

2002 Research Grants

**Dr. Doug Brooks Women's and Children's Hospital
North Adelaide, South Australia , Australia
"GGA proteins and I-cell disease"**

New diseases involving proteins that control vesicle traffic within cells are currently being recognized and many display a similar clinical presentation to that seen in lysosomal storage disorder patients. A new group of molecules called GGA proteins (Golgi localized, gamma ear containing, ARF binding proteins) have been shown to be involved in vesicle traffic within cells and interact with the mannose-6-phosphate receptors that normally target and transport lysosomal proteins to lysosomes. In this project, we planned to investigate patients characterized as having either I-cell disease or MLIII. Some of these patients will obviously have a deficiency in the enzyme N-acetylglucosamine 1-phosphotransferase that has been shown to cause I-cell disease. It is now recognized that mutations in the two separate subunits of this enzyme give rise to a similar syndrome. However, a number of patients with symptoms consistent with I-cell disease may also represent a disorder that has not been previously described. We hypothesized that a defect in one of the GGA proteins should give an identical clinical presentation to I-cell disease, based on the inability to correctly traffic mannose-6-phosphate receptors. We proposed two specific project initiatives: 1. Develop monoclonal antibodies to specific regions on GGA proteins to allow the study of the cell biology and intracellular traffic of normal and mutant GGA proteins. 2. The characterization of I-cell and MLIII patients to identify potential defects in GGA proteins and vesicle traffic.

We have generated a panel of monoclonal antibodies and a specific polyclonal antibody to each of the GGA proteins; GGA1, GGA2 and GGA3. The antibodies produced, show specific reactivity to the respective GGA proteins and at least one antibody from each set detected denatured protein and showed reactivity on Western blots. Several of the monoclonal antibodies have had the positional location of their antibody reactivity mapped onto the respective GGA protein (ie. epitope mapped). We have developed immune quantification assays for determining intracellular levels of GGA proteins in cell extracts and optimized each of the respective assays. We have analyzed a panel of fibroblast cells and shown similar total levels of each GGA protein in I-cell patient (n=8) and control cell (n=7) lines. We have also analyzed these cell lines by immunofluorescence using the GGA antibodies, together with a panel of antibodies to lysosomal membrane markers, to determine the intracellular distribution of these markers. The study suggests that the panel of I-cell lines investigated have different patterns of marker distribution to the normal controls. Moreover, within the I-cell patient group there were at least two distinct patterns of marker distribution. We are currently investigating this altered marker distribution in relation to patient severity and the molecular defect in each patient.

**Gliddon BL and Hopwood JJ
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"Enzyme therapy in the CNS of MPS IIIA Mice"**

MPS IIIA results from the deficiency of the enzyme known as sulphamidase. The major hurdle in being able to treat MPS IIIA patients is enabling this enzyme to get into the brain (the central nervous system, or CNS) to reduce the amount of storage in brain tissue. In this project we used a MPS IIIA mouse model to evaluate enzyme replacement therapy (ERT) on the development of CNS by investigating:

1. the use of two doses of sulphamidase. Enzyme was injected into the temporal vein of mice from birth and the effect this had on reducing CNS pathology was assessed.

We used a laboratory-made form of the human enzyme using a process known as recombination. The resulting enzyme is known as recombinant human sulphamidase (abbreviated to rhNS). The mice were separated into different groups and some were treated with enzyme while others were not. In one group, MPS IIIA mice were given an injection of enzyme into their temporal vein. The enzyme doses used in this group were either 1 milligram of enzyme per kilogram of body weight (mg/kg) or 5 mg/kg rhNS on day 0 of life; in a second group MPS IIIA and unaffected mice were not given enzyme. All mice were given a 2nd intraperitoneal injection at one-week of age and a 3rd and final injection into their tail vein at two-weeks of age. All mice underwent testing to measure their ability to function at 13- and 15-weeks of age. One important measure of function we used was the gait width test, which tested the mouse's ability to walk. Between 6-10 weeks of age MPS IIIA mice display a characteristic narrowing of gait width when compared to unaffected mice. Treatment with rhNS from birth normalized gait width at both ages tested. Both enzyme doses were equally effective.

