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Gene therapy for the mucopolysaccharidoses (review)

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Summary

The mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders in which the storage material is glycosaminoglycan. Each MPS is caused by the genetic deficiency of a single lysosomal enzyme. Due to the nature of these diseases and the characteristics of the enzymes that are deficient most of the MPS are good candidates for gene therapy. Studies in animal models have supported this contention and have shown that several different approaches to gene therapy for the MPS are possible. However, it is also clear that each of these approaches is limited by the currently available technology and that the development of new gene delivery technology is a priority.

I. Introduction

The lysosome is a small sub-cellular organelle which is one of the primary sites for the degradation of molecules such as proteins, nucleic acid, mucopolysaccharide and lipids. At least 40 different lysosomal hydrolases that are involved in these processes are known. The protein constituents of the lysosome are synthesised in the endoplasmic reticulum and traffic to the lysosome via the Golgi apparatus. For the soluble lysosomal enzymes trafficking is mediated by the presence of mannose-6-phosphate residues on the enzyme which are specifically recognised by the mannose-6-phosphate receptor resulting in targeting to endosomes and then lysosomes (Kornfeld and Mellman, 1989). The acidification of the endosome results in the release and subsequent recycling of the receptor. Other targeting mechanisms also exist, for example the LAMPs (lysosome associated membrane proteins) are targeted via a carboxy terminal tyrosine/glycine motif (Williams and Fukuda, 1990). Extracellular substrates for degradation are delivered to the lysosome via the endocytic pathway while intracellular substrates are delivered via autophagic vacuoles.

A large number of genetic diseases have been identified that are caused by deficiencies of any one of a number of specific lysosomal enzymes which results in the inability of the lysosome to degrade the substrate normally turned over by that enzyme. This results in the lysosomal accumulation of the undegraded substrate and the clinical development of a lysosomal storage disease. Examples of well known lysosomal storage diseases include Gaucher's disease (deficiency of glucocerebrosidase with resultant storage of glucocerebroside) and Tay-Sachs disease (deficiency of hexosaminidase A with resultant storage of GM2 ganglioside).

Lysosomal storage diseases that result from deficiency of a lysosomal transporter or other proteins that have an indirect effect on lysosomal enzyme activity are also known. Examples of the former include cystinosis, sialic acid storage disease and mucopolysaccharidosis type IIIC. Examples of the latter include sphingolipid activator protein deficiencies, I cell disease and multiple sulphatase deficiency.

The mucopolysaccharidoses (MPS) (Neufeld and Muenzer, 1995) are a group of lysosomal storage disorders (LSD) in which the material that is stored and excreted is glycosaminoglycan (GAG). There are ten known MPS disorders each of which corresponds to a unique single enzyme deficiency (**Table 1**). Each enzyme deficiency results in the storage and urinary excretion of one or more GAG types due to the obligatory exolytic nature of the enzymes involved in GAG degradation. In MPS types I and II both dermatan sulphate and heparan sulphate are stored and excreted, in MPS IIIA, IIIB, IIIC and IIID only heparan sulphate is involved, in MPS IVA keratan sulphate and chondroitin-6-sulphate, in MPS IVB only keratan sulphate,

in MPS VI only dermatan sulphate (it is thought that chondroitin-4-sulphate, which is also a substrate for N-acetylgalactosamine-4-sulphatase, the enzyme deficient in MPS VI, may be degraded by the action of an alternate enzyme, hyaluronidase) and in MPS VII chondroitin sulphate, heparan sulphate and dermatan sulphate are all stored. All of the MPS with the exception of MPS II are autosomal recessive disorders. MPS II is an X-linked recessive disorder. The genes involved in all of the MPS, with the exception of the gene encoding the Acetyl CoA: α-glucosaminide-N-acetyltransferase which is deficient in MPS IIIC, have been successfully isolated (**Table 1**) providing the basic raw material needed for gene therapy for these disorders.

