Studying the Role of Sialyltransferase ST6Gal-1 in Regulating Hematopoiesis Using Cyclophosphamide Induced Myelo-Suppression as a Model

Personal Summary

Before high school, I was never the one whose favorite subject was science. I loved to hang out with friends, read, and write; a future in science had never particularly appealed to me. My first week of high school changed that. Through the Science Research Program at my school, I have been able to have the amazing opportunity to work in a cancer research laboratory at Roswell Park Cancer Institute in Buffalo, NY, one full day a week, as well as 2-3 days after school, and 3-4 weeks during the summer. Before applying to and entering the program, I thought science was simply looking under a microscope at cells, a more complex version of what we did in biology class. Through my experience in the lab, science has become a portal to an unending source of knowledge, one that I know very little of. I've come to realize that science, the subject that never interested me, is really so cool. I began my time in the lab simply learning laboratory procedures and protocols, and from that point, proceeded onto more complex scientific projects. However, it wasn't until the fall of my junior year, that I truly felt the metaphoric light bulb go off in my head. Up to that time, I had had some trouble producing concrete results as well as finding an aspect of the work in the lab with which I worked the best. In my junior year, I finally found that aspect. I learned to love what I was doing, why I was doing it, and what the product could become. Though it was still frustrating at points, and very time intensive, I knew what I was doing was worth something.

The research project I have grown so attached to was the one I submitted to the Intel Science Talent Search. This project was based on previous studies done in my lab, and given to me to research further. Throughout my time in the lab, I received much assistance and guidance from my mentor, Dr. Joseph Lau, as well as the other members of the lab. With their help, I studied the relationship between a certain gene, ST6Gal-1, and blood cell production after chemotherapy treatment. Now, as a graduating senior, still working on putting the final touches on this project, it is amazing to think of how much my outlook on science has changed. I hope that in the future, I will be able to use what I've learned in the lab, to help me expand my horizons, making me even more excited about those parts of life which I think I have little interest in now.

Research

Introduction

Inflammation is the body's protective response to injury or infection. Unresolved or chronic inflammation can contribute to diseases such as asthma and arthritis. A vital part of inflammation is the production of white blood cells and their recruitment to the affected area. Hematopoiesis is the production of all blood cells, including white blood cells, and is necessary to replace cells normally lost from inflammatory responses and from normal cell turnover. Insufficient hematopoietic cell production can lead to anemias (inadequate red cells), uncontrolled bleeding (lack of platelets) or infection (lack of white blood cells). Dysfunction in the process of hematopoietic differentiation results in uncontrolled proliferation of blood cells, such as in leukemia.

Cytoxan is a chemotherapeutic agent commonly used in the treatment of leukemia (cancer of the blood) and lymphoma (cancer of lymphatic cells). To work,

Cytoxan is converted into active metabolites in the liver. These metabolites stop the proliferation of rapidly dividing cells (both cancer cells and hematopoietic progenitor cells), and enforce apoptosis, or programmed cell death. A serious side-effect of Cytoxan treatment is the depletion of white blood cells, rendering patients extremely susceptible to infection and uncontrolled bleeding. However, hematopoietic stem cells are spared because they are mostly in an inactive or non-proliferative state. After Cytoxan treatment, hematopoietic stem cells are activated, leading to the replacement of the white blood cells. The rapid recovery of hematopoiesis after chemotherapy is vital to minimize the dangers of infection and bleeding. Therefore, the recovery from Cytoxan is an excellent model to study hematopoiesis and the factors that affect it, including cytokines, growth and signaling factors and other drugs

The enzyme, ST6Gal-1 plays an important part in the inflammatory response. ST6Gal-1 belongs to a family of enzymes called sialyltransferases that catalyze the attachment of sialic acid, a sugar, to protein complexes, located on the surface of cells or released into the blood [3]. Most ST6Gal-1 is located inside cells, but there are significant amounts freely circulating in the blood. The role of the ST6Gal-1 in the blood remains unclear, though we hypothesize that circulatory ST6Gal-1 is important in inflammatory responses by regulating hematopoiesis, specifically through the production of white blood cells for inflammation.

