Primates

Perhaps only such an effect is observed in rats but higher non-human primates such as the monkey would be impervious to their affects. Zou et al. (2009) demonstrated this was not so. However, in contrast with the earlier rat studies, the primate monkeys needed to be overwhelmed by a high dosage of ketamine (20 mg/kg) for degeneration to occur. An initial intramuscular injection of 20 mg/kg was followed by continuous intravenous administration of 20-50 mg/kg h for 3, 9, or 24 hours. The monkeys were dispatched after a 6 hour period to allow the anesthetics to take effect. Ketamine was confirmed with liquid chromatography and mass spectrometry. Brain slices of 40 micrometers were prepared using a microtome and a polyclonal antibody that detects cleaved caspase-3, an effector of apoptosis. Out of all sections of the brain- the hippocampus, amygdala, cerebellum, cerebrum, thalamus, and striatum, apoptosis was concentrated mainly in the frontal cortex.

How long does a monkey need be exposed to ketamine for neurodegeneration to occur? Although three hours did not seem to cause neuoroapoptosis, exposure for more than nine hours did, while exposure for twenty-four hours resulted in long term cognitive deficits (Figure 3). Most welcome is the knowledge that the 3 hour period did not cause significant apoptosis, as this is the time frame for an average surgery.

Figure 3. The effect of Ketamine over time. Note the insignificant effect over 3 hours. (Source: Zou et al. 2009)



Prolonged subjection to a high dosage of ketamine in postnatal day 3 perinatal rhesus monkeys for 24 h, with 1, 10, or 20 μM of ketamine, an NMDA antagonist, caused neurodegeneration in the frontal cortical area (Wang et al. 2006). Significant change in mitochondrial metabolism, DNA factionalism, and the release of lactate dehydrogenase were also observed. These phenomena are all characteristic of apoptosis.

Even when ISO or ketamine was administered to 6 day old rhesus monkeys for just 5 hours to maintain a light surgical plane and all physiological parameters were controlled as they would be for a neonate patient, neurodegeneration ensued (Brambrink et al. 2010). Pronounced apoptotic neurodegeneration was especially apparent among the immature oligodendrocyte glial cells participating in myelination as well as neurons. ISO, a widely used anesthetic for maintaining a prolonged stable surgical plane, was shown to cause approximately 4 times more degeneration than ketamine.

Other studies discussed which regions of the brain were affected but Creeley et al. (2013) pinpoints the specific cells - neurons and oligodendrocytes just beginning to myellinate their axons - that were deleted. In this experiment, 120 day old fetuses of rhesus monkeys, (comparable to a late third trimester human fetus) and 6 day old neonates (similar to the 4-6 month old human brain) were subjected to propofol for 5 hours. A pronounced effect was detected in the subcortical and caudal areas in the fetus. The neonates caudal brain regions were affected less and neurocortical regional damage formed distinct laminar patterns.

Sun et al. (2012) showed that even adolescent monkeys, when administered ketamine regularly, developed permanent damage. This was evident from the apoptotic effectors present in the prefrontal cortex. Macaque monkeys demonstrated abnormal behavior in their walk, jump, climb, and general movements. The damage appeared permanent in the 6 month treated monkeys, while 1 month treatment and control monkeys had no such display upon TUNEL testing.

Can These Results be Extrapolated to Humans?

What can be done to determine definitively if this issue applies to the human fetus and neonates? How can such studies be done non-invasively in humans? While studies can be done retrospectively on humans who have already undergone treatment based on the incidence and statistical distribution of apoptotic neurodegeneration, there are many confounding factors such as the dosage amount and duration, acid-base disturbances, hypoxia, starvation, route of administration, developmental period, and the subtype receptors activated. There are many co-factors present in humans absent in animals. To further muddle the matter, pediatric patients typically are administered a combination of anesthetic agents such as benzodiazepines and/or anticholinergics, possibly reducing the amount of ketamine needed to maintain a surgical plane. Due to all these confounding factors, it is not clear if results from these studies can be extrapolated to humans. Perhaps if the epidemiological studies were well-constructed and accounted for various variables by setting up groups of similar circumstances and arrange for a control for each group, it would aid in determining whether the human brain is vulnerable to apoptotic devastation by anesthetics. Or, methods can be developed for noninvasive techniques to determine if in vivo neurodegeneration takes place in the developing brain.

Anesthetics - Similar to Ethanol?

Because of the similarities in the molecular pathways of intoxication and general anesthetic agents-ethanol is both an NMDA antagonist and a GABA agonist-Ikonomidou et al. (2000) suspected that that studies on the affects of ethanol on fetuses would be applicable to general anesthetics. Seven day old rats were given 2.5 g/Kg of 20% ethanol in saline, and compared to the control only given saline. TUNEL and silver staining showed only modest physiological cell death in the control while the subject group showed condensed pervasive sections of degeneration. Creeley et al. (2013) presented the same affect on neurons and oligondrocytes in the fetal macaque brain.

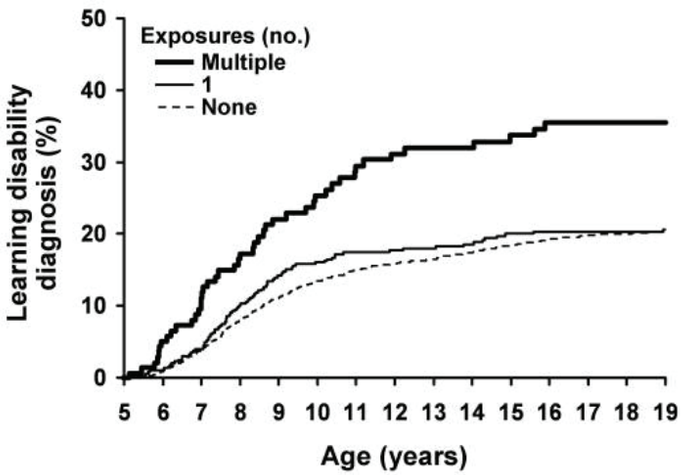
Ethanol has a debilitating effect at the molecular level. Above a 12 mol % (30.5 v/v %) threshold, desorption results from breaks in the lipid-water interface of the bimembrane, some of the lipid fragments amalgamate within the cell (Gurtovenko, Anwar, 2009). At the same time, components of the inner and outer leaflets of the lipid bi-

layer substitute one another's position, permanently upsetting the membrane structure. And this suggests that anesthetics may cause neurological damage just as ethanol causes developmental neurological damage to fetuses (fetal alcohol syndrome). Neurobehavioral deficiencies perceived to persist into adulthood include psychosis, hyperactivity, learning disabilities, and depression.

Anesthetics' Affects Persist into Maturity

Wilder et al. (2009), in a population based cohort study demonstrated retrospectively how children under 4 years of age subjected to an incidence of anesthesia were unlikely to be affected. However, 2 or more periods of general anesthetic application revealed a cognitive deficit as revealed by inferior scores on IQ and standardized achievement tests. Learning disabilities as characterized by poor math, reading and writing skills were apparent and they lagged continually further behind their peers as they advanced through school. By 19 years of age, 35% had a learning disability, representing a 15% rise against the general population (Figure 4). Because of the controversy regarding how to relate the vulnerable developmental stage of animals to their corresponding human stage, the experiment was repeated for children subjected to anesthesia for two or more instances. Similar results were generated. It is not clear if the anesthetics themselves or other factors were the cause.

