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Transfer of Proα2(I) cDNA Into Cells of a Murine Model of Human Osteogenesis Imperfecta Restores Synthesis of Type I Collagen Comprised of α1(I) and α2(I) Heterotrimers In Vitro and In Vivo

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Abstract The *oim* mouse is a model of human Osteogenesis Imperfecta (OI) that has deficient synthesis of proα2(I) chains. Cells isolated from *oim* mice synthesize α1(I) collagen homotrimers that accumulate in tissues. To explore the feasibility of gene therapy for OI, a murine proα2(I) cDNA was inserted into an adenovirus vector and transferred into bone marrow stromal cells isolated from *oim* mice femurs. The murine cDNA under the control of the cytomegalovirus early promoter was expressed by the transduced cells. Analysis of the collagens synthesized by the transduced cells demonstrated that the cells synthesized stable type I collagen comprised of α1(I) and α2(I) heterotrimers in the correct ratio of 2:1. The collagen was efficiently secreted and also the cells retained the osteogenic potential as indicated by the expression of alkaline phosphatase activity when the transduced cells were treated with recombinant human bone morphogenetic protein 2. Injection of the virus carrying the murine proα2(I) cDNA into *oim* skin demonstrated synthesis of type I collagen comprised of α1 and α2 chains at the injection site. These preliminary data demonstrate that collagen genes can be transferred into bone marrow stromal cells as well as fibroblasts in vivo and that the genes are efficiently expressed. These data encourage further studies in gene replacement for some forms of OI and use of bone marrow stromal cells as vehicles to deliver therapeutic genes to bone. J. Cell. Biochem. 83: 84–91, 2001.

Key words: osteogenesis imperfecta; collagen; gene transfer; bone marrow; gene therapy

Osteogenesis Imperfecta (OI) is a heterogeneous group of genetic disorders that affect connective tissue integrity, with bone fragility being the major clinical feature. Most forms of OI are the result of mutations in the genes that encode the proα1 and proα2 polypeptide chains of type I collagen, the major protein of bone [Kuivaniemi et al., 1991; Byers and Steiner, 1992; Prockop et al., 1993, 1994; Tilstra and Byers, 1994]. Because OI is an incurable genetic disease, cell and gene therapy are being investigated as potential treatments [Colige et al., 1993; Jaspal et al., 1994; Laptev et al., 1994; Wang and Marini, 1996; Grassi et al., 1997; Marini and Gerber, 1997; Pereira et al., 1998; Horwitz et al., 1999; Prockop and Kivirikko, 1999]. Gene therapy for genetic diseases involves replacement of a defective gene with a normal gene [Crystal, 1995; Evans and Robbins, 1995]. Although OI is a genetic disease, it is a complex genetic disease that affects most connective tissues in which type I collagen is the major structural protein. Most cases of OI result from mutations, which substitute the conserved

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glycine with a different amino acid [Prockop et al., 1993, 1994]. Glycine is the smallest amino acid that can occupy the restricted space in the center of the triple helix when the three chains assemble. Amino acids with bulky side chains in this position destabilize the triple helix resulting in the degradation of all the three chains. The antisense gene therapy approaches being investigated by some investigators for the treatment of OI are aimed at eliminating the collagen alleles that encode the defective collagen chains. The aim here is to convert a dominant negative mutation into a mild one [Colige et al., 1993; Jaspal et al., 1994; Laptev et al., 1994; Wang and Marini, 1996; Grassi et al., 1997; Marini and Gerber, 1997]. Once the mutant collagen alleles have been eliminated, normal collagen genes could be transferred to the cells. These approaches may lead to the synthesis of a sufficient number of normal collagen molecules to restore tissue integrity. In addition, OI mutations that involve absence or non-expression of mutant collagen genes could potentially be treated by supplying normal collagen genes.