Samples of mouse brain and liver were taken at different time points after injection to determine both the effect of the enzyme and its distribution in these tissues. We observed a reduction in the amount of storage in all brain regions studied except one, the olfactory bulb (a part of the brain that is involved in giving us our sense of smell) at four-weeks of age in both of the treated groups of mice. Once again, there was no difference between the 1 mg/kg and 5 mg/kg groups.

A constant problem in trying to obtain large numbers of MPS IIIA mice occurs because the mothers of MPS IIIA mice have a tendency to eat their young. In this study we mated female MPS IIIA mice treated with rhNS with untreated male MPS IIIA mice and found that enzyme replacement from birth improves the ability of MPS IIIA mice to maintain their young.

MPS IIIA mice have an average life span of 10-12 months. Ten-months after enzyme injections, all untreated MPS IIIA mice had either died or been euthanased due to health issues. Importantly, at this age MPS IIIA mice treated with enzyme were still surviving.

Our results show that enzyme replacement from birth is effective in normalizing gait abnormalities observed in MPS IIIA mice and in reducing the amount of storage in various regions of the MPS IIIA mouse brain. A single dose of enzyme injected from birth is also effective in improving the reproductive outcome of MPS IIIA matings, and in increasing the life span of MPS IIIA mice.

2. assessing whether or not CNS pathology is reversible by directly injecting enzyme into the

brain of mice at various stages of their development.

MPS IIIA mice aged 6-, 12- and 18-weeks were injected with rhNS directly into two regions of the brain, the hippocampus and cerebellum (regions of the brain involved in memory and motor function, respectively). The brains of these mice were assessed at 24-weeks of age. Treatment was shown to reduce the amount of storage and other neurodegenerative changes in widespread areas of the MPS IIIA brain.

Dr. Rob Wynn

Manchester Children's Hospital, Manchester, England

"Autologous Stromal Stem Cells as a target of genetic manipulation in the management of MPSII (Hunter syndrome)"

Stromal stem cells can be easily taken from bone marrow, expanded in the laboratory and differentiated into cartilage and bone cells. They have been shown to be able to participate in the repair of bone fracture and correct inherited skeletal disorders such as Osteogenesis Imperfecta. Hunter's syndrome or MPSII is caused by the absence of an enzyme called Iduronidate-2-Sulphatase (I2S). This enzyme is important for the degradation of glycosaminoglycans (GAGs). A lack of this enzyme results in a multisystem disorder with developmental delay combined with bone and joint disease. Very limited benefit follows hematopoietic stem cell transplantation of MPSII, especially with regard to the skeletal disorder. Enzyme replacement therapy is under evaluation at present but seems to present a similar outcome with regard to the skeletal disease.

In this project we proposed to use stromal stem cells as target cells to correct the enzyme defect in Hunter's syndrome, particularly in the skeleton as these cells could take part in the bone formation. We wanted to test the hypothesis that stromal stem cells from Hunter's Syndrome could be corrected using a retroviral vector containing the I2S gene, and that the genetically modified stromal stem cells could generate bone expressing this transgene.

So far we have isolated stromal stem cells from two MPSII patients. The cells were able to expand in culture and differentiate into bone cells in a similar way to stromal stem cells isolated from normal donors. We have modified stromal stem cells from both MPSII patients and normal donors using a retroviral vector containing a marker gene called Green Fluorescent Protein (GFP). Cells containing this gene will appear green when exposed to a fluorescent light allowing for quantification. Over 90% of stromal stem cells from MPSII patients could be modified to produce GFP in a similar way to cells from normal donors. However introduction of the I2S gene into stromal stem cells of MPSII patients using a similar retroviral vector to the one used to produce GFP resulted in very poor correction of the enzyme with levels at best of 2.7% of normal. We hypothesized that this was due to the large size of the I2S gene, which made it more difficult to produce enough viral particles for efficient correction of the stromal stem cells. We have designed a new vector that can produce both genes, GFP and I2S, at the same time. Cells that produce GFP can be seen and separated from the rest. Once the separation has occurred we expected all of those cells to produce I2S. Indeed with this system I2S corrected marrow stem cells from MPSII patients showed levels of I2S enzyme 18 times higher than marrow stem cells from normal donors. Our visiting scientist from Brazil, who started working on this project in February, is now in the process of measuring levels of GAGs after selection of the cells and

correction of the enzyme deficiency. She is also looking at the ability of the corrected stromal stem cells to produce and secrete the I2S enzyme to correct the surrounding cells.

