Therapeutic effects of Genistein, Minozac and Fosteum in a mouse model of Mucopolysaccharidosis type IIIB (Sanfilippo syndrome B)

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In this study, Genistein, Minozac, Fosteum, and a combination treatment of Genistein and Minozac were administered to NAGLU mice, a mouse model for Sanfilippo syndrome type B, to determine if they would attenuate the production of proinflammatory cytokine levels in the brain. The mouse model for this disease is deficient in N-acetyl-glucosaminidase (NAGLU), one of four enzymes responsible for the degradation of heparan sulfate. The cells of NAGLU mice, as well as those of humans affected with Sanfilippo syndrome type B, gradually become distended due to the accumulation of heparan sulfate in the cell’s lysosomes. This retarded degradation is associated with an increase in proinflammatory cytokines in the brain, which is thought to exacerbate the neurodegenerative effects of the disease. Either heterozygous or mutant mice were treated with Genistein, Fosteum, Minozac, or a combination of Genistein and Minozac. Brains were harvested after 28 days of treatments and their homogenates were run on a BioRad custom-array bead assay. Genistein decreased the levels of proinflammatory cytokines in the NAGLU mice. Conversely, Fosteum aggravated neuroinflammation. Minozac was ineffective, and the combination treatment of Genistein and Minozac was more effective than Minozac alone, but not as effective as Genistein alone.

BACKGROUND

Sanfilippo Syndrome

Mucopolysaccharidosis type IIIB (Sanfilippo syndrome type B, or MPS IIIB) is an autosomal recessive disorder characterized by the lysosomal accumulation of heparan sulfate (HS) (Neufeld and Muenzer, 1989). There are four forms of mucopolysaccharidoses (types I-IV), typified by a deficiency in one of the four enzymes necessary for the degradation of heparan sulfate. In MPS IIIB, the buildup occurs due to a deficiency in N-acetylglucosaminidase (NAGLU) (Heldermon, 2007).

Patients often present with symptoms in the central nervous system though enzyme deficiencies and lysosomal distensions are also systemic (Neufeld and Muenzer, 1989). In the first phase of the disorder, often occurring between one and four years of age, clinical patterns are associated with developmental delay, especially in language. The second phase of the illness typically begins between three and four years of age and is characterized by severe behavioral disturbances, including increasingly frequent and severe temper tantrums, marked hyperactivity, decreased attention span, aggression, panic attacks, and disturbed sleep patterns. Physical growth is usually normal. The third phase of the illness occurs at about ten years of age and begins with effects in balance and, consequently, motility, leaving many affected individuals wheelchair bound by their mid-teenage years. Swallowing difficulties are often common, as are frequent episodes of aspiration of both food and saliva (Cleary, 1993). Death usually occurs due to respiratory complications, heart failure, or infection (Heldermon, 2007). Therapy is limited to supportive care (Cleary, 1993).

NAGLU Mouse Model

A mouse model of Sanfillipo syndrome type B was created by replacing an 852-bp fragment within exon 6, the site within the mouse genome homologous to the human NAGLU gene, with a neo gene (Li, 1999). The mouse model was proved to be representative of the human condition through vigorous histological and behavioral testing (Heldermon, 2007).

Treatments

To date, no treatments have successfully eradicated lysosomal inclusions or corrected the clinical manifestations of MPS IIIB. The continuous administration of supraphysiologic levels of enzyme seem to quell the visceral expressions of the disease, but have little effects on the neurologic symptoms (Sands, 1994). Furthermore, a study by Heldermon explored possible treatment modalities in the form of gene therapy with intracranial (IC) AAV 2/5-hNAGLU and bone marrow transplant (BMT). Treatment with IC AAV was shown to be the most
efficacious in increasing lifespan (by an average of 112 days) and improving motor function as assessed by a rocking RotaRod. BMT was shown to be ineffective in improving lifespan or motor function. Combination treatments improved the sizes of lysosomal inclusions and hearing as measured by auditory brainstem response (ABR). Despite these results, the improvement of symptoms did not coincide with histological improvements (Heldermon, 2010).