To explore the above possibilities, we demonstrate here that transfer of a murine cDNA into cells deficient in the synthesis of proα2(I) chains results in the synthesis of normal type I collagen consisting of α1(I) and α2(I) heterotrimers with α1(I) and α2(I) chains in a 2:1 ratio. In addition injection of an adenovirus carrying the murine proα2(I) cDNA into the skin of mice deficient in pro α2(I) collagen synthesis results in the synthesis of type I collagen consisting of α1 and α2 heterotrimers at the injection site. The cDNA was transferred into bone marrow stromal cells that were harvested from a mouse model of human OI (oim) [Chipman et al., 1993; Balk et al., 1997]. The homozygous mice have deficient synthesis of proα2(I) chains [Chipman et al., 1993; Balk et al., 1997]. Cells from the mouse model of OI do not express proα2(I) chains due to a nucleotide deletion in the region that encodes the carboxyl-terminal domain of the pro α2(I) chain [Chipman et al., 1993]. This mutation leads to non-incorporation of proα 2(I) chains into heterotrimers resulting in the accumulation of α1 (I) homotrimers in tissues. The present findings demonstrate the feasibility of collagen gene transfer into bone marrow stromal cells and encourage further investigation in the use of these cells to deliver collagen genes to bone.

METHODS

Vector Construction

The recombinant adenoviral vector used in the present study is a first generation replication deficient type 5 adenovirus that lacks the E1 and E3 loci [Crystal, 1995; Yeh and Perricaudet, 1997]. The murine proα2(I) cDNA that was cloned in SP72 vector was released from plasmid by Hind III and Bam HI. The murine collagen cDNA was inserted into the E1 region, with expression under the control of the human cytomegalovirus (CMV) early promoter. High titer suspensions of the recombinant adenovirus were prepared following the methods described previously [Hardy et al., 1997; Yeh and Perricaudet, 1997]. The murine proα2(I) cDNA was inserted into the pAdlox shuttle plasmid under the control of the regulation of the CMV promoter. A confluent 10 cm dish of CRE8 cells (1.6×10^7) was plated into 6 cm dishes and grown at 37°C for 4 h. Transfection of these cells with pAdlox-murine proα2(I) cDNA was performed by the calcium phosphate precipitation method with 3 μg of ψ 5-helper virus DNA. The media were changed 16 h postinection. The transfected CRE8 cells were carefully fed daily until there were large scale plaques. The cells were isolated and released by allowing seven passages of the virus on Cre8 cells and freezing/thawing the viral lysate. The virus was purified using cesium chloride gradient ultra-centrifugation at 30,000 rpm. The virus was dialyzed against Tris saline buffer pH 7.4 and the titer was determined by optical density: viral particles = OD at $260\lambda \times$ Dilution/9.09×10^{-13}.

Bone Marrow Stromal Cell Isolation

Bone marrow stromal cells were established by flushing the marrow from the femurs of oim mice into T-25 flasks. The cells were maintained in culture without disturbance for 7 days and then non-adherent cells were removed. The adherent cells were maintained in culture with media changes every 2 to 3 days as described previously [Balk et al., 1997; Oyama et al., 1999].

Transduction of Bone Marrow Stromal Cells

Bone marrow stromal cells from oim mice were plated in 6-well plates in DMEM supplemented with 10% FBS and 50 μg/ml ascorbic acid. At confluence, the media were removed

and the cells were transduced by adding 750 μl of a high titre virus (5×10^{12} particles/ml) encoding the murine proα2(I) cDNA (Adeno-Collagen cDNA) at 50 and 100 MOI (multiplicity of infection). The cells were incubated with the virus for 2 h with agitation every 20 min. After 2 h, the transduced cells were transferred to DMEM supplemented with 10% FBS and 50 μg/ml ascorbic acid and incubated further for 24 h to allow the cells to recover. The cells were then analyzed for collagen production as outlined below.

Analysis of Collagen Synthesis

The transduced cells were transferred to serum free media supplemented with 50 μg/ml β-aminopropionitrile, 50 μg/ml ascorbic acid, and 10 μCi/ml of [^{3}H] proline (specific activity 1.92 TBq/mmol) to analyze for collagen synthesis using the methods described previously [Balk et al., 1997; Oyama et al., 1999]. Briefly, after 24 h of incubation at 37°C in 5% CO_2, the medium and cell layer were harvested and in some experiments combined, adjusted to 0.5 M acetic acid and treated with pepsin at 100 μg/ml for 4 h at 4°C. Solid NaCl was added to the pepsin digests to a final concentration of 3.0 M NaCl and stirred at 4°C for 18 h. The pepsin resistant chains were recovered by centrifugation and were then dialyzed against 0.05 M NH_4CO_3. The dialysates were freeze-dried and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) followed by autoradiography.