Previous studies have established a link between circulatory ST6Gal-1 and hematopoiesis. It has been shown that mice with decreased levels of ST6Gal-1 in the blood have greater hematopoietic cell production [2]. We hypothesized that circulatory ST6Gal-1 regulates hematopoiesis by inhibiting blood cell production. We predicted

that circulatory ST6Gal-1 would be depressed when blood cell production is required, and would be elevated when blood cell production is no longer above normal. The hematopoietic recovery after Cytoxan treatment was used as the model to study this hypothesis.

My research focused on questioning the relationship between ST6Gal-1 and hematopoietic white blood cell production after Cytoxan treatment. The study of this relationship between hematopoiesis and ST6Gal-1 is significant because it could potentially benefit those treated for leukemia by myelo-ablative drugs such as Cytoxan that severely decrease blood cell counts after treatment. Therefore, understanding ST6Gal-1's role could contribute to a faster recovery of hematopoiesis after chemotherapy of this kind, which could impact the lives of cancer patients. Understanding ST6Gal-1's relationship to hematopoiesis could also improve understanding of the inflammatory response.

Methods

The relationship between hematopoietic cell production and circulatory ST6Gal-1 was studied through the analysis of liver ST6Gal-1and white blood cells. Liver was studied because it is the primary location for ST6Gal-1 production in the body. Blood cell counts were used to examine hematopoietic recovery to ST6Gal-1 expression after Cytoxan treatment.

From previous studies done in my lab, we were interested in studying how ST6Gal-1 expression changes with Cytoxan treatment in relation to how white blood cell count changes. Based on our hypothesis that ST6Gal-1 inhibits hematopoiesis, it was

expected that ST6Gal-1 levels would decrease when white blood cell counts decreased, increase when cell counts increase, and return to normal when counts returned to normal.

In this study, liver tissue and blood were collected from 4-5 animals per group on days 0, 2, 4, 6 and 10 and 17 (Table 1). The animals were of two different genotypes: wild type and ST6Gal-1 deficient mice. Wild type mice were used as the control, as they are considered the “normal mice.” The two genotypes studied were wild type and ST6Gal-1 deficient mice. ST6Gal-1 deficient mice lack one of the promoters for the ST6Gal-1 gene, P1. P1 is one of the six independent promoter regions that regulate transcription of the gene. Liver ST6Gal-1 expression is primarily regulated through the P1 promoter [3]. These mice with altered P1(ΔP1) were created previously with a targeted deletion of the P1 promoter so that they produce ST6Gal-1, but on a much smaller scale than wild type mice [3]. Comparing the recovery from Cytoxan of ΔP1 to wild type mice provides insight about the effect of ST6Gal-1 on hematopoiesis, and can lead to more improvements in the recovery from Cytoxan.

To study liver ST6Gal-1 expression, messenger RNA (mRNA) was extracted from liver using Trizol and a Phenol-Chloroform purification, which ensures that the mRNA is completely pure. For each sample, the purity and concentration of mRNA was checked using nano-drop technology.

Through the process of reverse transcription, chromosomal DNA (cDNA) was made from the mRNA utilizing a Bio-Rad DNA synthesis kit. The cDNA produced is then used to analyze gene expression in the sample. In these studies, the analysis protocol

used was real time PCR (polymerase chain reaction). PCR is the process of replicating a small quantity of DNA numerous times for analysis using gel electrophoresis. In real time PCR, a quantitative version of PCR, a small amount of each sample is combined with water and SYBR-green, a dye that binds to the DNA. Throughout the heat cycles in real time PCR, the DNA is replicated numerous times, and gene expression is quantified at the end of each cycle. The amount of gene expression present at the end of the protocol is a summation of all the counts from each cycle. While regular PCR only gives results in terms of detection at the end of the procedure, Real Time PCR was used in these experiments because it gives both detection and quantification. A quantitative count of expression is better for analyzing and comparing expression levels.