Parallel studies investigating the best conditions for transplantation of the stromal cells in the bone are also ongoing. These studies should enable us to assess the potential of using genetically modified marrow stromal cells for the therapy of skeletal disorders in MPSII patients by the end of the year.

In July 2002, The National MPS Society awarded three research grants. The recipients receive \$30,000 for each of the two years of funding. Following are the reviews of the first year of research:

R"GGA proteins and I-cell disease"

Dr. Douglas Brooks, Lysosomal Diseases Research Unit, Department of Chemical Pathology, Women's and Children's Hospital, North Adelaide, South Australia

New diseases involving proteins that control vesicle traffic within cells, are currently being recognized and many display a similar clinical presentation to that seen in lysosomal storage disorder patients. A new group of molecules called GGA proteins (Golgi localized, gamma ear containing, ARF binding proteins) have been shown to be involved in vesicle traffic within cells and interact with the mannose-6-phosphate receptors that normally target and transport lysosomal proteins to lysosomes. In this project, we planned to investigate patients characterized as having either I-cell disease or MLIII. Some of these patients will obviously have a deficiency in the enzyme N-acetylglucosamine 1-phosphotransferase that has been shown to cause I-cell disease. However, a number of these patients may also represent a disorder that has not been previously described. We hypothesized that a defect in one of the GGA proteins should give an identical clinical presentation to I-cell disease, based on the inability to correctly traffic mannose-6-phosphate receptors. We proposed two specific project initiatives: 1. Develop monoclonal antibodies to specific regions on GGA proteins to allow the study of the cell biology and intracellular traffic of normal and mutant GGA proteins. 2. The characterization of I-cell and MLIII patients to identify potential defects in GGA proteins and vesicle traffic. Progress in the first year of this project has been excellent.

PROGRESS:

Development of monoclonal antibodies to specific regions on GGA proteins: There are three known GGA proteins. We have generated a panel of 22 monoclonal antibodies with reactivity to GGA1 (6), GGA2 (10) and GGA3 (6). All of the antibodies show specific reactivity to the respective GGA proteins and at least one monoclonal antibody from each set, detects denatured protein and shows reactivity on Western blots. Several of the monoclonal antibodies have had the positional location of the antibody reactivity mapped on the respective GGA protein (epitope mapped). We have also generated specific polyclonal antibodies to each of the three GGA proteins. These antibodies will allow the development of immunoquantification assays for determining intracellular levels of normal and mutant GGA proteins and facilitate a more sophisticated functional analysis of the different domains of these proteins. Preliminary analysis of the monoclonal antibodies has shown reactivity in immunofluorescence experiments and this should allow the proposed high resolution confocal imaging of cells from "I-cell" patients. In the second year of this project we plan to investigate the cell biology of GGA proteins in I-cell patients and identify patients with potential defects in GGA proteins.

"Enzyme therapy in the CNS of MPS IIIA mice"

Dr. Briony Gliddon, Lysosomal Diseases Research Unit, Department of Chemical Pathology, Women's and Children's Hospital, North Adelaide, South Australia

Two major experiments were included in the proposal,

- 1) Dose dependent ERT in MPS IIIA mice from birth, and
- 2) Direct injections of enzyme into the CNS of MPS IIIA mice at various ages

Summary of 1)