Figure 4: A population based cohort study demonstrating the long-term affect of multiple anesthetic incidence. (Source: Wilder et al. 2009)



But even if anesthetics are shown to definitively cause neurodegeneration, clinical medicine is not much further advanced. How can children who need surgery safely receive treatment? Anesthetic application to children during surgery cannot simply be abandoned; its use prevents neurotoxicity from developing in the brain as well as the harmful effects of stress, anxiety and chronic pain disorders. Exposure to prolonged or repeated painful stimuli lowers the threshold of pain processing, and alters development of the brain, cognitive functioning, and behavior. Moreover, surgical patients who were not administered anesthetics exhibited the very same pervasive neurodegeneration; nothing was gained by avoiding it. Anesthetic treatment resulted in attenuating the pain and protecting the developing brain. (Anand et al. 2004). Anesthetics also protect the brain from damage by preventing hypoxic and ischemic incidents, allowing the necessary oxygen-carrying blood flow to continue to nurture the brain.

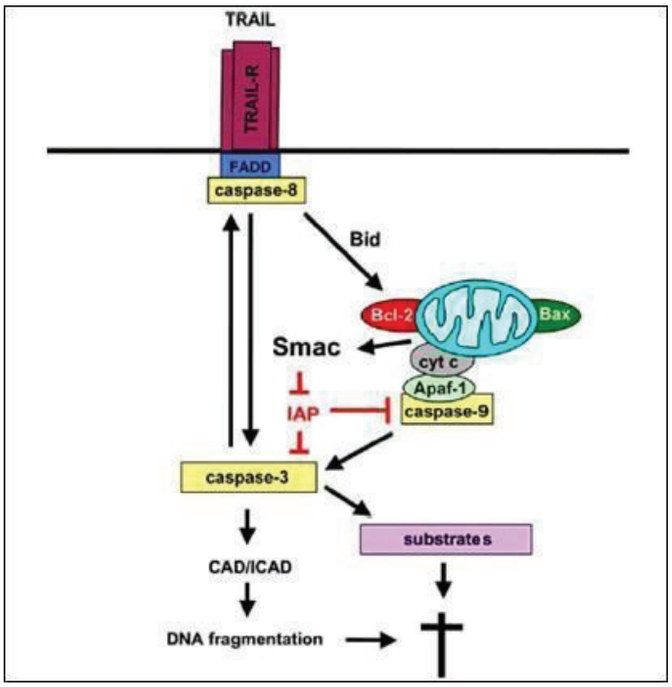
Apoptotic Mechanism

A possible mechanism for GA induced neuroapoptosis is via the mitochondrial pathway (Figure 5). Molecular stimulation prompts association of Bcl-2 with mitochondrial Bcl-2, leading to the release of cytochrome c. Cytochrome c in turn binds Apaf-1, effecting a change in its conformation. Apaf-1 association with procrease-9 allosterically activates it, in turn stimulating procaspase-3 and procaspase-7. Perhaps a cascade induced by GAs cause the mitochondria to release more cytochrome c, leading to activation of more caspases. Sanchez et al. (2011) demonstrated support for the theory of GAs affecting mitochondria when he discovered that long term use of GAs compromised its structure and function as well as lingering dysfunction in inhibitory synapses in neural signaling.

Figure 5 : ***(Printed on next page)*** A schematic summary of the apoptotic pathway. A stimulant triggers association of Bcl-2 with mitochondrial Bcl-2. The ensuing series of reactions activates caspases. (Source: Kalantri 2010)

Alternatively, Kuwana and Newmeyer (2003) suggests that GAs spur a release of a large concentration of Cl (2+) from the ER lumen which can in turn lead to an increase in permeability of mitochondria. The mitochondria

swells and the external membrane may burst. The cytochrome c will then disperse throughout the cell and the apoptotic cascade will begin. Bcl-2 can bring about apoptosis by controlling the release of Ca(2+), thereby regulating whether mitochondria will burst and discharge cytochrome c.



Possible Protection Against Apoptosis

A viable alternative to GAs must be developed or the underlying mechanisms better studied and understood so as to address the specific toxic pathway. When ISO and N2O were administered together, differential gene expression was observed in 45 pathways related to the brain, highlighting the difficulties researchers have in trying to pinpoint the exact pathway by which the apoptotic damage is caused and developing a drug to neutralize its affects (Wang et al. 2012).

Once the exact apoptotic pathway is determined, drugs can be developed to act in concert with the anesthetic to prevent the apoptotic cascade while maintaining the benefits of a sedative effect. Progress in labs has already been made in identifying a possible "antidote," L-carnitine. L- carnitine is an amino protein that supports oxidation of fatty acids and is essential in skeletal muscle metabolism. L-carnitine, when administered concurrent with GAs, significantly minimized the damage even for long periods under high dosages (Zou et al. 2008).

Di Marzio et al. (1997) reports that sphingolipids play a key role in apoptosis; Apoptosis may be arrested by inhibition of a sphingolipid activation cascade. L-carnitine in AIDS patients blocked sphingomyelinase, thus preventing sphingomyelin breakdown into phosphocholine and ceramide, an intracellular apoptotic effecting molecule. Ceramide exists in the mitochondrial pathway. When high levels are present, the respiratory chain is prevented and cytochrome c is released (Ghafourifar et al. 1999).

Mechanisms in neuropathic diseases also lead to initiation of the apoptotic cascade with cytochrome c released into the cytoplasm (Di Cesare Mannell et al. 2007). Treatment of rats with acetyl-L-carnitine reduced the amount of cytochrome c present in the cytosol and suppressed the apoptotic pathway. Unfortunately, L-carnitine, when tested, did not provide successful protection. Acetyl-L-carnitine also reduced the number of chromatin undergoing the irreversible condensation of chromatin characteristic of apoptotic cells (karyopyknosis).

While the exact apoptotic effecting pathway of GAs is uncertain, it is probable that it is related to both sphingomyelinase and the release of cytochrome c into the cytoplasm. Consequently, it is likely that both L-carnitine and acetyl-L-carnitine would aid in preventing apoptosis. It is premature to declare whether one would be more successful than the other in preventing neuroapoptosis while the basic pathways GAs trigger remain a mystery. Perhaps L-carnitine and acetyl-L-carnitine's shielding capacity is applicable to only specific mechanisms initiated by a specific anesthetic or a specific form of the anesthetic's administration.

The X-chromosome linked inhibitor of apoptosis protein (XIAP) acts as an important factor in the apoptotic pathway, inhibiting apoptosis by blocking the activity of caspase-3. When the GA sevuflorane was delivered to lung carcinoma cells for up to 6 hours, apoptosis resulted, blocking cell proliferation (Liang et al. 2011). Researchers observed down-regulation of XIAP expression while caspa-

se-3 levels rose. But administration of Acetyl-L-carnitine was shown to parallel a reduction of karyopyknosis, suggesting the induction of XIAP to block this occurrence (Di Cesare Mannell et al. 2007).

Conclusion

While recent studies of rodents and primates suggest that GAs may cause apoptosis in the developing brain by its dual mechanism as an NMDA antagonist and GABAa agonist, whether this applies to the developing human brain remains inconclusive. Better constructed epidemiological studies would probably be the best route to determine unequivocally if the developing human brain is vulnerable. Should it prove to be vulnerable, protection in the form of L-carnitine and Acetyl-L-carnitine may be used to neutralize the malevolent affects in future pediatric anesthesiology.

References

Anand KJ, Soriano SG. 2004. Anesthetic agents and the immature brain: are these toxic or therapeutic? Anesthesiology101(2):527-30.

Brambrink AM, Evers AS, Avidan MS, Farber NB, Smith DJ, Zhang X, Dissen GA, Creeley CE, Olney JW. 2010. Isoflurane-induced neuroapoptosis in the neonatal rhesus macaque brain.Anesthesiology112 (4):834-41.

Broad KD, Curley JP, Keverne EB. 2009. Increased apoptosis during neonatal brain development underlies the adult behavioral deficits seen in mice lacking a functional paternally expressed gene 3 (Peg3). Developmental Neurobiology 69(5):314-25.