Measured levels of ST6Gal-1 expression from real time PCR were compared to a control. The control used for this experiment is RPL (Ribosomal Protein 32). RPL is a housekeeping gene found in the ribosomal complex of every cell, and is therefore used as a basis of comparison to other genes.

Analysis of real time PCR results was obtained by comparing the RPL expression level in samples to that of the gene studied; in this case ST6Gal-1. Analysis of ST6Gal-1 expression was repeated to ensure the reliability of the data. Each sample was analyzed in duplicate, and the duplicates were then averaged together. Before averaging, all samples were also checked to ensure that duplicates were similar. At each time point, expression was compared to a day 0 point, using the T-statistic; all tests were two-tailed, and used a 5% alpha level. Similarly, I compared results of wild type animals to ΔP1 at each point using a T-test.

In order to conduct these experiments, I had the assistance of other members of my laboratory. They provided advice, and answered my questions and helped me whenever possible. The first set of experiments was planned mostly by other members of my lab. However, for the second set, I worked with my mentor and other members of my lab to plan the experiments: the number of animals used, the additional time point to be added, and the scheduling of when the experiments would take place. The ΔP1 and wild type animals were bred and grown by other members of my lab. A post-doctoral fellow and the lab technician in my lab sacrificed the mice and collected blood and liver tissue from them. The blood was immediately sent to another lab for CBC analysis.

All laboratory work done in these experiments was done by me. I performed mRNA extraction and purification, reverse transcription, and real time PCR on the liver tissue samples. I did the analysis of the data collected from these experiments with assistance from the post-doctoral fellow in my laboratory. I was trained to perform these procedures by other members in my lab. Throughout my project, they also provided answers to questions regarding protocols and laboratory technique.

Results

Results of the first study are shown in Figure 1. ST6Gal-1 expression was measured using liver mRNA. Compared to day 0, before Cytoxan was injected, there was a trend toward partial down regulation of ST6Gal-1 expression from day 2, up to day 10. Only day 6 and 10 expression levels were significantly different from day 0.

Results from the second set of experiments are shown in Figures 2 and 3. Trends for liver mRNA expression for wild type animals were similar to those from the

first set of experiments (Figure 2). However, because of the larger sample size for each time point, all measurements on days 2-17 were statistically significantly different from day 0. Because of the lack of up regulation of ST6Gal-1 up to 10 days after treatment in the first set of experiments, it was hoped that by 17 days, ST6Gal-1 expression would return. However, ST6Gal-1 expression remained significantly depressed compared to day 0, even at day 17.

For ST6Gal-1 deficient animals (ΔP1), as expected, day 0 expression was lower than in wild type mice. In the ΔP1 mice, only day 2 was statistically significantly different from day 0. In comparisons of wild type and ΔP1 animals, days 0, 2, and 4 were statistically significantly different; expression levels were close to the same at days 6, 10 and 17 for the two genotypes (Figure 2).

Results for white blood cells counts from the second set of experiments are shown in Figure 3. Counts decreased dramatically after Cytoxan treatment for the day 2 and 4 measurements. They then recovered rapidly and returned to normal by day 17. All time points in both WT and ΔP1 animals, except for day 17 were statistically significantly different compared to day 0. Comparing WT and ΔP1 mice at each time point, only days 6 and 10 were statistically significantly different. On day 6, white blood cell counts were higher in ΔP1 animals than in wild type. On day 10, the opposite was true; wild type animals had higher white blood cell counts than in ΔP1 animals.