MPS	Clinical syndrome	Enzyme	GAG Stored/Secreted	Gene Isolation (Reference)
MPS I	Hurler/Scheie	α-L-iduronidase	Dermatan sulphate, heparan sulphate	Yes (Scott et al., 1991)
MPS II	Hunter	Iduronate-2-sulphatase	Dermatan sulphate, heparan sulphate	Yes (Wilson et al., 1990)
MPS IIIA	Sanfilippo	Sulphamidase	Heparan sulphate	Yes (Scott et al., 1995)
MPS IIIB	Sanfilippo	N-acetylglucosaminidase	Heparan sulphate	Yes (Weber et al., 1996)
MPS IIIC	Sanfilippo	Acetyl CoA: α-glucosaminide-N-acetyltransferase	Heparan sulphate	No
MPS IIID	Sanfilippo	N-acetylglucosamine-6-sulphatase	Heparan sulphate	Yes (Robertson et al., 1988)
MPS IVA	Morquio	Galactose-6-sulphatase	Keratin sulphate, chondroitin-6-sulphate	Yes (Tomatsu et al., 1991)
MPS IVB	Morquio	β-D-galactosidase	Keratin sulphate	Yes (Morreau et al., 1989)
MPS VI	Maroteaux-Lamy	N-acetylgalactosamine-4-sulphatase	Dermatan sulphate, chondroitin-4-sulphate	Yes (Peters et al., 1990)
MPS VII	Sly	β-D-glucuronidase	Dermatan sulphate, heparan sulphate, chondroitin sulphate	Yes (Guise et al., 1985)

Table 1.

All of the MPS are progressive with many affected children appearing normal at birth. Severe cases are usually diagnosed within the first year or two of life. The clinical symptoms of the MPS vary but generally include several of the following, hepatosplenomegaly, skeletal changes (dystosis multiplex), stiff joints, corneal clouding, hirsutism, respiratory and cardiovascular dysfunction and central nervous system degeneration (Neufeld and Muenzer, 1995). The clinical phenotype of an individual patient with MPS is largely determined by the nature of the storage material and the severity of the enzymatic deficiency. For example MPS types IVA, IVB and VI in which the storage material is either keratan sulphate (IVA and IVB) or dermatan sulphate (VI) do not develop CNS pathology or associated symptoms while MPS types IIIA, B, C and D, in which only heparan sulphate is stored, have severe CNS disease and relatively mild somatic features. The tissues affected in each disease can be linked to the type and amount of GAG normally synthesised in the tissue. Established bone pathology and CNS deterioration are usually considered to be irreversible while much soft tissue pathology can be reversed. The most common causes of death in the MPS are due to cardiovascular and respiratory disease. Death normally occurs by or during the teenage years although affected individuals with "mild" disease may, in exceptional cases, live a near-normal life span.

A generally applicable treatment for the MPS must therefore be able to deliver replacement enzyme to a large number of sites in the body. On a cellular basis this is a reasonable proposition as the well characterised mannose-6-phosphate lysosomal targeting signal (Kornfeld and Mellman, 1989) and the complementary mannose-6-phosphate receptor provide an efficient mechanism for endocytosis, and the subsequent lysosomal targeting, of enzyme from the peripheral circulation (for example enzyme administered by intravenous injection or enzyme secreted from gene corrected cells after gene therapy). This mechanism provides the theoretical basis for enzyme replacement and gene replacement therapies and has now been well documented experimentally using recombinant sources of enzyme (Oshima et al., 1990; Anson et al., 1992b;

Bielicki et al., 1993; Unger et al., 1994; Bielicki et al., 1995; Islam et al., 1993). However some of the cells that need to be treated are only poorly exposed to the peripheral circulation. The prime example is the cells of the central nervous system which are separated from the circulation by the blood brain barrier. Another important example is the cells of the bone growth plate (such as chondrocytes) which are only poorly exposed to the circulation and show high levels of storage in many of the MPS. Enzyme replacement studies have shown that enzyme injected intravenously is very rapidly removed from the circulation with the major proportion being taken up by the liver. Other organs that receive relatively large amounts of enzyme are the spleen, kidney and lung (Sands et al., 1994; Kakkis et al., 1996; Crawley et al., 1997). Dose response studies in the MPS VI cat have demonstrated that the administration of large doses of enzyme from birth are required to have a major effect on moderating the development of skeletal pathology (Crawley et al., 1997). The amount of administered enzyme reaching the CNS in such studies is very small.

The experience with enzyme replacement studies suggests that gene therapy strategies that simply deliver enzyme to the peripheral circulation will not be a completely effective or generally applicable technology for treatment of the MPS. However, such strategies can theoretically be used to treat those MPS in which there is no CNS involvement such as types IVA, IVB and VI, or in the less severe forms of some of the other types in which CNS involvement is also not apparent, for example the Scheie form of MPS type I or mild forms of MPS type II. Most of these strategies are based on the genetic modification of cells that can be easily isolated, cultured and reimplanted such as fibroblasts and myoblasts. Of these most progress has been made with the use of fibroblasts.