Colquhoun D, Sivilotti LG (2004). Function and structure in glycine receptors and some of their relatives. Trends in Neurosciences 27(6): 337–44.

Creeley C, Dikranian K, Dissen G, Martin L, Olney J, Brambrink A. 2013. Propofol-induced apoptosis of neurones and oligodendrocytes in fetal and neonatal rhesus macaque brain.British Journal of Anaesthesia 110 (S1): i29–i38

Creeley CE, Dikranian KT, Johnson SA, Farber NB, Olney JW. 2013. Alcohol-induced apoptosis of oligodendrocytes in the fetal macaque brain. Acta Neuropathologica Communications 12;1(1):23.

Di Cesare Mannelli L, Ghelardini C, Calvani M, Nicolai R, Mosconi L, Vivoli E, Pacini A, Bartolini A. 2007 Protective effect of acetyl-L-carnitine on the apoptotic pathway of peripheral neuropathy. European Journal of Neuroscience 26(4):820-7.

Dikranian K, Ishimaru MJ, Tenkova T, Labruyere J, Qin YQ, Ikonomidou C, Olney JW. 2001. Apoptosis in the in vivo mammalian forebrain. Neurobiology of Disease (8)3:359-379

Di Marzio L, Alesse E, Roncaioli P, Muzi P, Moretti S, Marcellini S, Amicosante G, De Simone C, Cifone MG. 1997. Influence of L-carnitine on CD95 cross-lining-induced apoptosis and ceramide generation in human cell lines: correlation with its effects on purified acidic and neutral sphingomyelinases in vitro. Proceedings of the Association of American Physicians 109(2):154-63.

Ghafourifar P, Klein SD, Schucht O, Schenk U, Pruschy M, Rocha S, Richter C. 1999. Ceramide induces cytochrome c release from isolated mitochondria. Importance of mitochondrial redox state. The Journal of Biological Chemistry 274(10):6080-4.

Gurtovenko AA, Anwar J. 2009. Interaction of ethanol with biological membranes: the formation of non-bilayer structures within the membrane interior and their significance. Journal of Physical Chemistry B 113 (7):1983-92.

Huang L, Liu Y, Jin W, Ji X, Dong Z. 2012. Ketamine potentiates hippocampal neurodegeneration and persistent learning and memory impairment through the PKCγ-ERK signaling pathway in the developing brain. Brain Research 2;1476:164-71.

Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vöckler J, Dikranian K, Tenkova TI, Stefovska V, Turski L, Olney JW. 1999. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283(5398):70-4.

Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Hörster F, Tenkova T, Dikranian K, Olney JW. 2000. Ethanol-Induced Apoptotic Neurodegeneration and Fetal Alcohol Syndrome. Science 287(5455):1056-1060

Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF. 2003. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. The Journal of Neuroscience 23(3):876-82.

Kalantri P. 2010. Apoptosis: Concept, Mechanisms and Clinical Implications. Pharmaceutical Information, Articles and Blogs. http://www.pharmainfo.net/reviews/apoptosis-concept-mechanisms-and-clinical-implications

Kuwana T, Newmeyer DD. 2003. Bcl-2-family proteins and the role of mitochondria in apoptosis. Current Opinion in Cell Biology 15(6):691-9.

Liang H, Gu MN, Yang CX, Wang HB, Wen XJ, Zhou QL. 2011. Sevoflurane inhibits proliferation, induces apoptosis, and blocks cell cycle progression of lung carcinoma cells. Asian Pacific Journal of Cancer Prevention 12(12):3415-20.

Olney J, Labruyere J, Wang G, Wozniak D, Price M, Sesma M. 1991. NMDA antagonist neurotoxicity: mechanism and prevention. Science 254 (5037):1515–8.

Sanchez V, Feinstein SD, Lunardi N, Joksovic PM, Boscolo A, Todorovic SM, Jevtovic-Todorovic V. 2011. General Anesthesia Causes Long-term Impairment of Mitochondrial Morphogenesis and Synaptic Transmission in Developing Rat Brain. Anesthesiology 115(5):992-1002.

Sun L, Li Q, Li Q, Zhang Y, Liu D, Jiang H, Pan F, Yew DT. 2012. Chronic

ketamine exposure induces permanent impairment of brain functions in adolescent cynomolgus monkeys. .Addiction Biology [serial online] Available from: MEDLINE, Ipswich, MA. Accessed January 8, 2014.

Wang C, Sadovova N, Hotchkiss C, Fu X, Scallet AC, Patterson TA, Hanig J, Paule MG, Slikker W Jr. 2006. Blockade of N-Methyl-D-Aspartate Receptors by Ketamine Produces Loss of Postnatal Day 3 Monkey Frontal Cortical Neurons in Culture. Toxological Sciences 91(1):192–201

Wang C, Slikker W, Paule MG, Hanig JP, Sadovova N, Li QZ, Patterson TA, Shi L, Rainosek SW, Zhang J, Guo L, Liu, F. 2012. Inhalation Anesthesia-Induced Neuronal Damage and Gene Expression Changes in Developing Rat Brain. Systems Pharmacology 1 (-1)

Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL, Warner DO. 2009. Early exposure to anesthesia and learning disabilities in a population-based birth cohort. Anesthesiology 110(4):796-804.

Zou X, Sadovova N, Patterson TA, Divine RL, Hotchkiss CE, Ali SF, Hanig JP, Paule MG, Slikker W Jr, Wang C. 2008. The effects of L-carnitine on the combination of, inhalation anesthetic-induced developmental, neuronal apoptosis in the rat frontal cortex. Neuroscience 151(4):1053-65.

Zou X, Patterson TA, Divine RL, Sadovova N, Zhang X, Hanig JP, Paule MG, Slikker W Jr, Wang C. 2009. Prolonged exposure to ketamine increases neurodegeneration in the developing monkey brain. International Journal of Developmental Neuroscience 27(7):727-31.

Current Research of Extracorporeal Photopheresis and Future Applications

Chaim Lederer

Abstract

Photopheresis, also known as Extracorporeal Photopheresis (ECP) is making inroads in treatment of previously untreatable diseases. As the medical world has delved deeper into, Although the mechanisms of photopheresis are largely unknown, increasingly detailed studies have proven its efficacy. The lack of side effects has made photopheresis an ideal option for patients. The treatment is also versatile enough that it can be used as a mono-therapy or as a supplement to other traditional therapies. The use of photopheresis has been proven successful in the treatment of cutaneous T-cell lymphoma (CTCL) and graft-versus-host disease (GvHD), and is currently being administered for immune system disorders, bone marrow, or stem cell implantation, liver, heart, or lung transplants (where there is fear of rejection) and any

Introduction

Research in the field of Extracorporeal Photopheresis (ECP) commenced in 1982. Richard Edelson, MD, a Professor at Columbia University, had been experimenting with a therapy he invented as an alternative treatment for untreatable cancers. After many years of research, he achieved an astonishing clinical success in the treatment of two patients with extensive cutaneous T-cell lymphoma (CTCL). Previously, in the 1970's, Dr. Edelson noted that removing circulating cancer cells from patients with Sezary Syndrome (SS), a form of CTCL, by way of repetitive Leukapheresis, resulted in a temporary improvement in their skin disease. Leukapheresis is a procedure that removes abnormal white blood cells from the blood. It is a form of apheresis, which is the process of removing one particular constant from the blood and returning the rest to circulation. In the 1980's, Dr. Edelson collaborated with Therakos, a Johnson and Johnson company, to design a device to accomplish Leukapheresis while simultaneously exposing a small amount of the patients' white blood cells to Methoxsalen, a light sensitizing drug, and ultra violet (UVa) light to activate the medication.