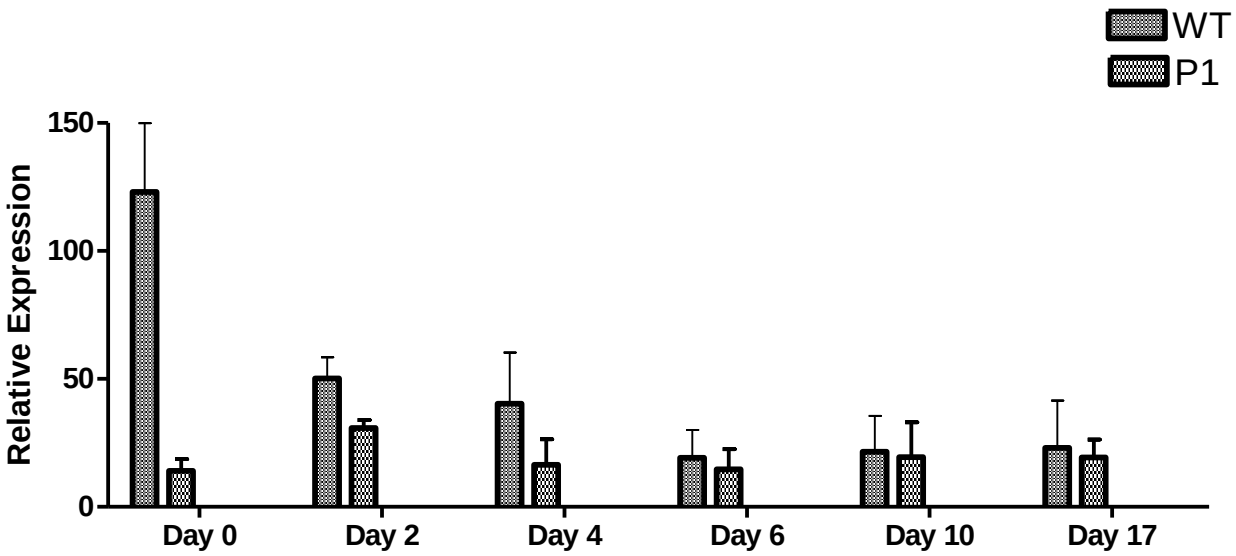
We had hypothesized that ST6Gal-1 expression would decrease after Cytoxan treatment, when white blood cell counts are low, and there is a need for increased hematopoiesis. As we expected, ST6Gal-1 expression did drop after Cytoxan. However,

white blood cell counts returned to normal by day 17. ST6Gal-1 expression continued to be low in both wild type and ΔP1 animals. There were differences in the response of white blood cell counts in wild type and ΔP1 animals, with ΔP1 animals having higher white blood cell counts on day 6 after treatment. This latter finding correlates with previous studies [2].

Illustrations

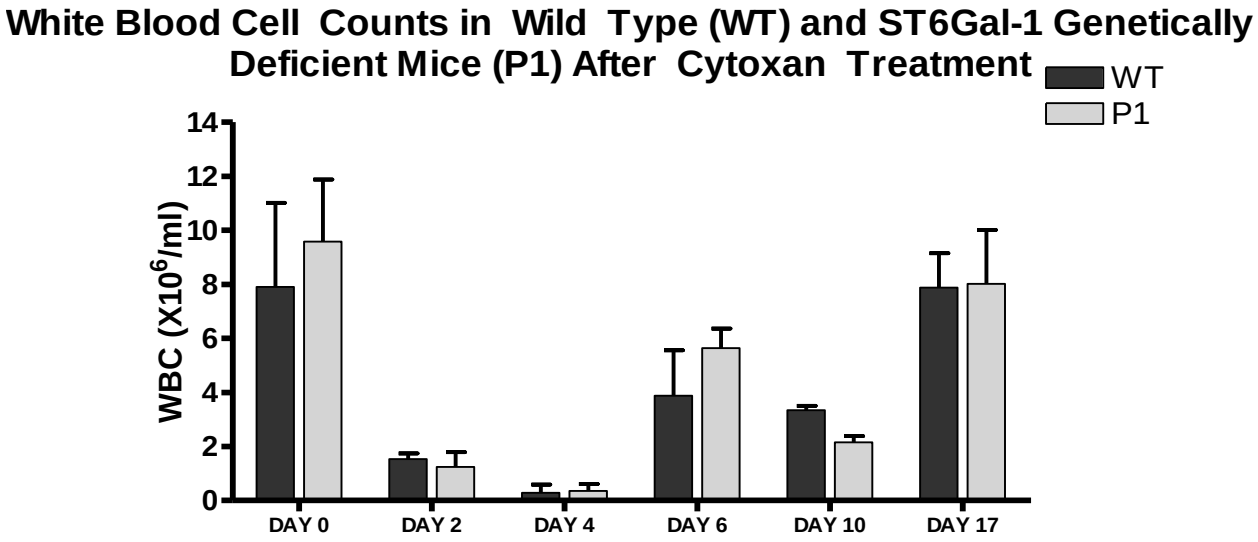
Figure1:

