0031-3998/05/5804-0816
PEDIATRIC RESEARCH
Vol. 58, No. 4, 2005
Printed in U.S.A.

46th ANNUAL MIDWEST SOCIETY FOR PEDIATRIC RESEARCH Scientific Meeting St. Louis, Missouri October 20–21, 2005

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BIOENGINEERING OF COAGULATION FACTOR VIII FOR IMPROVED SECRETION

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PURPOSE: Mechanisms that limit recombinant factor VIII (rFVIII) within heterologous systems (eg. commercial rFVIII production or gene therapy applications) include: 1) inefficient expression of FVIII mRNA, 2) inefficient folding of the primary translation product within the endoplasmic reticulum (ER) with retention by chaperone proteins and 3) a requirement for facilitated transport from the ER to the Golgi apparatus. The B domain of FVIII shares no homology with any identified protein, is dispensable for functional activity and is extensively glycosylated (18 asparagine (N)-linked glycosylation attachment sites). B domain-deleted (BDD)-FVIII generates higher concentrations of mRNA compared to full-length FVIII and therefore increased primary translation product but exhibits a reduced rate of secretion and increased intracellular retention. Bioengineering of the FVIII B domain effectively improves the trafficking of rFVIII through facilitated ER-Golgi transport.

METHODS: We have used a series of B domain variants to map key regions of the B domain that regulate mRNA levels and secretion efficiency. Each construct has increased B domain size (ranging from 29 to 774 amino acids (aa) and increased number of N-linked oligosaccharides (from 1 through 18).

RESULTS: BDD-FVIII, FVIII wild-type (WT) and FVIII B domain variants were transfected into COS and CHO cell lines and the media harvested for analysis of FVIII activity and antigen. The addition of even a few N-linked oligosaccharides within a short B domain spacer (optimal at 226 aa and 6 N-linked oligosaccharides) improves secretion of BDD-FVIII approximately 5 to 10-fold. With additional increase in B domain size and oligosaccharide content, secretion efficiency declined in a stepwise fashion to similar levels observed for FVIII WT. An alternative construct was prepared in which the human FVIII B domain was replaced with the FVIII B domain from puffer fish (*fugu rubripes*). The fugu FVIII B domain is 224 aa and shares only 6% sequence identity to the human FVIII B domain, yet has a high density of N-linked oligosaccharides (11). This construct was secreted as efficiently as the 226aa/N6 human construct.

CONCLUSIONS: The secretion efficiency of FVIII can be regulated by the size and oligosaccharide content of the B domain. The results suggest that it is primarily the presence of N-linked oligosaccharides that improves secretion efficiency. FVIII bioengineered for improved secretion will be an alternative for gene therapy strategies as well as recombinant FVIII production in manufacturing or transgenic strategies.

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ALTERATIONS IN EXPRESSION OF NEUROTROPHINS AND THEIR RECEPTORS IN RESPONSE TO HYPEROXIC LUNG INJURY

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Neurotrophins and their receptors have important roles in survival of neural tissues that may extend to cells of non neural origin. Their presence and response to hyperoxia-induced lung injury has not been fully characterized. We randomized four day old rat pups to breath either room air or hyperoxia (F_IO_2 ≥95%) for 1, 3, 6 and 10 days. By Western blot we found a significant decline in the hyperoxic-exposed group of Neurotrophin-3 (NT-3) and its high affinity receptor TRK-C, beginning at day 3 with maximal decline at day 6. NT-3 and TRK-C are widely distributed in the lungs both in the airways and air sacs as seen by immunohistochemistry. We repeated the experiment using human fetal lung fibroblast under culture conditions exposed to 95% oxygen and found the same results. NT-3 and its high affinity TRK-C receptor level declined significantly by 4 hours and were undetectable by 12 hours of exposure as determined by Western blot. We conclude that hyperoxia decreased lung expression of NT-3 and its receptor. The findings may have implications for lung growth and development.

3

RITUXIMAB THERAPY IN CHILDREN WITH CHRONIC REFRACTORY IMMUNE CYTOPENIA: LONG TERM EFFICACY AND IMMUNE ANALYSIS.