Immunity: Background Information

The immune system consists of differentiated hematopoietic cells that are created from bone marrow hematopoietic stem cells (HSC). HSCs separate into various types of blood components such as erythroid cells, platelets, myeloid cells (such as neutrophils, basophils, eosinophils, monocytes), mast cells, natural killer cells, dendritic cells, natural killer T cells, thymus derived cells (T cells), and B cells depending on the environment and stimuli (Lensch, 2012). These immune cells arrange host protection by responding to foreign antigens after the cells mature. However, a proper maintenance of cellular and molecular balance is crucial for these immune responses to proceed. Once this delicate balance is askew, normal immunity is disrupted and diseases with increased susceptibility to infection arises.

Within the immune system, the Dendritic cells (DC) are antigen-presenting cells. The DCs, also known as accessory cells, play an integral role in both inborn immunity and adaptive immunity. The cells link inborn and adaptive immunity by activating B and T-cells through the presentation of antigens on their cell surfaces (Novak, et al, 2010). When B-cells are triggered by DCs, an immune response consisting of secretion of antigen specific antibodies occurs. Simultaneously, DCs activate CD4+ helper and CD8+ T-cells that define and stimulate T-cell responses specific to each antigen (Banchereau, Steinman, 1998). Depending on the nature of the antigen, different CD4+ T-helper (Th) responses such as Th-1, Th-2, Th-17, and T-regulation can occur. Th-cells also secrete cytokines that promote cell interactions with additional cells, which

Chiam Lederer graduated in May 2014 with a B.S. in Biology.

are then dispatched to the infected site. For example, the Th-1 response is mediated by CD4+ helper T-cells and CD8+ T-5 cells that secrete IFN-γ, IL-2, and IL-12 to fight against viruses. The CD4+ helper cells then "help" to invoke a cytotoxic attack against the virus, mainly through CD8+ cytotoxic T-cells (Steinman, Hemmi, 2006) (Kadowaki, 2007) .

Located in the immune system, T-regulatory cells (T-regs) regulate a wide variety of immune cells such as CD4+, CD8+, B-cells, natural killer T-cells, and antigen presenting cells (APC) both in vitro (artificial environment) and in vivo (natural environment). These cells make up 5-10% of the total agranulocytic cells found in the body's blood (Sakaguchi, 2008). They have the distinct ability to regulate reactions in the immune system, preventing immune diseases such as allergies and autoimmunity. T-regs have often been recognized as the cause for failure of cancer vaccines. On the other hand, T-regs also have potential use for patients with irregular immune systems. In autoimmune patients, an increase in T-regs may be an ideal method of treatment since their immune system is compromised and these cells could help regulate it. Similarly, the removal of T-regulatory cells may assist in increasing a cytotoxic response to tumor antigens.

Cutaneous T-cell Lymphoma (CTCL)

Cutaneous T-cell lymphoma (CTCL) is a heterogeneous group of non-Hodgkin's peripheral T-cell lymphomas that mainly affect the skin. Two of the most common forms of CTCL are Mycosis Fungoides (MF) and Sézary Sydrome (SS). They are distinguished by malfunctioning CD4+ T-cells and impaired immunity. Together they encompass approximately 70% of all CTCL cases. About 1,500 new cases of MF/SS are reported yearly, and there are currently 16,000-20,000 individuals living in the United States with MF/SS (Criscione, Weinstock, 2007). CTCLs are incurable and thought to arise from uncontrolled reproduction and accumulation of atypical mature helper, memory clonal T-lymphocytes (Vonderheid, Bernengo, 2003).

Sézary Syndrome (SS)

Discovered and introduced by Albert Sézary in 1938, Sézary Syndrome (SS) is a form of leukemia consisting of cells with cerebriform shaped nuclei, itching, exfoliative dermatitis, and adenopathy (inflammation of the lymph nodes). Patients with SS do not have a good prognosis; there is an average survival rate of only 3 years (Scarisbrick, et al, 2001). SS patients have been found to have a high rate of Staph colonization. Secondary and hospital acquired infections, most often from Staphylococcus aureus (Staph) sepsis due to an impaired immune system, breaks in the skin and use of catheters are particularly fatal in SS patients. The cause of SS is unknown and the diagnosis is very difficult due to its similarities to other skin ailments. It was originally believed that SS was derived from MF. However, MF and SS can be differentiated since MF cells are effector memory T-cells and SS cells are central memory T-cells. With Sèzary cells, they are also found in both the blood and skin with CD4+ T cells lacking the expression of CD-26 or CD-7. (Campbell, et al, 2010)

Graft-versus-Host Disease (GvHD)

Graft-versus-host disease (GvHD) is an immune system disorder that limits the use of stem cell and bone marrow transplant therapies. The donor immune cells used in the transplant recognize host cells as being foreign, based on human leukocyte antigen (HLA) mismatch. The donor cells mount an immunological attack, resulting in damage to organs including the skin, gut, liver, eyes, or lungs.

There are two types of GVHD, acute GVHD and chronic GVHD. Symptoms occurring within 100 days post-transplant are described as acute GVHD and any occurrence of symptoms beyond 100 days are characterized as chronic GVHD. An analysis of acute GVHD showed that amongst the patients affected 81% had skin involvement, 54% had gastrointestinal involvement, and 50% had liver involvement (Martin, et al, 1990). Candidates for transplant therapies have a plethora of ailments which can lead to incrased numbers of antigen presenting cells resulting from damages produced by a multitude of factors such as underlying disease and its treatmentvia chemotherapy, radiation, and infections. In order to prepare the host's body for transplantation, the patient has to undergo a procedure called total body irradiation. Total body irradiation is used to suppress the immune system, thus preventing the patient's immune system from destroying implanted cells that are foreign to the body. During this procedure,

inflammatory cytokines such as IL-1 and tumor necrosis factors (TNF) are secreted from the patient's tissues. Cytokines, which can potentially cause damage to the gastrointestinal tract via endothelial apoptosis, may increase the severity of GvHD if the microbial products such as bacteria attack the blood system. Activation of the immune system or tolerance by the body is controlled by APC's that are activated by these secretions (Roncarolo, et al, 2001).

In the cells there is a major histocompatibililty complex (MHC). The MHC is a set of molecules on the surface of the cells which facilitates the interactions of leukocytes (WBCs). CD4+ and CD8+ T-cells, and cytokines are the main disease facilitators. In every individual, the MHC takes on its own unique genetic coding. One of the primary functions of the MHC is the recognition of foreign antigens invading the immune system. Since the MHC is highly polymorphic, it has the ability to recognize countless types of antigens. It then binds peptide fragments taken from the pathogens and displays them on the cell surface for recognition by the T-cells. The MHC presents the antigens to the T-cell receptor (TCR), and the T-cells are activated or deactivated via an interaction between the TCR and the MHC. In the case of GvHD, everything is awry because the grafted T-cells fail to recognize the MHC proteins on host cells. Due to the MHC mismatch, the graft T-cells attack the host cells thinking they are foreign even though they are non-antigenic. Thus, the MHC mismatch contributes to the disrupted normal immunity leading to GVHD.

Extracorporeal Photopheresis (ECP)

Extracorporeal Photopheresis is a pioneering treatment with minimal side effects, which improves the quality of life, and has increased the survival rates for persons with select incurable diseases. Based on clinical trials, the procedure was approved by the FDA for treatment of erythrodermic Leukemic-CTCL (L-CTCL) (Edelson, et al, 1987). At the time, response rates between 54% and 66% had been reported in L-CTCL patients with about 10% complete responders (Jiang, et al, 1999) (Crovetti, et al, 2000). This opened up a new world of possibilities in the treatment of cancers. Most cancer treatments come with a large amount of side effects along with the possibility of a cure. This treatment had the potential of accomplishing the latter, with almost no risk of side effects.