These findings show that replacing NAGLU through ERT, gene therapy with IC AAV, or BMT is not sufficient to correct MPS IIIB. Therefore, additional pathways must be investigated in order to provide more comprehensive treatment modalities. Marked neuroinflammation in MPS IIIB brains has been thought to play a role in disease progression (Heldermon, 2010). Glycosaminoglycans (GAGs) such as heparan sulfate have been shown to incite inflammatory events within the cell, including cytokine and chemokine production, leukocyte recruitment, and the maturation of inflammatory cells (Taylor and Gallo, 1996). Prednisone, an immunosuppressant, has been shown to yield the best results in regards to an increase in lifespan, decrease in GFAP concentration, improvement in motor function, and attenuation in the immune response as assessed by T-cell activation and leukocyte/lymphocyte concentration (DiRosario, 2009).

**Administered Drugs**

Genistein is an isoflavone and a tyrosine kinase inhibitor. Previous literature has shown Genistein to be effective in decreasing the production of heparan sulfate in MPS IIIB mice (de Ruijter, 2007). Also known as 4’, 5, 7-trihydroxyisoflavone, Genistein acts on several growth factor receptors, the most important of which is the epidermal growth factor receptor (EGFR), which mediates the production of heparan sulfate in the cell. The level of GAG production has been shown to be attenuated by receptor inactivation via tyrosine kinase inhibition (Jakobkiewicz-Banecka, 2007).

Minozac is a central nervous system (CNS) penetrant molecule that has demonstrated the ability to decrease neuroinflammation in mouse models of traumatic brain injury and electroconvulsive shock-induced seizures. Minozac’s ability to diminish the neuroinflammatory response was shown through the decreased expression of glial fibrillary acidic protein (GFAP), as well as the astrocyte marker S100B (Chrzascz, 2010).

Fosteum is a combination of Genistein, cholecalciferol (vitamin D3), and citrated zinc bisglycinate. It is an FDA-approved dietary supplement used to manage osteopenia and osteoporosis (Burnett, 2011). Zinc and cholecalciferol have anti-inflammatory implications in other diseases such as rheumatoid arthritis and chronic kidney disease (Prasad, 2009; Mathias, 2010).

**Hypothesis**

We hypothesize that the administration of these drugs, separately or in tandem, will decrease proinflammatory cytokine levels in the brains of NAGLU mice. We hypothesize that the combination treatment of Genistein and Minozac will be the most effective.

**SUBJECTS AND METHODOLOGY**

**Animals**

A mouse colony of MPS IIIB mice was transferred to the University of Florida from Washington University in St. Louis, Missouri in 2009. The colony was maintained through strict sibling mating. Mutant and heterozygous males were crossed with heterozygous females. The genotypes of the pups were determined via PCR of the NAGLU gene on exon 6 with the neomycin insertion. Results were verified using a substrate-based fluorescence assay to test for the presence of N-acetylglucosaminidase (Heldermon, 2007).

**Experimental Design**

Two-hundred-twenty mice, half mutant and half heterozygous, were randomly sorted into five treatment groups: control, Genistein, Fosteum, Minozac or Genistein, and Minozac in combination. These treatments were administered to mutant (-/-) and heterozygous mice (+/+) at two time points: six weeks (“new mice”) or six months (“old mice”) of age. Twenty two mice were in each treatment cohort, with eleven at each age checkpoint. Each study lasted for 28 days. Table 1 describes the treatment setup.

Mice in the control group were fed chow that was soy-free. Genistein and Fosteum were administered through fortified chow that was also soy-free.
Minozac was dissolved in normal saline solution (0.9 %) at a concentration of 0.15mg/100µl and was injected subcutaneously into the specified mice daily. Each mouse in a cohort receiving Minozac received 5mg of the drug per kilogram of its body weight (Chrzaszcz, 2010), which was weight adjusted weekly throughout the experiment. The amount of chow eaten each day was measured to ensure normal food consumption.