Alkaline Phosphatase Activity

To determine whether the transduced cells retained their osteogenic potential, the transduced cells were plated in 48-well plates and treated with 100 ng/ml of rhBMP-2 every the other day for six days. On the 7th day, the cells were subjected to freeze thaw cycles and the lysates were analyzed for alkaline phosphatase activity (ALP) using the Sigma ALP assay kit as described previously [Balk et al., 1997].

Expression of proα2(I) Chains In Vivo

Oim mice were anesthetized and were then injected intradermally with 5×10^9 viral particles of the Adeno-proα2(I) cDNA in 50 μl of GBBS (Gey's balanced salt solution). The injected sites were marked with ink for easy identification. Saline was injected at different locations into the skin of the oim mice that received the Adeno-collagen cDNA vector and served as controls. The mice that were injected with the adenovirus encoding the collagen gene were sacrificed at 7 days after injection and tissues were harvested from the virus injected sites as well as the saline injected sites. Collagens were extracted from the harvested tissues by mild pepsin digestion and analyzed by SDS–PAGE.

Analysis of the Proteins in the Cells and Medium Conditioned by the Transduced Cells

To determine whether the collagens synthesized by the transduced cells were efficiently secreted, the distribution of the collagens synthesized by the transduced cells in the medium and in the cells were determined. After cell transduction with the adeno-collagen cDNA, the cells were incubated at 37°C, in 5% CO_2 in presence of the [^{3}H] proline for 24 h. After 24 h, the medium was removed and to the cell sheet, water was added, and the cells were subjected to freeze thaw cycles two times. The medium proteins and the proteins in cell lysates were separately digested with 100 μg/ml of pepsin in 0.5 M acetic acid for 2 h. The pepsin resistant chains were precipitated with 3 M NaCl and analyzed by SDS–PAGE followed by autoradiography.

RESULTS

The murine proα2(I) cDNA was originally cloned in SP72 vector [Phillips et al., 1991, 1992]. The proα2(I) cDNA was removed from the plasmid by Hind III and Bam HI restriction enzymes and the proα2(I) cDNA was then inserted into the E1 locus of the type 5 replication deficient adenovirus (Fig. 1). The murine proα2(I) cDNA is expressed off the human CMV early promoter. A high titre recombinant virus of 5.18×10^{12} particles/ml was generated and was used to transduce the murine bone marrow stromal cells deficient in the proα2(I) collagen synthesis.

Transduction of Bone Marrow Stromal Cells and Collagen Analysis

Initially, bone marrow stromal cells were transduced with the Adeno-proα2(I) collagen cDNA at different MOIs to determine the optimal conditions that resulted in the maximal synthesis of type I collagen comprised of α1(I) and α2(I) chains in a correct ratio of 2:1. Trans-

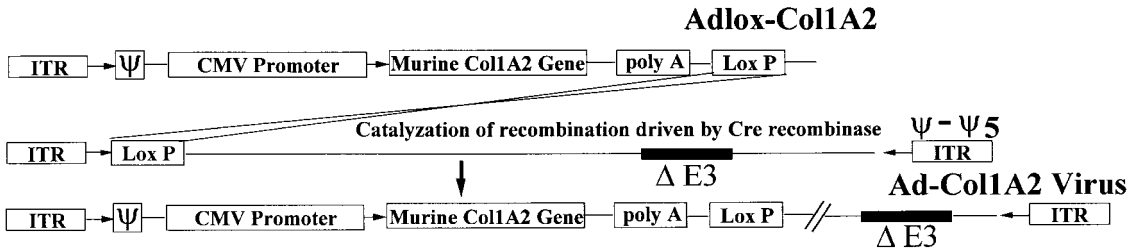

Fig. 1. Schematic diagram of the Adeno-proα2(I) cDNA construct. The murine proα2(I) cDNA was inserted into the E1 locus of the adenovirus. The collagen gene is under the control of the CMV early promoter.