II. Fibroblast mediated gene therapy for the MPS

Fibroblasts can be readily isolated from skin biopsies and grow well *in vitro* for a limited number of generations before senescing (the so-called Hayflick limit). The number of generations that a cell will grow for will vary from individual to individual but is usually between ten and twenty five (Hayflick and Moorhead, 1961). During *in vitro* culture fibroblasts are amenable to genetic modification with retroviral or plasmid expression vectors (Veelken et al., 1994; Elder et al., 1997). Retroviral vectors with reasonable titres can be used to effectively transduce 100% of the cells in a primary fibroblast culture and vectors are available that result in high levels of expression (Miller and Rosman, 1989; Hantzopoulos et al., 1989). Many studies have demonstrated successful *in vitro* genetic correction of fibroblasts from MPS patients and the ability of enzyme secreted by gene corrected cells to cross-correct (unmodified) MPS cells (Wolfe et al., 1990; Anson et al., 1992a; Bielicki et al., 1996; Taylor and Wolfe, 1994; Braun et al., 1993). These results have provided a foundation for attempts to reimplant genetically modified fibroblasts such that they serve as “enzyme factories” *in vivo*. Most of these studies have been done in laboratory mice, either in naturally occurring mouse models of MPS such as the MPS VII (gus^{mps}) mouse (Moullier et al., 1993) or in *nude* mice (Salvetti et al., 1995) in which expression of a human protein is tolerated and can be followed with specific reagents (such as monoclonal antibodies). More recently knockout mouse models for MPS I (Clarke et al., 1997) and VI (Evers et al., 1996) have also been generated. These models appear to accurately reflect much of the pathology of the corresponding human conditions and can provide a good basis for evaluating forms of therapy although the short time span over which symptoms develop in the mouse (which can be related to the short maturation time of mice) can be a severe limitation when testing some gene therapy protocols. A good example of this is the observation that the skeletal pathology found in the MPS VII mouse develops over the same time frame (approximately 4-6 weeks) as that required for bone marrow engraftment and haematopoietic repopulation; it is therefore impossible to analyse the effect that bone marrow transplantation, or any post-natal gene therapy procedure aimed at the PHSC, has on development of bone pathology as the pathology is already established by the point at which haematopoietic repopulation is complete.

Gene transduced fibroblasts expressing β-glucuronidase or α-L-iduronidase which have then been reimplanted in the form of neo-organs have been evaluated in the MPS VII (Moullier et al., 1993), and *nude* (Salvetti et al., 1995) mice, respectively. The neo-organ is formed by incorporation of the fibroblasts into a collagen gel containing PTFE fibres as a structural matrix. After implantation into the peritoneal cavity the neo-organ becomes vascularised enabling secreted enzyme to enter the circulation. An alternative method of implantation is to deposit a cell mass under the renal capsule (Heartlein et al., 1994). The results of the neo-organ experiments in mice using gene vectors expressing lysosomal enzymes have shown that only low levels of enzyme result from the treatment with most enzyme accumulating in the liver and spleen. In the MPS VII mouse the levels of enzyme reached levels likely to be therapeutic only in these two tissues (Moullier et al., 1993). Expression of β-glucuronidase from neo organs has also been evaluated in (normal) dogs. Implantation of one to six neo organs resulted in low levels of enzyme in the liver for at least 340 days (Moullier et al., 1995). One major limitation of the neo organ technology therefore appears to be the low levels of enzyme synthesised by the gene-corrected fibroblasts after re-implantation. In addition it is now also clear that the reimplanted fibroblasts have a limited *in vivo* life span (Kruger et al., 1997), probably due to apoptosis of the implanted cells, and that this is most likely responsible for a significant proportion of the decline in expression levels seen over time after implantation of gene-corrected fibroblasts (Moullier et al., 1993, Scharfmann et al., 1991;

Hoeben et al., 1993; Naffakh et al., 1995). This suggests that the technological imperative is to develop vectors which direct much higher levels of stable expression *in vivo* and to modify the apoptotic response of the cells, perhaps by over-expression of genes known to inhibit apoptosis (Kruger et al., 1997). In addition it would be helpful to evaluate this technology in more realistic larger animal models of the MPS and lysosomal storage disorders, such as some of the available cat (Haskins et al., 1992), dog (Schuchmann et al., 1989; Occhiodoro and Anson, 1996; Stoltzfus et al., 1992; Kaye et al., 1992) and caprine (Pearce et al., 1990; Thompson et al., 1992) examples that have been described.