In the ECP procedure that was created by the Therakos company, blood is drawn from the arm or a central catheter, and a very small amount (5x109) of the patients white blood cells (3-5%) are separated and collected for irradiation or apheresis. The blood is centrifuged to separate the leukocyte enriched blood portion from the red blood cells in plasma. The rest of the blood is automatically returned to the bloodstream. The collected white blood cells are then treated with a medicine called Methoxsalen/Uvadex. The drug is activated when it is exposed to ultra-violet (UV) light. The medicated white blood cells are exposed to UVa light, with a strength of 1.5 J/cm^2. The function of the medication is to covalently bind and cross-link DNA, leading to apoptosis of treated cells. These cells are now known as Methoxsalen treated buffy coat cells. The buffy coat cells are then re-infused back into the patient . so that they can cause an immune response. In the body, the spleen and the liver now pick up the Methoxsalen treated white blood cells. The day after ECP treatment, the treated cells undergo apoptosis. Even though the mechanism is not understood, these cells are phagocytized by antigen presenting cells (APC) that are causing disorder in the body. It is theorized that the phagocytosis causes a regulation of immune responses by altering the APC function. The APC's now become immunological tolerant inducing or tolerogenic to the immune system. The phagocytosis causes a decrease in the secretion of pro-inflammatory cytokines and effector T-cells. Additionally, it causes an increase in anti-inflammatory cytokines such as TGF-beta and IL-10. This process and reaction causes a boost in the activation and stimulation of T-regulatory cells which in turn leads to a healthy regeneration and rebalancing of the T-cells and other immune cells in the body. Additionally, blood that has been irradiated halts lymphocytes from causing harm to patients who have compromised immune systems. ECP does not cause any known harm to the body, and the blood does not become radioactive or toxic.

Since there was some understanding as to how ECP was able to achieve these positive results, it brought about the possibility of expanding its usage for other severe diseases. These diseases include: multiple sclerosis

(MS), systemic sclerosis, Crohn's disease, rheumatoid arthritis, type-1-diabetes, and autoimmune diseases such as lupus. Trials have also begun in cases of whole transplanted organ rejection. In CTCL patients, ECP has shown the successful destruction of malignant T-cells via apoptosis and the differentiation of monocytes into effective antigen presenting dendritic cells. These two outcomes concurrently lead to a decrease of the tumor load and initiation of antitumoral immune response. (Garban, et al, 2012).

Studies

Studies have shown the efficacy of ECP treatment in patients with early-stage mycosis fungoides (MF). The studies were conducted with ECP as a mono-therapy and as a supplement to the traditional cancer therapies. The patients underwent treatment of ECP for two days every four weeks over a time span of six months. Those that had a partial response continued the treatment of ECP for the full six months and those without an initial response added oral bexarotene and/or interferon α. These studies produced astonishingly positive results.

Participants included nineteen patients with early-stage MF (7 men, 12 women; 16 white, 3 African American) with an average age of 63.5 years (range, 46-85 years). 42% of the participants responded to the ECP by itself with an average amount of 12 ECP sessions over 12 months (8/19; including 7 partial response, 1 complete response). Seven of the patients with stabilized MF at 3 months received additional bexarotene treatment (3/5; 1 complete response) or were administered bexarotene plus interferon α (1/2), and 57% (4/7) responded to the updated treatment. Side effects were limited to those expected with standard chemotherapy treatment. The effects included nausea, vomiting, and diarrhea.

The studies proved that ECP is effective for patients with early-stage MF alone or in combination with drugs and included improved quality of life. It has shown a response of as high as 42% with ECP treatment alone (Talpur, et al, 2011)

Another study was on the efficacy of ECP on steroid-dependent acute GVHD. A complete resolution was attained in 82% of patients with skin involvement, 61% with liver involvement, and 61% with gut involvement. These patients received ECP on 2 consecutive days every one to two weeks, until there was a noticeable response and continued every two to four weeks until it reached a maximum response. The next stage conducted was a study of the relationship of mortality and survival. The 4-year survival rate for those in whom acute GVHD had a complete response was 59% (Kumar, 2011).

When ECP was first introduced into the medical world, it was only proven to work on CTCL and GvHD. Due to the success, the research community continued looking to see which other ailments the treatment could be applied to. This has become a worldwide concerted effort in attempt to make headway in previously incurable diseases and clinical trials have begun on the effect of ECP on many different unrelated or atypical diseases..

Another case where the curative effects of ECP can be observed is through studies involving patients afflicted with Crohn's disease, who are incapable of responding to alternative treatments such as immune-suppressants and/or anti-TNF therapies. After twelve sequential weeks of treatment, the success of the individual's results were evaluated based on the patient's decrease in their Crohn's Disease Activity Index (CDAI) of less than or equal to 100 points, or the remission which can be characterized by an index of less than 150 points. From the 28 patients that participated in the study, half successfully responded to the treatment with 13 subjects reacting at week 6, and 7 of them obtaining remission after the 12-week interval. From the 12 patients who chose to advance with a 12-week extension study, 9 continued to effectively respond to the treatment. Through their study, clinicians were capable in discerning ECP as a productive means in which patients with Crohn's disease were able to attain desirable results (Abreu et. al.. 2009).

Conclusion

Beginning with the initial successes in the 1980's, extracorporeal photophersis has been a medical breakthrough in a field where there rarely is a cure found with limited or no side effects. The difficulty of understanding how it achieves its results has not been a deterrent to further expansion of its usage. Today, ECP is at the forefront of clinical trials bringing hope to many with lim-

ited options or where the cure is sometimes just as deadly as the disease, as is the case in chemotherapy and radiation treatments. With its versatility, ECP becomes an additional option even for patients already using other therapies. Trials have produced extremely promising results for diseases with little or no hope. To date, ECP has only been approved for treatment in CTCL and GvHD, but current trials are expanding its usage. ECP has promising potential to be the cure of the future pertaining to immune system related diseases.

References

Abreu MT, Tirpitz Cv, Hardi R, Kaatz M, Assche G v, et. al., (2009). Extracorporeal photopheresis for the treatment of refractory Crohn's disease: Results of an open-label pilot study. Inflammatory Bowel Diseases, 15:6, 829-836.

Banchereau J, Steinman RM, (1998). Dendritic cells and the control of immunity.

Nature, 392, 245-252.

Campbell JJ, Clark RA, Watanabe R, Kupper TS, (2010). Sezary syndrome and mycosis fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical behaviors. Blood, 116, 767-771.

Criscione VD, Weinstock MA, (2007). Incidence of cutaneous T-cell lymphoma in the United States. Archives of Dermatology, 143, 854-859

Crovetti G, Carabelli A, Berti E, Guizzardi M, Fossati S, De Filippo C, Bertani E, (2000). Photopheresis in cutaneous T-cell lymphoma: five-year experience. Int J Artif Organs, 23, 55-62.

Edelson R, Berger C, Gasparro F, Jegasothy B, Heald P, Wintroub B, Vonderheid E, Knobler R, Wolff K, Plewig G, (1987). Treatment of cutaneous T-cell lymphoma by extracorporeal photochemotherapy. Preliminary results. New England Journal of Medicine, 316, 297-303.

Garban F, Carras S, Drillat P, Jacob MC, Fabre B, Callanan M, Courby S, Makowski C, Cahn JY, Gressin R, (2012). Extracorporeal photopheresis as a curative treatment strategy in non epidermotropic T-cell lymphoma and large granular lymphocyte leukemia. Retrieved from the web 12/24/2013. Ann Oncol. http://annonc.oxfordjournals.org/content/early/2012/03/15/annonc.mds014.full

Greinix HT, (2009). The Role of Extracorporeal Photopheresis in Graft-vs-Host Disease. Asia-Pacific Journal of Oncology and Hematology, 3, 1-5.