duction of the cells at 50 MOI, resulted in the synthesis of type I collagen comprised of α)1(I) and α2(I) chains in approximately 2:1 ratio (Fig. 2, lane A). When the cells were transduced with the virus at 100 MOI, the cells did not survive, only the cells that did not pick up the virus survived (Fig. 2, lane B). Cells from normal littermates synthesized type I collagen consisting of α1(I) and α2(I) heterotrimers while the cells from oim mice synthesized α1(I)-homotrimers as expected (Fig. 2, lanes C and D respectively).

Collagen Secretion by the Transduced Cells

To determine whether the transduced cells secreted the collagen efficiently, the distribution of the collagens synthesized by the transduced cells in the medium and in the cells was determined. As Figure 3 shows, the α1(I) homotrimers synthesized by the oim cells, were efficiently secreted into the medium conditioned by the cells (Fig. 3, lane C), very little collagen was detected in the cell fraction (Fig. 3, lane D). Analysis of the collagens synthesized by the transduced cells also demonstrated that the type I collagen synthesized by the cells was efficiently secreted into the medium with very little collagen remaining within the cells (Fig. 3, lanes A and B). These data demonstrate clearly that gene transduction did not interfere with the ability of the cells to process and secrete the synthesized collagens efficiently.

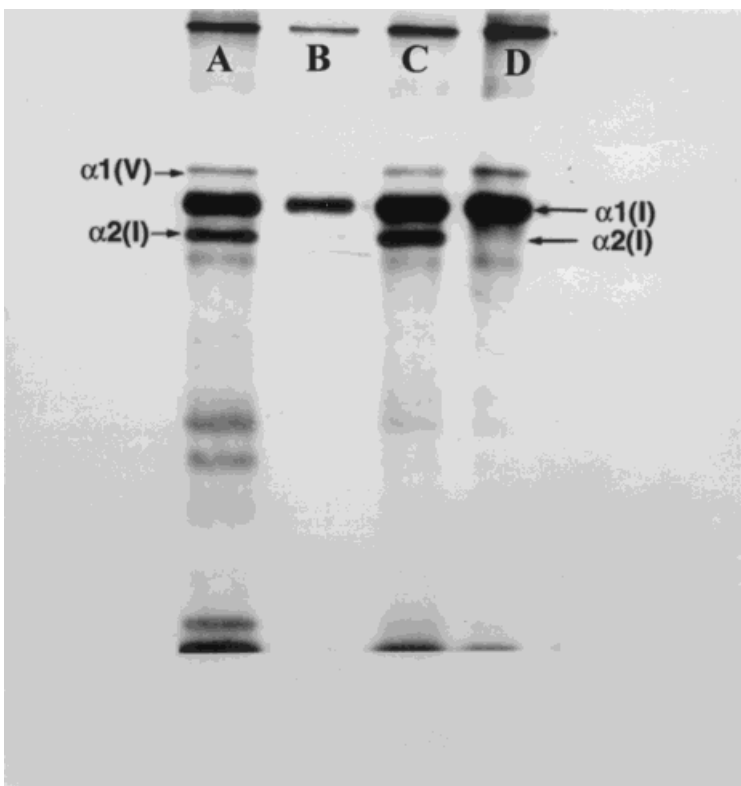

Fig. 2. Autoradiograph of pepsin resistant collagen chains synthesized by non-transduced and transduced bone marrow stromal cells from *oim* mouse. *Oim* mouse cells were transduced with the Adeno-proα2(I) cDNA at 50 and 100 MOI. At 50 MOI the cells synthesized α1(I) and α2(I) chains in the correct ratio of 2:1, (**lane A**) like the normal littermates (**lane C**). At 100 MOI the cells that picked up the virus died (**lane B**). The non-transduced cells synthesize α1(I) chains as expected (**lane D**). Extensive degradation products are evident in lane A, presumably due to the non-incorporated α2(I) chains.