III. Myoblast-mediated gene therapy for the MPS

Myoblasts are also considered as potential candidates for gene correction (Blau and Springer, 1995; Miller and Boyce, 1995). In this instance both *ex vivo* and *in situ* approaches to gene transduction into myoblasts have been considered (Salvatori et al., 1993; Sajjadi et al., 1994) although neither has been systematically tested with regard to the MPS or any other of the lysosomal storage diseases. There is a single study of myoblast mediated gene therapy, using an *ex-vivo* approach, in the MPS I dog (Shull et al., 1996). The muscle is a well vascularised tissue, is very metabolically active, and so appears well suited to the synthesis of large amounts of gene product. In addition a large mass of muscle is available for gene correction. The biology of the muscle therefore appears compatible with the requirements for its use as a target for MPS gene therapy in an analogous approach to that outlined above for fibroblast-mediated gene therapy. However, the *ex vivo* approach suffers from the poor efficiency with which myoblasts can be reimplanted into muscle while the *in situ* transduction requires vectors that are able to transduce non-replicating cells. Recent data suggests that recombinant adeno-associated virus vectors are promising vehicles for efficient gene transfer into myoblasts *in situ* (Fisher et al., 1997).

The one study of myoblast-mediated gene therapy in the MPS I dog (Shull et al., 1996) used an *ex vivo* approach in which cultured myoblasts were transduced with retrovirus carrying the α-L-iduronidase gene. Unfortunately the re-implantation of the gene corrected cells generated an immune response against α-L-iduronidase (the MPS I dog carries a null mutation (Stoltzfus et al., 1992) which was correlated with a rapid decrease in the levels of enzyme and the number of myoblasts containing the α-L-iduronidase gene.

The general applicability of myoblast-mediated gene therapy for the MPS needs to evaluated further especially as there now seems to be a vector, AAV, that is able to efficiently transduce myoblasts *in situ* (Fisher et al., 1997). It is possible that the myoblast may well be more suited for long-term expression of introduced genes than fibroblasts, especially if it is re-implanted into, or left in, its natural environment, the muscle fibre. This, and the use of muscle specific promoter elements, may allow myoblast mediated gene therapy to avoid the problems of down regulation of expression and apoptosis of transduced cells associated with fibroblast mediated gene therapy (see above).

IV. Bone marrow stem cell mediated gene therapy for the MPS

The long established use of bone marrow transplantation both in animal studies and in clinical treatments has demonstrated that in general terms the bone marrow is perhaps the ideal cell population for gene therapy. The biology of the haematopoietic system, in which a small population of very primitive haematopoietic stem cells (HSC) are used as the basis for the continuous generation of extremely large numbers of the variety of mature cells found in the periphery, means that by targeting this small stem cell pool for gene transfer a large population of gene-corrected cells can be continuously generated for the lifetime of the individual. More specifically bone marrow transplantation studies in animal models of (Taylor et al., 1992; Birkenmeier et al., 1991; Hoogerbrugge et al., 1988a; Breider et al., 1989; Gasper et al., 1984; Walkley et al., 1994) and patients with (Cowan, 1991; Hopwood et al., 1993; Hoogerbrugge et al., 1995) lysosomal storage disorders provide strong evidence that gene correction of the haematopoietic system is likely to be a viable approach for gene therapy of the LSD in general and the MPS in particular (Walkley et al., 1996). Ideally the development of a screening system for affected newborns (Meikle et al., 1997; Sweetman, 1996) combined with cord blood banking (Broxmeyer, 1995) would provide an opportunity to effect treatment before the development of clinical symptoms. Because of the irreversible nature of the skeletal and CNS pathology in the MPS, treating affected individuals presenting with clinical symptoms is always going to be less than 100% effective. In addition it is clear that the endogenous levels of lysosomal enzymes synthesised and secreted by the haematopoietic system are generally not high enough to be completely corrective, gene therapy therefore must be optimised in terms of expression levels if it is to offer improved efficacy over bone marrow transplantation.

One of the animal models that has provided evidence for the potential efficacy of bone marrow transplantation and hence HSC mediated gene therapy is the fucosidosis dog. It is this animal model that we are using, in collaboration with