After 28 days, the mice were sacrificed. Eight of eleven in each group were sacrificed primarily by carbon dioxide and secondarily by cervical dislocation according to UF IACUC regulations. The brains of these mice were excised and flash frozen in liquid nitrogen. Three of eleven in each group were prepared for subsequent immunohistochemical analysis.

Assays

Cytokine concentrations in the brains of the sacrificed mice were quantified using a BioRad custom array bead assay. Tissues were prepared and the assay was run according to the company’s protocol.

Statistical Analysis

For untreated controls, significance was determined using 1-way ANOVA comparing mutants and heterozygote mice. The efficacies of each treatment group were analyzed by a 2-way ANOVA, which analyzed the effects of age and genotype within a treatment group. When t-tests indicated that a value was close to significant, an additional 1-way ANOVA, which analyzed the effects of treatment within genotype, was performed.

RESULTS

Basic Fibroblast Growth Factor

Fibroblast growth factor-2 (FGF-2) was significantly upregulated in mutant new and old mice compared to their heterozygous counterparts, as assessed by 1-way ANOVA. Concentrations in new heterozygous mice averaged 761.53 pg/ml and those in old heterozygous mice averaged 752.91 pg/ml. In contrast, the average concentrations found in the new and old mutant mice were 2030.38 pg/ml and 2345.46 pg/ml, respectively (p<0.01). See Figure 1.

During initial assessment of treatment by 1-way ANOVA, Genistein was effective in significantly decreasing the average concentration of FGF-2 to 1656.24 pg/ml in old mutants (p<0.05). As assessed by 2-way ANOVA, comparing the effect of treatment on each group compared to age-matched controls, Minozac did not appear to have any effect on the levels of proinflammatory cytokines. The combination treatment of Genistein and Minozac showed a trend of slightly decreasing FGF-2 concentration, but this attenuation was not significant.

Fosteum was significantly upregulated FGF-2 in new mutant mice (p<0.0001), new heterozygous mice (p<0.0001), old mutant mice (p<0.05), and old heterozygous mice (p<0.001). See Figure 2.

Macrophage Inflammatory Protein 1α

Analysis by 1-way ANOVA comparing untreated mutant and heterozygote animals revealed that MIP-1α was significantly upregulated in old MPS IIIB mice (p<0.05). See Figure 1.
Though none of the drugs tested significantly decreased the concentration of MIP-1α, certain trends were observable. New mutant mice expressed an average concentration of 437.79 pg/ml, and old mutant mice expressed an average concentration of 503.19 pg/ml. Genistein decreased concentrations of MIP-1α in new and old mutant mice to 382.90 pg/ml and 427.27 pg/ml, respectively. However, this decrease was not significant. The combination treatment of Genistein and Minozac showed a similar decrease to that described in FGF-2. Neither of these attenuations was statistically significant. 2-way ANOVA demonstrated that Fosteum increased the concentration of this proinflammatory cytokine significantly in new mutant mice (p<0.01), new heterozygous mice (p<0.0001), and old heterozygous mice (p<0.05). See Figure 3.

Other Proinflammatory Cytokines

The other four cytokines that were tested (TNF-α, VEGF, IL-6 and IL-1α) did not yield significant results with respect to upregulation in untreated mutants as compared to age-matched heterozygotes. See Figure 1.