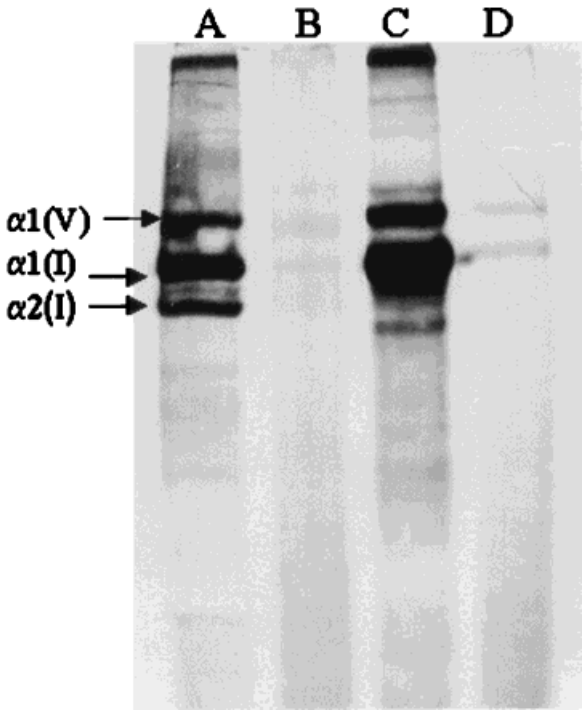

Fig. 3. Autoradiograph of the collagens in the medium conditioned by the cells and within the cells transduced with the Adeno-proα2(I) cDNA. Over 90% of the collagens synthesized by the transduced cells are present in the medium (**lane A**), similarly most of the collagens synthesized by the non-transduced cells are present in the medium (**lane C**). Very little collagen is present within the cells (**lanes B** and **D**).

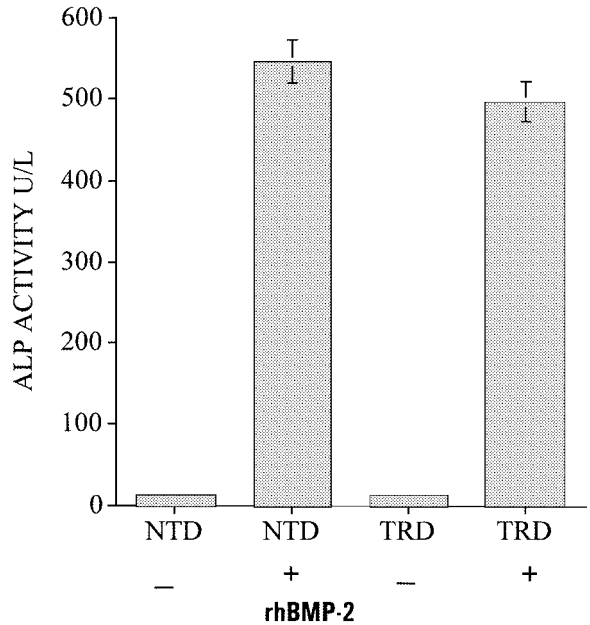

Fig. 4. Alkaline phosphatase activity of the transduced cells. The cells transduced with the proα2(I) cDNA (TRD) exhibit levels of ALP activity equivalent to that of the non-transduced (NTD) cells when treated with rhBMP-2. The non-transduced and transduced cells exhibit low levels of ALP activity without rhBMP-2 treatment.

Alkaline Phosphatase Activity

The murine bone marrow stromal cells established from oim mice were shown previously to exhibit alkaline phosphatase activity when treated with rhBMP-2 [Balk et al., 1997]. To assess whether the transduced cells retained the potential to differentiate toward osteoblastic lineage after gene transduction, cells were treated with rhBMP-2 and then analyzed for ALP activity. The bone marrow stromal cells transduced with the Adeno-proα2(I) collagen cDNA at 50 MOI were divided into two portions. One aliquot of the cells was analyzed for collagen synthesis and the other aliquot was analyzed for the ALP activity. Analysis of the ALP activity of the transduced cells demonstrated that the cells exhibited ALP activity that was equivalent to that of non-transduced cells (Fig. 4). These data demonstrated that gene transduction did not alter the osteogenic potential of the transduced cells.