Progress on this study has been very good, with most of the preparatory work now complete. Based on successful results with enzyme replacement therapy (ERT) of 1mg/kg rhNS intravenous injections into mucopolysaccharidosis type IIIA (MPS IIIA) mice from birth, it was decided to follow on from these experiments using enzyme doses 0.2, 1.0 and 5.0 mg/kg recombinant human sulphamidase (rhNS) to determine which dose gives greatest efficacy. Before enzyme can be used for therapy in the mice it is necessary to produce and purify large amounts of enzyme to a high quality suitable for injection into mice. This has now been achieved. We have also developed various tests to evaluate and compare the behavior and learning abilities of MPS IIIA and unaffected control mice and therefore critically assess the efficacy of ERT in MPS IIIA mice. Male MPS IIIA mice display an aggressive behavioral phenotype from 8-12 weeks of age. It was observed in the preliminary experiment whereby 1mg/kg rhNS was injected into MPS IIIA mice from birth that this aggressive phenotype was delayed, a positive result of the therapy. At that stage we had no test to quantify this aggression in the mice and it remained only qualitative. Consequently we are designing a test known as the resident-intruder test, which is commonly used to measure aggression in mice. This test will be employed in the dose-dependent ERT experiment. A large number of mice (80 MPS IIIA and 40 unaffected) are needed for this study to make the behavioral testing statistically significant. This requires an extensive breeding program to obtain such a large number of mice, in which it is necessary that they be age matched (ie born at least in the same week). Breeding has been ongoing throughout enzyme purification and behavioral test analysis and we are nearing our final round of breeding whereby injections will commence into the mice.

Summary of 2)

The study described in the grant has been completed. The aim of this study was to determine whether enzyme replacement directly into the CNS of adult mice can prevent, delay or even reverse the functional deficits and pathological features of MPS IIIA in mice. In preliminary experiments one group of mice received enzyme treatment through intracerebral injections into both the hippocampus and cerebellum, (the sites related to pathology and behavior deficits in MPS IIIA mice) at 6 weeks of age, the other at 12 weeks of age and another at 18 weeks of age. All animals were sacrificed at 24 weeks of age. In both 6 and 12 week treated animals, but not 18 week treated mice, enzyme treatment reduced the formation of axonal spheroids, anomalies found in the diseased brains of untreated MPS IIIA mice. This suggests that spheroids which seem to form between 12-18 weeks of age are irreversible. Enzyme treatment also abolished the neurodegenerative changes seen in the retrosplenial cortex of untreated MPS IIIA mice. The observation of a reduction in the number of lysosomal storage vacuoles in treated mice appeared to be related to time post treatment, where mice treated most recently ie: at 18 weeks old at treatment, 24 weeks old at sacrifice showed the greatest reduction in storage vacuoles. This suggests clearance of storage by enzyme occurs rapidly but that in the absence of continued enzyme delivery, storage vacuoles reappear. These preliminary results are very encouraging. We

plan to further enhance these studies with a greater number of mice and to compare the behavior and learning abilities of these treated mice with untreated controls.

Autologous Marrow Stromal Cells as a Target of Genetic Manipulation in the Management of MPS II.

Dr. Robb Wynn, Consultant Hematologist, Manchester Children's Hospitals, Manchester, England

We are grateful for the opportunity to report on the progress we have made during the first year of this two year grant from the National MPS Society Inc. We believe we have made very considerable progress in following the schedule of research as laid out in our original research schedule, and therefore towards achieving the aims of our research.

a) Isolation

Expansion and differentiation of Mesenchymal Stem Cells (MSCs) from patients with MPSII We have attempted to do this in two ways in our laboratory as we had originally stated. We have used plastic adherence and compared the properties of MSCs from MPSII patients with MSCs from patients with other MPS and from age-matched controls. We have also attempted to isolate MAPCs (Multipotent Adult Progenitor Cells) as described by Reyes et al in Minneapolis. We have not been able to isolate MAPC in this manner ? these difficulties have been shared by scientific groups elsewhere (personal communication). The isolation and use of MAPCs have in this last year been patented by the Minneapolis investigators and we have therefore concentrated on MSCs isolated by plastic adherence.

We have obtained MSCs from MPS II patients, although the number of patients has been less than anticipated as participation in ERT renders the patient ineligible for marrow donation, according to the terms of those trials. Our Research Ethics requires that the marrow be done under general anaesthesia during significant other surgery.

We do demonstrate that:

- the number of MSCs in MPS II marrow is comparable to age-matched control subjects - see Fig 1. The CFU-f assay expresses the number of MSCs per 10⁶ nucleated cells in the marrow
- the expansion of the MSC II MSC population is similar to that obtained from age-matched control MSCs ? see Fig 2.