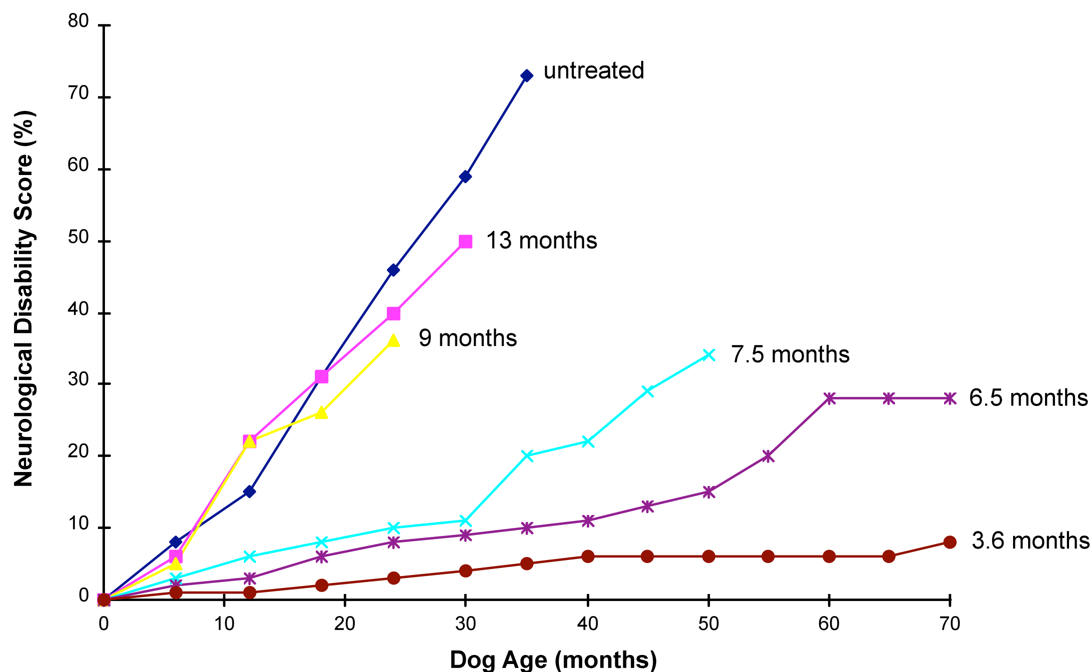

Figure 1. Effect of bone marrow transplantation on the development of canine fucosidosis.

The neurological disability score (y axis) is a quantitative assessment score for the development of the clinical disease associated with deficiency of α-L-fucosidase in the English Springer Spaniel. Each line represents an animal that received a transplant of normal allogeneic bone marrow at the age (in months) indicated. The results clearly show that bone marrow transplantation at 3.6 months is almost completely effective at preventing disease progression. Untreated animals usually require euthanisation at approximately three years of age.

the group at Westmead hospital (NSW, Australia), to evaluate this approach to gene therapy with special reference to the treatment of central nervous system pathology. Canine fucosidosis results from a 14 bp deletion at the end of the first exon of the gene encoding α-L-fucosidase which in turn results in a frameshift in the reading frame and a truncated protein (Occhiodoro and Anson, 1996). The clinical course of the disease is a progressive central nervous system deterioration manifesting first as mild hypermetria and ataxia then more overt and pronounced stance and gait defects and finally a severe mental and motor deterioration (Taylor et al., 1987; Taylor and Farrow, 1988). Euthanasia is normally required before approximately 40 months of age. Roseanne Taylor's studies of allogeneic bone marrow transplantation in the fucosidosis dog (Taylor et al., 1986; Taylor et al., 1988; Taylor et al., 1992) have convincingly demonstrated that this procedure results in significant enzyme replacement in a wide variety of tissues, including the central nervous system. Enzyme levels of up to approximately 50% of normal result in peripheral tissues and levels of up to approximately 25% of normal in the CNS. The accumulation of enzyme activity in the CNS is significantly slower than in peripheral tissues. This is thought to be a reflection of the slow accumulation of donor derived cells of haematopoietic origin in the CNS (see below). Assessment of the clinical course of the disease shows that if the bone marrow transplant is done before the development of significant pathology it also has a profound effect on the clinical progression of the disease as measured by an objective score of neurological disability. Animals receiving transplants at 3-4 months are almost completely normalised both in terms of the clinical progression of the disease and in terms of lifespan. In contrast bone marrow at later stages where overt disease pathology was already apparent had little effect on the continuing course of the disease (**Fig. 1**).

In the fucosidosis dog we therefore have an animal model that demonstrates that gene correction of the pluripotent haematopoietic stem cell can result in correction of the central nervous system disease associated with the

MPS. It is therefore an ideal model for developing and testing (autologous) stem cell mediated gene therapy which aims to reproduce and improve on the results from allogeneic bone marrow transplantation. It tells us when we need to do the procedure, how much enzyme we need the cells to make and what results we may expect from a positive experiment.