Jiang SB, Dietz SB, Kim M, Lim HW, (1999). Extracorporeal photochemotherapy for cutaneous T-cell lymphoma: a 9.7-year experience. Photodermatol Photoimmunol Photomed, 15, 161-165.

Kadowaki N, (2007). Dendritic cells: a conductor of T cell differentiation. Allergology International, 56, 193-199.

Kumar A, (2011). Using Extracorporeal Photopheresis in the Treatment of Graft-Versus-Host Disease. Retrieved from the web 1/19/14. http://www.onclive.com/publications/obtn/2011/june-2011/using-extracorporeal-photopheresis-in-the-treatment-of-graft-versus-host-disease/2

Lensch, M Wiliiam, (2012). An evolving model of hematopoietic stem cell functional identity. Stem Cell Reviews and Reports, 8, 551-560.

Martin PJ, Schoch G, Fisher L, Byers V, Anasetti C, Appelbaum FR, Beatty PG, Doney K, McDonald GB, Sanders JE, (1990). A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. Blood, 76, 1464-1472.

Ni X, Zhang C, Talpur R, Duvic M. Resistance to activation-induced cell death and bystander cytotoxicity via the Fas/Fas ligand pathway are implicated in the pathogenesis of cutaneous T cell lymphomas. J Invest Dermatol 2005;124:741-50.

Novak N, Koch S, Allam JP, Bieber T., (2010). Dendritic cells: bridging innate and adaptive immunity in atopic dermatitis. The Journal of Allergy and Clinical Immunology, 125, 50-59.

Roncarolo MG, Levings MK, Traversari C, (2001). Differentiation of T regulatory cells by immature dendritic cells. The Journal of Experimental Medicine, 193, F5-9.

Sakaguchi S, Yamaguchi T, Nomura T, Ono M, (2008). Regulatory T cells and immune tolerance. Cell, 133, 775-787.

Scarisbrick JJ, Whittaker S, Evans AV, Fraser-Andrews EA, Child FJ, Dean A, Russell-Jones R, (2001). Prognostic significance of tumor burden in the blood of patients with erythrodermic primary cutaneous T-cell lymphoma. Blood., 97, 624-630.

Steinman RM, Hemmi H, (2006). Dendritic cells: translating innate to adaptive immunity. Current Topics in Microbiology and Immunology, 311, 17-58.

Talpur R, Demierre MF, Geskin L, Baron E, Pugliese S, Eubank K, Zic JA, Miller DR, Tharp M, Bohjanen K, Duvic M, (2011). Multicenter photopheresis intervention trial in early-stage mycosis fungoides. Clin Lymphoma Myeloma Leuk, 11(2), 219-27.

Vonderheid EC, Bernengo MG, (2003). The Sezary syndrome: hematologic criteria. Hematology/Oncology Clinics of North America, 17, 1367-1389.

Biomarkers in NSCLC Epidermal Growth Factor Receptor Mutations

Suzane Freidman

Abstract

Lung Cancer is the most common global cause of cancer related deaths in men and women (Markus, Alain, 2013). As standard radiation and chemotherapy have proved ineffective, novel target therapies are in the midst of development. This review will analyze the success of the inhibitor drugs targeting the Epidermal Growth Factor Receptor (EGFR) mutation, commonly found amongst Lung Cancer patients. Numerous studies and reviews are utilized to determine the cause of the 10% success rate currently exhibited for these drugs. The L858R and E746-A750 point mutations and deletions respectively, were found prevalent in responsive patients as well as clinical-pathological features such as female gender, Asian descent, non-smoking history, and Adenocarcinoma. Adenocarcinoma was found almost exclusively in responsive patients and non-smoking history is proposed to have an independent correlation to EGFR mutations (Kosaka, et.al. 2004). These prevailing features can be used as biomarkers to predict the responsiveness of a patient population, leading to efficient and successful distrigution of the EGFR inhibition drug to Lung Cancer patients.

Introduction

The Epidermal Growth Factor Receptor (EGFR), also known as ErbB1 or Her1 in humans, was first discovered by Stanley Cohen in the 1970's (Yarden, Sliwkowski, 2001). Since then, our understanding of the protein and its critical role in numerous forms of cancer has advanced in enormous strides. Advances in this area have proven to be fruitful, and much hope is put into future development for cancer cures. EGFR is now understood to be one of four members of the ErbB interactive family of Tyrosine Kinase (TK) Proteins. EGFR, as well as ErbB 2, 3, and 4 (Her 2, 3, 4) are phosphorylation inducing proteins on the Tyrosine portion of the receptors (Yarden, Sliwkowski, 2001). EGFR carries out numerous signal transduction pathways which result in cellular proliferation, metastasis, and apoptotic aversion, all clearly oncogenic activities. Consequently, EGFR commonly causes numerous types of cancers whenever-expressed in epithelial tissue throughout the body (Heist, Christiani, 2009). In Non Small Cell Lung Cancer (NSCLC) specifically, EGFR over expression is the most common cause, leading to much research and analysis of this protein, and its mutations in regard to lung cancer. Numerous drug therapies have been developed to target the specific mutations that were found as causes of NSCLC. Understanding of the structure and mechanism of this protein is crucial to the development of biomarkers to target specific populations containing the mutation to allow for further development of target therapy for cancer.

Methods

Various databases were utilized to gather papers on research studies and reviews on the subject matter to analyze it and determine possible biomarker methods and their efficiency. Articles were found in Google Scholar, Touro Library Database, and Rutgers University Database. These papers were analyzed and used to come to the conclusions explained in the discussion of this paper.

Results:

EGFR

EGFR is a tri-domain membranous receptor protein consisting of an extracellular ligand-binding domain mainly comprised of cysteine, a hydrophobic transmembranous area, and an intracellular Tyrosine Kinase Domain (Yarden, 2001). The Tyrosine Kinase domain is made up of a juxtamembrane region, an area for TK activity, and a C - terminal domain (Markus, Alain, 2013). A number of ligands contain an affinity to EGFR including the Transforming Growth Factor-α (TGF-α), Epidermal Growth Factor (EGF) and Amphiregulin (Figure 1) (Huang, Harari,1999). All EGFR ligands contain around 50 amino acids including six

Suzzane Freidman graduated in May 2014 with a B.S.Honors in Biology.

cysteine residues in β-sheet formation and are highly regulated by numerous signal transduction pathways throughout the cell (Yarden, Sliwkowski, 2001). TGF-α is the most common ligand (Vealel, et. al. 1987). Following EGFR binding, dimerization of EGFR occurs to form a homodimer with an identical receptor or to form a heterodimer with a different protein of the ErbB family (Isobe et. al. 2005). Heterodimers are stronger than homodimers with a smaller rate of ligand dissociation, increased period of tie at the cell surface, and increased mitogenic capabilities. Dimerization leads to autophosphorylation of Tyrosine residues of the cytoplasmic region of the protein itself. The specific regions activated on the Kinase domain, and consequently the specific pathways activated, are dependent on the ligand's identity as well as EGFR's dimerization partner. EGFR's activation allows it to bind to cytoplasmic messenger proteins which initiate various signal transduction cascades resulting in cell proliferation, metastasis, and apoptosis aversion (Yarden, Sliwkowski, 2001).