We have also observed that the differentiation properties of the MPS II marrow are the same as age-matched MSCs. Taken together these data are consistent with our underlying hypothesis that MPS II MSCs are suitable targets for genetic manipulation.

b) Production of IDS retroviral producers

We have commenced work on what we had originally scheduled for the second year of the grant. We have confirmed that MSCs from MPS II patients are as good a target for genetic manipulation as other MSC clones in our hands. We have used A GFP vector (fig 4, below) to

obtain the same transduction frequency (up to 80-%).

We have gone on to prepare retroviral packaging cell lines and we have screened several of these clones by measurement of the IDS enzyme. We have several clones that have enzyme activity above the control we are continuing this process. MSCs of the MPS II patients will then will be exposed to retrovirus and enzymatic correction of the deficient tissue obtained.

c) The year ahead, and our research plans

In this year we plan to consolidate the research achievements of our first year. We have appointed a Brazilian scientist to join us for one year. We hope to have some more original marrow from MPS II patients to confirm our preliminary observations that the numbers and properties of these cells in MPSII is the same as that in age-matched controls. We will optimize the packaging cell line and transduction of MPS II MSC cells and confirm in vitro correction of the condition. In parallel we have other projects that are examining the homing of these cells to the sites where they will be required (CNS, bone) as well as their functional potential in vivo in models of MPS disease.

We are grateful to the Society for giving us the opportunity to pursue this research and, as we continue our studies, we hope to publish our work to allow others to learn from what we have achieved.

Research Final Reviews

Annual report on the grant: "Evaluation of Neonatal Gene Transfer in Dogs and Cats with MPS" funded in 2003 in a joint venture by the Ryan Foundation and the National MPS Society to Dr. Mark Haskins at the University of Pennsylvania and Dr. Katherine Parker Ponder at Washington University.

1. With your grant funds and the help of Dr. Emil Kakkis, we now have a dog colony of six heterozygous MPS I females and two heterozygous MPS I males to breed to produce affected pups. While not as many breeding animals as we need, it has been a good start.
2. Dr. Ponder made a retrovirus vector with the canine cDNA to treat affected MPS I pups.
3. While waiting for affected pups, we treated seven MPS I-affected kittens with the dog iduronidase vector. These animals expressed canine iduronidase for about 2 months before the serum activity was lost. Our best estimate is that the cats mounted an immune response to the liver cells making the dog protein. We are continuing to explore ways to prove what happened, and to clone the cat cDNA to make a cat-specific vector.
4. Dr. Ponder has treated a cohort of MPS I mice, with very encouraging results. When she gave a medium dose of vector to newborn mice, they had high serum α -L-iduronidase activity (1000 units/ml, with normal levels 1 unit/ml) and a normal hearing threshold and response to light in the retina at 8 months of age. In addition, echocardiograms and the bones appeared normal at this time. In contrast, the untreated MPS I mice were abnormal in all of these categories. Alpha-L-iduronidase activity was well above normal in all organs in the treated animals. Lysosomal storage was reduced in peripheral organs.
5. We have now treated six MPS VI kittens with a retroviral vector made by Dr. Ponder that contains the normal cat 4-sulfatase cDNA (a gift from Dr. John Hopwood). Two of the kittens are eight months old and have about 10 times the normal serum sulfatase activity, which is very encouraging. The cats have even higher liver sulfatase activity, much improved facial features, are less growth retarded, have improved skeletal radiographs, but continue to have cloudy corneas. The remaining 4 kittens are only 3 weeks post-treatment.
6. Last week we had our first MPS I puppy born, and she was treated with intravenous retrovirus vector containing the canine iduronidase cDNA. It is too early to tell if she is expressing enzyme, or what the effect will be, but we are optimistic.
7. The best news of all is that a grant to the NIH entitled "Gene Therapy for MPS I" that Dr. Ponder and I wrote a year ago was funded. If it had not been for the initial support of the Ryan Foundation and the National MPS Society

that provided us with a way to gather preliminary data, the grant application would surely not have been successful.

Thank you,

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