Other animal models of lysosomal storage diseases have also provided evidence for the potential efficacy of bone marrow transplantation and have provided some evidence for the mechanism by which bone marrow transplantation results in enzyme replacement in the CNS. In the α-mannosidosis cat bone marrow transplantation also halts disease progression and results in clearance of storage material from neuronal tissue (Walkley et al., 1994). In this case bone marrow transplantation was done at an age (8 to 12 weeks) by which time overt clinical symptoms are already apparent. Despite this the procedure appeared to be clinically efficacious. Histological staining for α-mannosidase activity revealed the presence of enzyme positive neurones, glial cells and cells associated with blood vessels. Bone marrow transplantation studies in the twitcher (galactosylceramidase deficiency/Krabbe's disease) mouse has provided similar results, BMT results in enzyme replacement in the CNS and decrease of the levels of stored substrate, is partially effective in preventing clinical disease, triples the life span and donor marrow derived cells can be detected in the CNS of transplanted animals (Hoogerbrugge et al., 1988a, b). These were identified as glial cells. Transplantation of bone marrow marked with a retroviral vector containing the human glucocerebrosidase gene has confirmed the penetration of the CNS by bone marrow derived cells in mice. In this instance immunohistochemical analysis using a monoclonal antibody to human glucocerebrosidase revealed the presence of perivascular and parenchymal microglia of donor origin (Krall et al., 1994). As this study was done in normal mice it suggests that colonisation of the CNS by donor derived cells is not related to the presence of a pathological state, however it is possible that it is related to the effects of the bone marrow transplantation procedure itself. Experiments in animals receiving no radiation or chemical pre-conditioning could resolve this point.

Clinical trials of allogeneic bone marrow transplantation in patients suffering from various of the MPS are also somewhat encouraging although with important caveats. Bone marrow transplantation in patients with MPS I for instance, clearly moderates the progression of the disease (Hopwood et al., 1993, Hoogerbrugge et al., 1995) but is equally clearly not curative, especially for skeletal problems, the development of which seem to be little affected by the procedure. Similarly bone marrow transplantation in MPS VI will moderate some of the soft tissue pathology but has no discernible affect on the skeletal pathology (Hoogerbrugge et al., 1995). The success of bone marrow transplantation in the fucosidosis dog has led to this procedure being trialed clinically (Vellodi et al., 1995). Bone marrow transplantation is curative for non-neurological Gaucher's disease (Ringden et al., 1995, Hoogerbrugge et al., 1995) but in this instance it should be noted that the cells responsible for symptomatology, macrophages, are a haematopoietic lineage.

Taken together the results obtained in animal models and clinical trials of bone marrow transplantation clearly show that targeting of the PHSC for gene transfer is likely to be an effective way of achieving enzyme replacement in the CNS in lysosomal storage diseases including the MPS.

However, it should also be noted that certain of the lysosomal storage diseases, for example Batten's disease (Lake et al., 1995) and GM1 gangliosidosis (O'Brien et al., 1990), do not appear to respond to bone marrow transplantation. In the case of Batten's disease this may be due to the physical nature of the protein involved which may be membrane bound (The International Batten Disease Consortium, 1995) preventing secretion and hence cross-correction of other cells. Other possible reasons for lack of clinical efficacy include low levels of enzyme secretion by haematopoietic cells colonising the CNS or the existence of pre-existing storage and/or pathological damage that cannot be reversed. The reasons for the variability in response of LSD and MPS to BMT need to be studied further and may help define when this approach to gene therapy will be appropriate.

The only convincing HSC mediated gene therapy experiment relevant to the LSD has been done in the MPS VII mouse (Wolfe et al., 1992). In the MPS VII mouse retroviral mediated transfer of the β-glucuronidase gene into HSC resulted in significant enzyme replacement in one animal in bone marrow (26% of normal), spleen and lymph node (6%), thymus and liver (2%) and lung and liver. This resulted in reduced storage in tissues where enzyme was detected. A second animal analysed had lower levels of enzyme and enzyme was only detectable in the bone marrow and spleen (<1% of normal). Experiments addressing HSC transduction in larger animal models and humans have been frustrated by the very low levels of long term HSC transduction obtained (Bienzle et al., 1994; Kiem et al., 1995; Donahue et al., 1996; Xu et al., 1995; van-Beusechem et al., 1995; van-Beusechem and Valerio, 1996; Hanania et al., 1996) indicating the presence of technical limitations in the vector systems used. The low level of transduction of long term repopulating HSC seen in non-murine animals and in humans appears to result from a basic incompatibility between the vector system used (murine leukemia virus (MLV) based retroviral vector systems) and the biology of primitive HSC. MLV and gene transfer systems based on MLV have been shown to be incapable of infecting/transducing non-cycling cell populations (Roe et al., 1993, Miller et al., 1990). In addition it has been convincingly shown that the most primitive HSC populations are extremely quiescent, both *in vivo* and *in vitro* (Stewart et al., 1993; Hao et al., 1995; Berardi et al.,

1995; Hao et al., 1996). Extensive attempts to overcome this incompatibility between MLV vectors and the biology of the primitive HSC by stimulating the latter with cytokines to induce cell cycling have been made (Donahue et al., 1996; Xu et al., 1995; van-Beusechem et al., 1995; van-Beusechem and Valerio, 1996; Hanania et al., 1996). However, the results of this approach to achieving high levels of gene transfer have been disappointing. Long term haematopoietic cell culture systems have also been used as the basis for transduction protocols (Bienzle et al., 1994; Kiem et al., 1995) and while appear in some instances (Bienzle et al., 1994) to give somewhat better results have not convincingly resulted in efficient transduction of the most primitive HSC.