A statistically significant attenuation of these proinflammatory cytokines of mice in the treatment groups was not observed. However, weak trends were again noted. Genistein consistently decreased levels of TNF-α, IL-6 and IL-1α; this trend was not observed with VEGF. Minozac significantly increased the concentrations of IL-6 (p<0.05) and IL-1α in old mutants (p<0.05). See Figures 4 and 5. Minozac did not seem to affect levels of TNF-α or VEGF. The combination treatment of Genistein and Minozac either remained near baseline levels or slightly decreased levels of proinflammatory cytokines. Attenuations seen with the combination treatment did not reach statistical significance. See Figures 4–7. Fosteum intensified the expression of all six of the surveyed cytokines. Fosteum treatment resulted in increased concentrations of TNF-α in new mutants (p<0.001), new heterozygotes (p<0.01), old mutants (p<0.01) and old heterozygotes (p<0.01). See Figure 4. IL-1α was upregulated in new heterozygotes (p<0.01) and old mutants after the administration of Fosteum. See Figure 5. Likewise, Fosteum treatment led to an upregulation of IL-6 in new mutants (p<0.05). Minozac exhibited a trend of not altering or worsening the expression of these cytokines. See Figure 6. VEGF was increased in new mutants (p<0.0001), new heterozygotes (p<0.0001), old mutants (p<0.01) and old heterozygotes (p<0.01) following the administration of Fosteum. See Figure 7.
Figure 1. Baseline cytokine concentrations in the brains of new and old MPS IIIB heterozygous and mutant mice based as assessed by the BioRad Custom Cytokine Array Bead Assay. * indicates significance as determined by 1-way ANOVA comparing cytokine concentration in age-matched mutant and heterozygote brains.
Figure 2. Concentration of FGF-2 in 10-week-old (new) and 7-month old (old) mice as assessed by the BioRad Custom Array Bead Assay. Cytokine concentration was determined across four different treatment groups: Genistein, Fosteum, Minozac, and combination Genistein+Minozac. * indicate significance as determined by 2-way ANOVA against age & genotype matched untreated controls. Blue * indicate significance as determined by a 1-way ANOVA against untreated age matched heterozygote controls. Red * indicate significance as determined by a 1-way ANOVA against its corresponding matched, untreated group.
Figure 3. Concentration of MIP-1α in 10-week-old (new) and 7-month old (old) mice as assessed by the BioRad Custom Array Bead Assay. Cytokine concentration was determined across four different treatment groups: Genistein, Fosteum, Minozac, and combination Genistein+Minozac. * indicate significance as determined by 2-way ANOVA against age and genotype matched untreated controls. Blue * indicate significance as determined by a 1-way ANOVA against untreated heterozygote controls.
Figure 4. Concentration of TNF-α in 10-week-old (new) and 7-month old (old) mice as assessed by the BioRad Custom Array Bead Assay. Cytokine concentration was determined across four different treatment groups: Genistein, Fosteum, Minozac, and combination Genistein+Minozac. * indicate significance as determined by 2-way ANOVA against age and genotype matched untreated controls.
Figure 5. Concentration of IL-1α in 10-week-old (new) and 7-month old (old) mice as assessed by the BioRad Custom Array Bead Assay. Cytokine concentration was determined across four different treatment groups: Genistein, Fosteum, Minozac, and combination Genistein+Minozac. * indicate significance as determined by 2-way ANOVA against age and genotype matched untreated controls.
Figure 6. Figure 6 shows the concentration of IL-6 in 10-week-old (new) and 7-month old (old) mice as assessed by the BioRad Custom Array Bead Assay. Cytokine concentration was determined across four different treatment groups: Genistein, Fosteum, Minozac, and combination Genistein+Minozac. * indicate significance as determined by 2-way ANOVA against age and genotype matched untreated controls.
Figure 7. Concentration of VEGF in 10-week-old (new) and 7-month old (old) mice as assessed by the BioRad Custom Array Bead Assay. Cytokine concentration was determined across four different treatment groups: Genistein, Fosteum, Minozac, and combination Genistein+Minozac. * indicate significance as determined by 2-way ANOVA against age and genotype matched untreated controls.
DISCUSSION

Though none of the treatments proved to significantly downregulate proinflammatory cytokines, the upregulations observed in FGF-2 and MIP-1 support the idea that neuroinflammation may indeed be playing a role in the pathology of MPS IIIB.

FGF-2 is important in cell growth and differentiation and has implications in angiogenesis and tissue healing. FGF-2 binds to the cell surface tyrosine kinase receptor, fibroblast growth factor receptor 1 (FGFR-1). In order to be endocytosed, this complex must be stabilized by heparan sulfate (Quarto, 1994). We speculate that the accumulation of heparan sulfate in the brains of MPS IIIB mice may enhance the expression of FGF-2 via a positive feedback mechanism.