ST6Gal-1 Expression in Liver after Cytoxan Treatment in Wild Type (WT) and ST6Gal-1 Genetically Deficient (ΔP1) Mice



There was significant decrease in liver ST6Gal-1 expression after Cytoxan treatment at each time point, compared to day 0, up through day 17 for the wild type (WT) mice. For ST6Gal-1 deficient mice (ΔP1), only day 2 was statistically significantly different from day 0. In comparisons of the two genotypes on each day, days 0 through 4 wild type samples were statistically significantly different from their ΔP1 counterparts.

Figure 2:



The recovery of white blood cell counts after Cytoxan treatment was similar to that shown in previous studies [2]. Lowest counts were reached at day four, and returned to normal levels by day 17. Days 6 and 10 were statistically significantly different comparing ΔP1 to Wild Type animals. Compared to day 0 counts, days 2-10 in ΔP1 and Wild Type were statistically significantly different.

Discussion

The purpose of this study was to examine the relationship between hematopoietic production following myelo-suppresion (suppression of myeloid hematopoietic stem cells), and ST6Gal-1 expression. We hypothesized that ST6Gal-1 regulates blood cell production, inhibiting hematopoiesis. By analyzing ST6Gal-1 expression after chemotherapy treatment, we examined if there was an inverse relationship between liver ST6Gal-1 expression and hematopoiesis.

Previous work done has focused on the effect of ST6Gal-1 on inflammation. It was found that down regulation of ST6Gal-1 increases inflammatory response, by increasing inflammatory cell counts at the site of inflammation. Counts of granulocytes, a type of white blood cells, decreased dramatically after Cytoxan treatment, and then returned to normal after 5 days [2]. In my studies, we focused on this relationship using the Cytoxan myeloablation model, measuring not only white blood cells, but also ST6Gal-1 expression in the liver.

Results from the first set of experiments showed a statistically significant decrease in ST6Gal-1 expression from day 0 to day 17after Cytoxan treatment, but no increase back to normal levels after the initial drop in expression. There are several plausible explanations for the lack of up-regulation. Although the number of mice in each group was larger, the sample size could be too small. It is also possible that it takes the animals longer than ten days to fully recover. Thus, it may be that ST6Gal-1 did not return to normal until after the end of the measurement period. Additionally, Cytoxan is processed in the liver where ST6Gal-1 is primarily produced. The toxicity of Cytoxan could possibly be affecting the ability of the liver to produce ST6Gal-1.

In the blood, white blood cell counts returned to normal by day 6. Despite the lack of up regulation of ST6Gal-1 expression, there was recovery of hematopoiesis. A benefit in this study was the use of two different genotypes, wild type and ΔP1, allowing us to examine the effects of Cytoxan on differing levels of ST6Gal-1 in mice. In the two genotypes, days 0-4 were significantly different from one another, while days 6-17 were not.