In summary HSC mediated gene therapy appears likely to be a viable approach for the treatment of the MPS but must aim to improve on bone marrow transplantation in terms of the degree of enzyme replacement afforded by the procedure so enzyme replacement is both more complete and more rapid. This will depend on the development of an efficient transduction system for the long term repopulating HSC. In addition it is likely that hyper-expressing vectors will need to be developed. Given that only qualified success has been achieved with bone marrow transplantation in MPS patients we feel that gene therapy procedures must, if at all possible, be evaluated in relevant animal models before clinical use is considered.

Our first attempts to develop stem cell mediated gene therapy in the fucosidosis dog have been based on the use of retroviral vector systems derived from MLV. In an attempt to improve on the generally disappointing results of others in regard to transducing the canine PHSC we used multiple cytokines to stimulate the bone marrow during transduction. These experiments were unsuccessful and will not be discussed here. However, we like many others have come to the conclusion that for this approach to gene therapy to succeed several challenges have to be faced in terms of the gene transfer technology used. It is apparent that for successful stem cell gene therapy we need a vector with certain features including:

i. An ability to efficiently transduce non-cycling cell populations (the stem cell pool is generally regarded as quiescent).

ii. An ability to stably integrate into the host cell genome (so that the transduced gene is retained as the stem cell population divides and expands to produce large numbers of differentiated cells).

iii. If transduction is receptor-mediated the vector particle must recognise a receptor expressed at reasonably high levels on stem cells.

iv. High level and stable expression of the transduced gene.

v. If the vector is viral-based it must be completely defective, of high titre, and demonstrably safe.

In my view these criteria can only be met by a viral vector, of the known viruses retroviruses and adeno-associated virus appear to be the obvious contenders. At present it is unclear if adeno-associated virus can be developed into an efficient, helper free integrating vector system for the PHSC while MLV based retroviral vectors are unable to effectively transduce quiescent cell populations. However in almost all other regards retroviral systems appear to be well suited for transduction of the haematopoietic stem cell. Integration of the virus into the host cell chromosome is a normal part of the viral life cycle, retroviruses appear to be easy to pseudotype with envelopes not only from other retroviruses (Eglitis et al., 1988; Wilson et al., 1989) but also from other viral groups (Ory et al., 1996) suggesting that if a suitable envelope/receptor combination can be found it can be adapted to a retroviral system. In addition the development of third generation retroviral packaging systems have shown that it is possible to produce recombinant retrovirus with a very small probability of contamination with replication competent virus (Markowitz et al., 1988; Cosset et al., 1995). This leaves two problems, the inability of Murine Leukemia virus based vectors to transduce quiescent cell populations (Miller et al., 1990) and the question of what levels of expression can be achieved by retroviral constructs *in vivo* in haematopoietic lineages. The first of these appears to be a more fundamental question and in some ways the second is undefined until the first can be overcome, I will therefore disregard it for the present.

Is there an answer to the conundrum presented by the incompatible biology of the HSC and MLV? Attempts to manipulate HSC into cycle to facilitate transduction with MLV based vectors have been largely unsuccessful (outside of the laboratory mouse) (Donahue et al., 1996; Xu et al., 1995; van-Beusechem et al., 1995; van-Beusechem and Valerio, 1996; Hanania et al., 1996), there is little effect on transduction efficiency either due to inefficient recruitment of the most primitive HSC into cycle and/or due to concomitant induction of cell cycling and differentiation. *In vitro* studies of human haematopoietic progenitor cells suggest that the most primitive cells are only very slowly recruited into the cell cycle even when cultured with extremely potent combinations of cytokines (Hao et al., 1996). At present this avenue would therefore appear to be effectively closed although new developments in the understanding of stem cell biology and regulation may one day make it viable. Very recently it has been shown that a combination of Flk3 ligand and thrombopoietin appears to support the amplification and self renewal of very primitive haematopoietic progenitors in cord blood (Piacibello et al., 1997) although it is not clear if this includes the true long term repopulating HSC. However this culture system would provide an ideal milieu for efficient retroviral transduction.