Once internalized, FGF-2 participates in the signal transduction of various pathways, many of which are inflammatory (Quarto, 1994). Previous studies have demonstrated that, when expression is juxtaposed alongside other proinflammatory cytokines (especially TNF-α), FGF-2 acts acutely and synergistically to augment the recruitment of polymorphonuclear (PMN) leukocytes, monocytes, and T-cells (Zitterman, 2006). Other studies have demonstrated that the internalization of FGF-2 enhances the activity of the plasminogen activator system, as measured by an increase in urokinase plasminogen mRNA. Plasminogen activation plays a significant role in tissue remodeling, cell migration (Quarto, 1994), and inflammation (Cunningham, 2009). The activation of the pathway was significantly upregulated in models of chronic inflammation, induced by inoculation with a murine prion disease (ME7) (Cunningham, 2009).

Our study also demonstrated a significant increase in Macrophage Inflammatory Protein 1α (MIP-1α) concentration in old mutant mice compared to old heterozygous mice. Genistein’s ability to decrease the MIP-1α was nearly significant. MIP-1α is involved in the induction of leukocyte chemotaxis, especially in regards to B lymphocytes, CD8+ T cells, NK cells, and eosinophils. It has also been shown to induce the release of histamine from basophils and induce the expression of CAMs (Cook, 1996).

FGF-2 and MIP-1α, amongst several other proinflammatory cytokines, are targets of interest for reducing neuroinflammation in MPS IIIB. The efficacies of the drugs tested in this study were assessed to determine whether they could contribute to the alleviation of the sequelae associated with this disorder.

Genistein has been used to treat MPS IIIB via substrate reduction. It has been shown to decrease GAG production and lysosomal storage in mouse models of MPS IIIB. Our results demonstrated a trend of decreasing neural proinflammatory cytokine levels in mutant mice treated with Genistein. It significantly decreased FGF-2 in old mutant mice compared to untreated old mutant mice. Genistein inhibits the autophosphorylation of the EGF tyrosine kinase receptor, thereby initiating a kinase cascade that leads to the downregulation of transcription factors used for HS synthesis (Jakobkiewicz-Banecka, 2009). The efficacy of this treatment is debated. A recent double blind study demonstrated a small yet still statistically significant reduction in GAG concentration in urine and plasma following the oral administration of Genistein to patients with MPS IIIB. However, the drug had no effects on behavior or hair morphology, a common treatment efficacy marker for MPS IIIB (de Ruijter, 2012). Though the idea of substrate reduction is appealing, our results seem to indicate that this approach is only slightly successful for improving neuroinflammation.

In contrast to the attenuating effects on neuroinflammation described in a mouse model of traumatic brain injury and electroconvulsive-induced seizures (Chrzaszczyk, 2010), this study found Minozac to be wholly ineffective in mitigating levels of neural proinflammatory cytokines. The aforementioned Minozac trial administered the drug intraperitoneally and acutely. Our trial administered the drug subcutaneously and chronically. Our failure to observe an increase in levels of proinflammatory cytokines may be caused by the mode of administration of the drug or, perhaps, by the development of tolerance to the drug.

Fosteum, a form of Genistein, additionally fortified with zinc bisglycinate and cholecalciferol (vitamin D3), increased concentrations of neural proinflammatory cytokines across all cytokines measured. It is speculated that the cholecalciferol infused in the Fosteum may have had an adverse effect on the murine species. Increased levels of cholecalciferol aid in calcium absorption, which can cause hypercalcemia and, consequently, calcification throughout their organ systems (Marshall, 1984). It is possible that sub-toxic levels of cholecalciferol may have exacerbated stress and inflammation in the brains of MPS IIIB mice.

Sanfilippo syndrome is a disease with a multifaceted and unclear pathology. Treatment must not only target the underlying problem (i.e., NAGLU deficiency) but also the secondary effects of neuroinflammation. This research aimed to assess the ability of Genistein, Minozac, Fosteum, and a combination of Genistein and Minozac on the levels of proinflammatory cytokines in the brains of MPS IIIB mice. Our study has shown that Genistein has a small effect in decreasing proinflammatory cytokines, but has failed to identify Minozac or Fosteum as viable treatment options.
REFERENCES