Raf-MEK-ERK Mitogen-Activated Protein Kinase Cascade

The most common EGFR signal transduction pathway is the Raf-MEK-ERK Mitogen-Activated Protein Kinase Cascade (Figure 2). This cascade contains a set of its own mutations throughout the steps of the pathway which are leading causes of cancer as well. Mammalian cascades involving Mitogen-Activated Protein Kinases (MAPK's) are part of a three step signaling process. The MAPK Kinase Kinase (MAPKKK) signals the MAP Kinase (MAPKK) which then signals the MAPK (Roberts, Der, 2007). In the EGFR MAPK cascade, active EGFR stimulates Raf, the MAPKKK, through association with adaptor protein Grb2 which recruits SOS-1 nucleotide exchange factors to the cell membrane. SOS-1 activates Ras proteins by exchanging the GDP bound to Ras for GTP. The activated Ras recruits Raf to the cell membrane and activates it through a complex process involving phosphorylation (Friday, Adjei, 2008). The Raf MAPKKK phosphorylates and activates MEK 1 and 2, the MAPKK's, which phosphorylate the MAPK's-Extracellular signal-regulated kinases (ERK 1 and 2). Activated ERK's go on to regulate numerous proteins and transcription factors, resulting in cell proliferation and survival. Although this pathway has previously been considered linear, in reality it involves many complex interactions with other pathways which regulate the different steps, mainly Raf Activation. Studies have shown that mutations in each step along the path of this complex pathway are present in a substantial percentage of cancers and other disorders. This complexity can be demonstrated with an example involving EGFR. EGFR is involved in the upstream and downstream regulation of the MAPK pathway. EGFR functions upstream by signaling the Ras protein in which case an EGFR mutation or over expression can cause hyperactivation of the MAPK pathway. Additionally, one of the products of ERK signaling is TGF-α and other EGFR ligands whose over expression can lead to hyperactivation of the pathway as well. In order to combat the numerous mutations which present themselves throughout this pathway, intense study and research is currently devoted to the development of Raf inhibitors, MEK inhibitors, Ras inhibitors, and EGFR inhibitors- our area of interest (Roberts, Der, 2007).

EGFR Inhibitors

As a result of the large proportion of EGFR mutations among Non Small Cell Lung Cancer, much effort has been devoted to the research and development of inhibitor drugs which combat the neoplasia resulting from the over-expression of the EGFR proteins. Novel target therapies in the midst of development can aim at the specific EGFR oncogene as opposed to affecting all the cells in the body with chemotherapy, which has proven itself relatively ineffective in treatment of lung cancer (Vealel, et. al. 1987). Various research groups are exploring distinct methods of inhibiting the hyperactivity of the EGFR pathway. This inhibition is relatively harmless because the EGFR pathway does not play an essential role in the bodies of healthy adults, its inhibition causing a rash or diarrhea at most (Fukuoka, et. al. 2003). Aside for the EGFR signal transduction pathway inhibitors discussed earlier, there are two forms of inhibitors of the EGF receptor itself currently in the form of marketable drugs. These two forms of inhibitors are monoclonal antibodies and tyrosine kinase inhibitors. Researchers are currently attempting to find the optimal combination of these two target therapies together with standard radiation and chemotherapy for each case of Lung Cancer. Monoclonal antibodies, such as Ce-

tuximab, are large molecules that act from outside of the cell. These antibodies inhibit EGFR activation by binding close enough to the ligand-binding site that it can block the ligand from binding to the receptor (Friday, Adjei, 2008).

Our inhibition drugs of interest are Tyrosine Kinase Inhibitors. Tyrosine kinase inhibitors are small molecules with the ability to move through the cell membrane in order to interact with the cytoplasmic EGFR domain. Tyrosine kinase inhibitors exhibit competitive inhibition, binding to the tyrosine residues of the EGF receptor, thereby blocking ATP from binding and activating the EGFR and its downstream effectors. This inhibition of the EGFR pathway has demonstrated clinical response and has prolonged life expectancy of EGFR mutation patients. It is believed that the tyrosine kinase inhibitors discontinue the translation of EGFR effectors which had induced cellular proliferation and metastasis, thereby reducing the excessive cell growth present in the malignancy (Massutí, 2003). Furthermore, it was proposed that tyrosine kinase inhibition induces apoptotic cell death by upregulation of Bim, a pro-apoptotic protein. Another strong possibility is that tyrosine kinase inhibitors induce cell death by inhibiting mTOR, an autophagy-inhibitor protein whose activity is normally increased by EGFR activity. Autophagy is the process of which the cellular organelles are swallowed up by lysosomal vesicles, but this process is normally regulated by the EGFR pathway. With the EGFR pathways halted, this inhibition is weakened and autophagy becomes more prevalent amongst the malignant cells causing the desired cell death (Markus, Alain, 2013).

Two TKI drugs currently on the market are Gefitinib/Iressa and Erlotinib/Tarceva. In the randomized, double blind, Phase II trial of Gefitinib, the results were promising. Included in the study were 210 advanced NSCLC patients who had been treated with chemotherapy once or twice and had received platinum treatment. They were chosen to receive a daily oral dose of either 250-mg or 500-mg Gefitinib. Tumor response rates were approximately 19% and the symptom improvement rate of evaluable patients was approximately 40%. Adverse effects were minimal in both cases consisting main of diarrhea and skin rashes (Fukuoka, et. al. 2003).

The Erlotinib results seem optimistic as well. The international phase III Erlotinib trial was randomized, double blind, and placebo controlled with a 2:1 ratio of 150mg daily dose of Erlotinib to the placebo group. The placebo was considered ethical because further chemotherapy would not have benefitted these patients whose first or second bout of chemotherapy had failed. Response rate was around 9% in the group who received Erlotinib and less than 1% in the placebo group. Median survival was prolonged by two months (Shepherd, et. al. 2005).

EGFR Mutations

The control that EGFR contains over cell proliferation and metastasis makes it a key protein for oncogenic mutation. Hyperactivity of the EGFR pathway in any step of its complex network can lead to neoplasia. This can happen through mutation, over expression, or amplification of EGFR, its ligands, or a protein involved in the EGFR pathway.

In a study done in Japan (Kosaka, et. al. 2004), tumor samples were obtained from 277 randomly selected patients (Figure 3). Exons 18-21, the first four exons that code for the Tyrosine Kinase domain, were amplified and underwent molecular analysis, as these areas had been previously shown to contain the EGFR mutations in conducted studies. Mutations in this area of the tumor's EGFR gene were found in 111 patients (40%). There were 52 in-frame deletion mutations, 54 point mutations, and 5 duplication/insertion mutations. The deletions were all around the five amino acids ELREA from codons 746-750 of exon 19. Approximately half were simple deletions and half were deletions coupled with point mutations or insertions. A Thymine to Guanine exchange at the second nucleotide of codon 858 in exon 21 was found in 85% of the point mutations, leading to an exchange of Leucine for Arginine. In summary, 91% of the EGFR mutations occurred as deletions around E746-A750 or as L858R point mutations. These two forms of mutations have come up in numerous studies conducted in this area, proving to be strongly correlated with EGFR mutational Non-Small Cell Lung Cancer (NSCLC).

Clinical Features Correlated to EGFR Aberration

As numerous studies were conducted, specific patterns of clinical and pathological features were found to

be present in the lung cancer patients involved in the studies. Female gender, non-smoking history, Asian descent, and Adenocarcinoma were all found to be directly related to EGFR mutations in NSCLC. EGFR mutations are almost completely exclusive to adenocarcinomas in all conducted studies. In the Japanese study, only one out of 111 EGFR mutated patients had nonadenocarcinoma. In a similar study conducted in Italy (Marchetti, et. al.2005), EGFR mutations were found exclusively in the adenocarcinoma patients. Smoking history showed significantly high statistical correlation to EGFR mutations as well. Fifty nine percent of the patients in the Italian study with mutated EGFR proteins were nonsmokers and 41% were smokers or former smokers. The study in Japan takes this smoking correlation a step further after completing more advanced statistical analysis solely on the Adenocarcinoma patients. They divided the smokers into three categories depending on the length of the period of time spent smoking, and it was discovered that the higher the exposure to smoking, the lower the percentage of EGFR mutation was found in that subgroup of patients, refuting the hypothesis that smoking is related to NSCLC.