In Vivo Transduction of Oim Bone Marrow Stromal Cells

To determine whether the collagen gene could function in vivo, Adeno-proα2(I) collagen cDNA was injected intradermally into the oim mice. Analysis of the collagens from the tissue harvested from the sites into which the virus was injected, demonstrated presence of type I collagen consisting of α1(I) and α2(I) heterotrimers (Fig. 5, lanes B and F). The sites that were injected with saline did not show any presence of the protein band that migrates in the position of α2(I) (Fig. 5, lanes C and E). These data demonstrated that direct injection of the virus encoding the proα2(I) cDNA resulted in the synthesis of type I collagen comprised of α1(I) and α2(I) heterotrimers by the skin fibroblasts at the injection sites. The type I collagen extracted from the tissue harvested from the adeno-proα2(I) cDNA injected sites contained high content of α1(I) homotrimers because the extracted collagens contained α1 (I) homotrimers that were already deposited in the tissue. Nevertheless the data demonstrate that a sufficient number of proα2(I) chains were

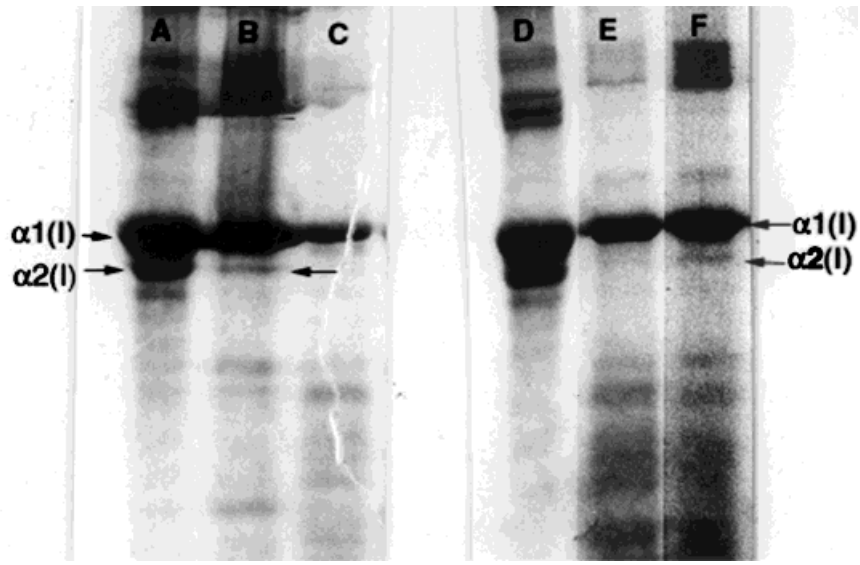

Fig. 5. In vivo expression of the α2(I) chains. SDS–PAGE of the collagens extracted by pepsin digestion of the tissues harvested from two *oim* mice injected with the Adeno-proα2(I) cDNA. Tissues from the mouse injected with the virus (**lanes B** and **F**) show presence of α2(I) chains. The saline injected sites (**lanes C** and **E**) do not show presence of α2(I) chains. **Lanes A** and **D**, pepsin extracted type I collagen from bovine bone.

newly synthesized in vivo that became incorporated into type I collagen heterotrimers and were detected by SDS–PAGE.