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***Purpose*:** Rituximab (anti-CD20 monoclonal antibody) successfully depletes mature B cells and can inhibit antibody mediated destruction of blood cells.. We evaluated efficacy and immune functions following rituximab in children with hematologic cytopenias, refractory to or dependent on conventional therapy. This study was designed to evaluate (1) safety and efficacy of rituximab (2) B cell mediated immune functions for one year following therapy.

***Methods*:** Children with chronic (>6 months) autoimmune hematologic cytopenias received rituximab - 375 mg/ m^2 weekly for 4 doses. In absence of response at dose three, 3 more doses at 750 mg/m^2 were administered. Follow up tests included blood counts, immunoglobin levels (Ig), tetanus toxoid antibody (Tab) titers and lymphocyte subset assay at 1, 3, 6, 9, and 12 months. All immunizations were held during the 12 month study period.

Results: 28 patients were treated for thrombocytopenia (ITP) (21), hemolytic anemia (AIHA) (5), and neutropenia (2). The drug was well tolerated by all patients. A second and third administration at yearly intervals was required in one patient with hemolytic anemia. Doses were escalated in four patients and 2 responded. The response rate was 50% and was better in patients with ITP and AIHA. Partial response was noticed in four patients. Response was sustained during the follow up period. No patient developed infections or other late complications. Peripheral blood B cell numbers were depleted at one month and recovered between six and nine months following therapy. T cells were unaffected. Ig G, M, A were lowest between three and six months and subsequently recovered. Tab titres were lowest at 6 months and spontaneously recovered

***Conclusions*:** Rituximab is safe for the treatment of ITP and AIHA. Dose escalation may be indicated in some cases. B-cell mediated immune functions recover by one year. This treatment option should be considered in children prior to invasive interventions such as splenectomy.

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IRON STATUS, REM DEPRIVATION AND SPATIAL WATERMAZE PERFORMANCE IN RATS.

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Iron deficiency anemia (IDA) has long-lasting effects on cognition and behavior in humans, even into adolescence. Sleep disturbances are common in adolescence and rapid eye movement (REM) sleep is thought to be important for memory consolidation. No study has assessed for an additive effect of REM deprivation (REMD) on learning and memory after IDA during development.

Objective: To assess the effect of REMD on spatial learning and memory in rats that were iron sufficient (IS) or iron deficient (ID) during development.

Methods: IS (13) or ID (11), 40-day-old Sprague Dawley rats were randomly assigned (within diet group) to REMD or no REMD (REM-sated - REMS) treatments. ID rats began an IS diet on day 1. Hematocrit and serum iron were measured for all rats every 3–4 days. REMD consisted of 6 hours during the light cycle for 20 consecutive days. All rats had 8, 1-minute daily swim trials in the Morris watermaze and latency, speed, path length, and thigmotaxis (tendency to stay along the wall) were measured. On day 21, a single 1-minute probe trial was performed. Measures were compared by diet and treatment using Student's t-test and repeated measures ANOVA with significance $p<0.05$.

Results: ID hematocrit and serum iron were significantly lower than IS rats on d1 ($F=67.773$, $p<0.001$; and $F=76.235$, $p<0.001$, respectively), but reached IS levels within one week. Overall, IS rats swam faster than ID rats ($F=6.487$, $p<0.02$) and REMD rats swam faster than REMS rats ($F=7.077$, $p<0.005$). Controlling for swim speed, ID-REMD rats had shorter latencies than ID-REMS rats (weeks 2–3) ($F=3.498$, $p<0.04$). Latencies did not differ with REMD treatment for IS rats. There was a trend for ID-REMD rats to have a shorter path length compared to ID-REMS rats and for IS-REMD rats to have a longer path length than IS-REMS rats ($F=2.721$, $p<0.08$). Path length in the platform quadrant was greater for ID-REMD versus ID-REMS rats (week 3), and for IS-REMS versus IS-REMD rats (week 2) ($F=5.042$, $p<0.02$). Overall, ID rats had greater thigmotaxis than IS rats ($F=14.910$, $p<0.002$). REMD reduced thigmotaxis for ID rats (d13–20, $F=6.755$, $p<0.005$) but did not affect this measure for IS rats. On the probe trial, there were no significant differences by diet or REMD treatment.

Conclusion: Iron deficiency during development adversely affects performance in a spatial learning task. Paradoxically, REM deprivation appears to improve performance for formerly ID rats. Further work is needed to understand whether cognitive or non-cognitive factors underlie this improvement.