Results from these studies support our hypothesis that ST6Gal-1 regulates hematopoiesis, because levels decrease after Cytoxan treatment, when increased hematopoiesis is required. However, ST6Gal-1 levels remained low, and continued to be low even after blood cell counts return to normal. Further work is needed to understand factors related to ST6Gal-1 recovery.

Conclusions from this research concerning ST6Gal-1's effect on hematopoiesis could potentially be used to improve recovery from chemotherapy treatment. The research done concerning the relationship between hematopoietic production and ST6Gal-1 expression is also essential in learning more about ST6Gal-1's role in the inflammatory process. Results from studies done on wild type animals can be more closely compared to the effect that Cytoxan has on blood cell production in humans, and can lead to improvements in the recovery process from Cytoxan.

Conclusions and Future Directions

From data collected in these experiments, we concluded that ST6Gal-1 expression in liver appears to have a regulatory effect on hematopoietic production after chemotherapy treatment. However, because ST6Gal-1 levels did not return to normal in the time frame, further studies will need to be done to understand if ST6Gal-1 has a role in inhibiting hematopoietic production and to understand the factors leading to white blood cell recovery.

If we understand ST6Gal-1's effect on hematopoiesis, a drug could be created which would inhibit ST6Gal-1, and allow the body to produce more blood cells more

quickly after treatment. This drug could potentially aid in the prevention of uncontrolled bleeding and infection after Cytoxan.

Future directions for this research include using other models to study the relationship between hematopoiesis and STGal-1, because of the possible toxicity Cytoxan has to liver ST6Gal-1. Fluorouracil (5FU), a less toxic chemotherapy drug used to treat leukemia will be used to see if ST6Gal-1 levels are similarly affected by this other chemotherapeutic agent. 5FU bypasses being processed in the liver. Therefore, the liver toxicity from Cytoxan treatment that may affect ST6Gal-1 levels in the liver would not affect expression after 5FU treatment. Both 5FU and Cytoxan treatments cause myelo-suppresion, which ensures they can be compared.

In the long-term, we would like to continue to see if ST6Gal-1 expression changes similarly in humans. To do this, we will collect blood samples from patients following chemotherapy, and measure ST6Gal-1 expression in the blood through sialyltransferase assays. From studies done with humans, we hope to see a similar response to that in mice. By studying the response in humans, more can be learned about how ST6Gal-1 levels are affected by Cytoxan with the hope of improving treatment.

References

1. Hematopoietic Stem Cells. In Stem Cell Information [World Wide Web site]. Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services, 2009 [cited Monday, September 20, 2010] Available at <http://stemcells.nih.gov/info/scireport/chapter5>

2. Nasirikenari, M, Segal BH, Ostberg JR, Urbasic A, Lau JT. "Altered granulopoietic profile and exaggerated acute neutrophilic inflammation in mice with targeted deficiency in the sialyltransferase ST6Gal-1." *BLOOD*. 108.10 (2006): 3397-405.

3. Jones, MB, Nasirikenari M, Feng L, Migliore MT, Lau JTY. "Role for Hepatic and Circulatory ST6Gal-1 Sialyltransferase in Regulating Myelopoiesis." *Journal of BIological Chemistry*. 285.32 (2010):25009-17.

4. Nasirikenari M, Chandrasekaran E.V., Matt KL, Segal BH, Lau JTY. "Altered eosinophil profile in mice with ST6Gal-1 deficiency: an additional role for ST6Gal-1 generated by the P1 promoter in regulating allergic inflammation." Journal of Leukocyte Biology. 87.3 (2010): 457-66.

5. Varki A, Lowe JB. "Biological Role of Glycan." *Essentials of Glycobiology.* National Center for Biotechnological Information, n.d. Web. 20 Sep 2010. <http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=glyco2∂=ch6>.