DISCUSSION

The data presented here have demonstrated clearly that collagen genes can be efficiently transferred into cells to substitute for the defective genes and that the genes are expressed with high efficiency in vitro as well as in vivo. In the present study we have shown that transfer of a murine proα2(I) gene into bone marrow stromal cells deficient in the collagen gene results in the synthesis of type I collagen molecules comprised of α1(I) and α2(I) chains in a 2:1 ratio. The type I collagen synthesized by the transduced cells was efficiently secreted indicating that gene transduction did not affect collagen secretion. The attractive feature of delivering collagen genes into bone marrow stromal cells is that these cells have potential to give rise to a variety of cells including osteoblasts [Caplan, 1991; Pereira et al., 1995; Prockop, 1997; Oyama et al., 1999; Pittenger et al., 1999]. The hypothesis is that, if the cells are used as vehicles to deliver the collagen genes to bone, if transplanted in vivo, they would self-renew and therefore provide treatment for life. This is supported by studies in which bone marrow was infused systemically into irradiated and nonoblated mice and shown to populate and to persist in different tissues of the recipient mice including bone [Stewart et al., 1993; Pereira et al., 1995, 1998; Nilsson et al., 1999; Oyama et al., 1999]. In addition a clinical trial in which children with severe OI were infused with allogenic bone marrow cells, demonstrated that the cells persisted in bone and presumably led to a reduction in the fracture rate of the bones of the marrow recipients [Horwitz et al., 1999]. We have also previously shown that bone marrow stromal cells that were transduced with the Lac-Z gene and infused in the oim mice bones, persisted in the bones of the recipient mice and appeared to differentiate into osteoblasts in vivo [Oyama et al., 1999].

The most common mutations in OI patients are point mutations that substitute the conserved glycine with a charged amino acid or amino acid having a bulky side chain that destabilizes the triple helix [Prockop et al., 1993, 1994]. These types of mutations lead to synthesis of abnormal α chains, which may associate with normal chains. The defective molecules may either be degraded intracellularly or they may be secreted and assembled into defective collagen fibrils in the extracellular matrix [Prockop, 1990; Niyibizi et al., 1992].

Supplying the normal gene in this case may not alleviate the problem. However collagen mutations in which an allele is absent or not expressed may be amenable to gene therapy. As shown in the present study, the cells harvested from oim mice do not express the proα2(I) chains, the cells however synthesized type I collagen consisting of α1(I) and α2(I) chains in a correct ratio of 2:1 in vitro when they were transduced with the Adeno-proα2(I) cDNA. These data demonstrated that collagen null mutations could be treated by supplying normal collagen genes to replace the defective genes. Several investigators are examining the use of antisense gene therapy approaches as an attempt to eliminate or decrease the expression of the mutant alleles [Colige et al., 1993; Jaspal et al., 1994; Laptev et al., 1994; Wang and Marini, 1996; Grassi et al., 1997; Marini and Gerber, 1997]. An antisense RNA or DNA molecule complimentary to the mRNA for the mutant protein binds to the target RNA to prevent its translation [Woolf et al., 1990]. Antisense oligonucleotides tightly bind to the target RNA, intracellular enzymes recognize paired mRNA with DNA and digest them [Wang and Marini, 1996]. The aim here is to convert a dominant negative mutation into a mild one. In this context a normal collagen gene could be supplied after eliminating the mutant allele. In addition even in dominant negative mutations, over expression of normal collagen gene may lead to the synthesis of a sufficient normal collagen molecules to perhaps normalize tissue function.

The ratio of α1(I) to α2(I) synthesized in vitro by the transduced cells was close to 2:1. The data suggest that sufficient proα2(I) chains were synthesized to engage all the α(I) chains, resulting in type I collagen heterotrimers, consisting of α1 and α2 chains in a ratio of 2:1. Presumably the proα2(I) chains that were not incorporated were degraded since α2(I) chains cannot form triple helices by themselves. Whether the unincorporated α1(I) chains are also degraded is not clear. Although in the present study, an adenoviral vector which gives transient gene expression was used for the delivery of the collagen gene, these preliminary data demonstrate conclusively that bone marrow stromal cells may be good candidates for the delivery of the collagen genes to the skeletal tissues. For sustained gene expression, retroviruses are the viruses of choice. Retroviruses however, require dividing cells to achieve high efficiency of transduction [Crystal, 1995]. High efficiency of transduction of bone marrow stromal cells with retroviruses has been achieved by using repeated transfections of the same cells with the same gene of interest [Marx et al., 1999]. It is therefore possible to achieve high gene expression in bone marrow stromal cells that are transduced with retroviruses carrying the collagen genes. Although an adenoviral vector was used to transduce the cells, the present data encourage further studies in the use of the bone marrow stromal cells as targets to deliver collagen genes or other therapeutic genes to bone. Further studies will focus on the use of retroviruses and bone specific promoters to deliver collagen genes to bone using bone marrow stromal cells.