In terms of gender, in both the Japanese and Italian studies as well as numerous other studies conducted on this subject, the females were a significantly larger percentage of the NSCLC patients found with the EGFR mutation. However, because female patients are usually nonsmokers as well as Adenocarcinoma type cancer patients, the Japanese study performed logistic regression analysis to determine if gender contributed independently to EGFR mutation. The result demonstrated that only Adenocarcinoma histology and smoking status contributed independently to EGFR mutation, while female gender was an outcome of the other features that were present amongst females in a higher percentage than males. Similar results were found in many other studies as well. Furthermore, an added observation was made on the small number of patients under the age of 50, menopause age in Japan. It was found that the EGFR mutation in these younger patients did not lean toward the female gender, further discounting theories of hormonal activity affecting the EGFR protein in females.

Japanese NSCLC patients were confirmed numerous times for having a higher proportion of EGFR mutation than American NSCLC patients (Kosaka, et. al. 2004). However, this feature is likely a result of other commonly found features in Asian populations. There are a significantly lower percentage of NSCLC patients with a history of smoking in Japan than in America. In the Japanese study, 83% of the female and 10% of the male patients were non-smokers, which is typical of Japanese patients. The conductors of this study note that in a parallel American study, however, 15% of the female and 6% of the male patients were never-smokers. These results were characteristic of many studies which evaluated American versus Asian smoking history.

Discussion

There has been great success in the area of EGFR target therapy, as well as with target therapy in general as a cure for cancer. However, in order to continue in the path toward success, clearer biomarkers must be established which can help to narrow down the population to which the drug is originally administered. Currently, the percentage of response to EGFR TKI's is only around 10% (Fukuoka, et. al. 2003, Shepherd,et. al. 2005). The current goal of the ongoing research in this area is to hone in on this 10% of patients, using research and experimentation to solve the mystery of their success. Eventually, as the response to each target therapy is clearly understood, each patient will be given his or her optimal drug combination, and greater response rates will be seen with cancer patients worldwide.

An integral step toward this goal is the determination of objective patient features for each target therapy which will signal success (or failure) in the inhibition of the gene of interest. A standard, quantitative scoring system for assessment must be established to determine if the target therapy is, in actuality, blocking what it was intended to block and if that blockage elicits a positive clinical response. New technology is being developed to assist the research in this area such as cDNA Arrays which will allow the identification of genes regulated by anti-EGFR therapies. Protein Arrays could be used to explore the proteins involved in the EGFR pathways (Baselga, Arteaga, 2005).

Once clear signs of drug success have been estab-

lished, it is necessary to explore the correlation between clinical-pathological patient biomarkers and drug success (or failure). Research is being conducted to explore the patterns of clinical patient features which are more prevalent among patients who responded to the drug. The two forms of biomarkers being researched amongst NSCLC patients are genetics and clinical characteristics. The genetic sequence mutations amongst EGFR NSCLC patients are somewhat clear. The two main mutations are the E746-A750 deletion as well as the L858R point mutation. However, the clinical patient features commonly prevalent in EGFR mutation patients must be examined further. In order to accomplish this goal, there are those who believe that a "No tissue-No trial" rule should be enacted in which a patient whose molecules of interest are unclear in the tissue would be excluded from the trial. This way, only patients who will help research further its goal of establishing a connection between drug response and biomarkers would be granted the drug (Baselga, Arteaga, 2005).

In terms of clinical features, it seems that the two characteristics which independently correlate to EGFR mutation are adenocarcinoma type cancer and non-smoking history. Female gender prevalence in drug response is a likely outcome of its correlation to Adenocarcinoma and non-smoking history. The results of the logistic regression analysis in many studies and trials did not point to female gender with an independent correlation to drug response. Additionally, the analysis of the pre-menopause patients in the Japanese study points to the same conclusion; female gender does not independently correlate with EGFR inhibition response rate. In the Gefitinib trial, no pharmacokinetic differences were found between men and women (Fukuoka, et. al. 2003).

The prevalence of drug response in Asian populations does not hold its own when scrutinized with more complex methods of statistical analysis. Upon observing a higher response rate for Japanese patients in the Gefitinib trial, a pharmacokinetic analysis was conducted which did not reveal any differences between Japanese and non-Japanese patients. Additionally, after an extremely complex logistic analysis with multiple variables involved, Asian descent response rates were not considered statistically significant (Fukuoka, et. al. 2003).

However, the connection between EGFR mutation and the independent characteristics of adenocarcinoma and smoking history are pretty firmly established. Fukuoka (2003) postulates that the slow growth of adenocarcinomas may lend itself to successful drug response.

Conclusion

The true link between prevalent clinical traits and drug response rate can only be understood with a deeper understanding of the complex EGFR network of signal transduction pathways and cross talk found amongst them. This will be possible with the use of technology such as genetic and protein arrays as well as analysis of future studies. This understanding, coupled with the knowledge of clear biomarkers, will allow for target drug administration to specific populations and higher response rates in EGFR inhibitor drugs. Target cancer drugs will eventually be administered to the small percentage of the population who will likely respond to the drug, thereby efficiently creating a higher success rate of target therapy.

References

Baselga J, Arteaga CL. Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. Journal of Clinical Oncology. 2005; 23(11): 2445-2459.

Friday BB, Adjei AA. Advances in targeting the Ras/Raf/MEK/Erk mitogen -activated protein kinase cascade with MEK inhibitors for cancer therapy. Clinical Cancer Research. 2008; 14:342-346.

Fukuoka M, Yano S, Giaccone G, et al.!! Multi-institutional randomized phase II trial of Gefitinib for previously treated patients with advanced non–small-cell lung cancer. Journal of Clinical Oncology. 2003; 21(12): 2237-2246.

Heist R S, Christiani D. EGFR-targeted therapies in lung cancer: predictors of response and toxicity. Pharmacogenomics. 2009; 10(1): 59-68.

Huang SM, Harari PM. Epidermal growth factor receptor inhibition in cancer therapy: biology, rationale and preliminary clinical results. Investigational New Drugs. 1999; 17(3): 259-269.

Isobe T, Herbst RS, Onn A. Current management of advanced non-small cell lung cancer: targeted therapy. Seminars in Oncology. 2005; 32(3): 315-328.

Kosaka T, Yatabe Y, Endoh H, et al.!!! Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Research. 2004; 64: 8919-8923.

Marchetti A, Martella C, Felicioni L. EGFR mutations in non–small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential impli-

cations on pharmacologic treatment. Journal of Clinical Oncology. 2005; 23(4): 857-865.

Markus DS, Alain CB. Epidermal growth factor receptor mutations in lung adenocarcinoma. Laboratory Investigation. 2013.Volume and pages?

Massutí B. EGFR signaling pathway. Fifth Congress on Lung Cancer. 2003;142-145

Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene. 2007; 26: 3291–3310.

Shepherd FA, Pereira JR, Ciuleanu T, et al.!!! Erlotinib in previously treated non–small-cell lung cancer. The New England Journal of Medicine. 2005; 353: 123-132.

Vealel D, Ashcroft T, Marsh C, Gibson GJ, Harris AL. Epidermal growth factor receptors in non-small cell lung cancer. British Journal of Cancer. 1987; 55:513-516.

Yarden Y. The EGFR family and its ligands in human cancer: signaling mechanisms and therapeutic opportunities. European Journal of Cancer. 2001; 37:s3-s8.

Yarden Y, Sliwkowski M X. Untangling the ErbB signaling network. Nature Reviews Molecular Cell Biology. 2001; 2(2): 127-137.

Notes

Notes

Lander College of Arts and Sciences
A Division of Touro College

Flatbush Campus
1602 Avenue J
Brooklyn, NY 11230
718.252.7800
www.touro.edu