The other avenue that is being explored by an increasing number of groups including ourselves is to look for alternative retroviruses that do not have the same limitations

as Murine Leukemia virus. Members of the lentivirus family appear to be able to infect at least some populations of quiescent cells, a prime example of this is the ability of human immunodeficiency virus (HIV) to infect terminally differentiated macrophages *in vivo* (Koenig et al., 1986). *In vitro* studies have extended this observation (Lewis et al., 1992) and shown that the ability of HIV to infect non-cycling cell populations is mechanistically linked to nuclear localisation sequences in the matrix protein (Bukrinsky et al., 1993) and Vpr (Heinzinger et al., 1994). Several groups have demonstrated the feasibility of making recombinant viral vectors from HIV and shown that the recombinant virus produced has the same ability to infect non-cycling cells (Reiser et al., 1996; Naldini et al., 1996; Poeschla et al., 1996). It remains to be seen whether safety issues associated with the use of HIV as a gene vector can be convincingly addressed and whether such vectors are more effective in transducing the haematopoietic stem cell pool or if other factors are limiting. However it is clear that alternatives for vector development do exist and need to be carefully evaluated in relevant animal models and pre-clinical studies. In addition other important factors, such as the distribution of different envelope receptors and mechanisms of gene regulation in vectors need to be investigated at both the basic research and applied levels. Large outbred animal models of human disease will be invaluable in defining the important parameters involved in making this approach to gene therapy a success and in proving its viability as a clinical treatment.

V. Other approaches and future directions

A. Direct treatment of the CNS in the mucopolysaccharidoses via gene replacement

As the CNS is considered one of the more intractable sites of pathology in the CNS some consideration has been given to developing therapeutic strategies aimed narrowly at correcting CNS pathology. Two approaches have been considered:- introduction of (genetically modified) cells into the CNS or direct gene modification of CNS resident cells. Both appear to be applicable to the treatment of the MPS, however although much generic work has been done on the development of gene transfer vectors suitable for direct transduction of the CNS there is no published work on the use of these systems with MPS genes or in MPS animal models. Transplantation of an immortalised neural progenitor cell line expressing β-glucuronidase into the brains of MPS VII mice has been reported and is effective in preventing lysosomal storage (Snyder et al., 1995). Similar results have more recently been demonstrated with fibroblast implants (Taylor and Wolfe; 1997) although there is clearly a problem with the stability of expression of the transduced gene.

B. Prenatal gene therapy

It is known that even though in most instances of the MPS there is no overt clinical presentation at birth lysosomal storage already exists and can also be found for a significant period prenatally. Prenatal gene therapy may therefore be required for completely effective treatment of some severe instances of the MPS. This form of therapy can be approached in several ways. The most immediate technology is that of prenatal allogeneic bone marrow transplantation in the pre-immune phase of foetal development (Cowan and Golbus, 1994, Touraine, 1996) (approximately the first trimester). Although this is not strictly gene therapy this approach has already been used for the treatment of X-linked severe combined immunodeficiency (Flake et al., 1996; Wengler et al., 1996) and could be extended by using allogeneic marrow that has been transduced with a gene vector to boost expression of the required enzyme. The use of neural progenitors to treat the brain (see above) is also applicable to the developing foetus. The other approach is to directly introduce gene vectors into the foetus to allow *in vivo* gene correction to occur (Clapp et al., 1995). This can either be aimed at the developing haematopoietic system or other tissues.

VII. Conclusion

Clinically gene therapy for the MPS has barely reached its infancy. The only ongoing clinical trial at present (July 1997) is for MPS type II in which peripheral T lymphocytes are the target for gene transduction (Whitley et al., 1996). However enzyme replacement studies, bone marrow transplantation in animal models, and in the clinic, and proof of concept experiments for gene correction all suggest that if some clearly defined technical challenges can be met gene therapy has the potential to be an effective treatment for the MPS. In my view gene transfer into the haematopoietic stem cell is likely to be the most effective and generally applicable approach, unfortunately this approach probably faces the most severe technical challenges. However, there are a number of other approaches that are clearly applicable to many of the MPS disorders that may be easier to develop to the point of clinical applicability. All these approaches need to be further developed and carefully evaluated in animal models before clinical trials proceed.

Pre-symptomatic screening of newborns for LSD is also a prerequisite for effective clinical treatment. Screening strategies based on measuring lysosomal markers using ELIZA type assays (Meikle et al., 1997), or storage material by tandem mass spectrometry (Sweetman, 1996), are being developed.

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