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REFERENCES

Balk ML, Bray J, Day C, Epperly M, Greenberger J, Evans CH, Niyibizi C. 1997. Effect of rhBMP-2 on osteogenic potential of bone marrow stromal cells from an Osteogenesis Imperfecta mouse. Bone 21:7–15.

Barsh GS, Byers PH. 1981. Reduced secretion of structurally abnormal Type I procollagen in a form of Osteogenesis Imperfecta. Proc Natl Acad Sci USA 78:5142–5146.

Byers PH, Steiner RD. 1992. Osteogenesis Imperfecta. Ann Rev Med 43:269–282.

Cao M, Westrhausen-Larson A, Niyibizi C, Kavalkovich K, Georgescu HI, Rizzo CF, Hebdar P, Stefanovic-Racic M, Evans CH. 1997. Nitric oxide inhibits the synthesis of type II collagen without altering COL2A1 mRNA abundance: prolyl hydroxylase as a possible target. Biochem J 324:127–138.

Caplan AI. 1991. Mesenchymal stem cells. J Orthop Res 9:641–650.

Chipman SD, Sweet HO, McBride DJ, Davisson MT, Marks SC, Shuldiner AR. 1993. Defective pro-α2(I) collagen synthesis in a recessive mutation in mice: a model of Osteogenesis Imperfecta. Proc Natl Acad Sci USA 90:1701–1705.

Colige A, Sokolov BP, Nugent P, Baserga R, Prockop DJ. 1993. Use of an antisense oligonucleotide to inhibit expression of a mutated human procollagen gene (COL1A1) in transfected mouse 3T3 cells. Biochemistry 32:7–11.

Crystal RG. 1995. Transfer of genes to humans: early lessons and obstacles to success. Science 270:404–410.

Evans CH, Robins PD. 1995. Possible orthopaedic applications of gene therapy. J Bone Joint Surg Am 77:1103–1114.

Grassi G, Forlino A, Marini JC. 1997. Cleavage of collagen RNA transcripts by hammerhead ribozymes in vitro is mutation-specific and shows competitive binding effects. Nucleic Acids Res 25:3451–3458.

Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. 1997. Construction of -adenovirus vectors through Cre-lox recombination. J Virol 71:1842–1849.

Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. 1999. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with Osteogenesis Imperfecta. Nat Med 5:309–313.

Jaspal S, Hillan SL, Prockop DJ. 1994. Partial rescue of a lethal phenotype of fragile bones in transgenic mice with a chimeric antisense gene directed against a mutated collagen gene. Proc Natl Acad Sci USA 91:6298–6302.

Kuivaniemi H, Tromp G, Prockop DJ. 1991. Mutations in collagen genes: Causes of rare and some common diseases in humans. FASEB J 5:2052–2060.

Laptev AV, Lu Z, Colige A, Prockop DJ. 1994. Specific inhibition of expression of a human collagen gene (COL1A1) with modified antisense oligonucleotides. The most effective target sites are clustered in double-stranded regions of the predicted secondary structure for the mRNA. Biochemistry 33:11033–11039.

Marini JC, Gerber NL. 1997. Osteogenesis Imperfecta: rehabilitation and prospects for gene therapy. JAMA 277:746–750.

Marx JC, Allay JA, Persons DA, Nooner SA, Hargrove PW, Kelly PF, Vanin E, Horwitz EM. 1999. High-efficiency transduction and long-term gene expression with a murine stem cell retroviral vector encoding the green fluorescent protein in human marrow stromal cells. Hum Gene Ther 10:1163–1173.

Mulligan RC. 1993. The basic science of gene therapy. Science 260:926–932.

Nilsson SK, Dooner MS, Weier HU, Frenkel B, Lian JB, Stein GS, Quesenberry PJ. 1999. Cells capable of bone production engraft from whole bone marrow transplants in non-ablated mice. J Exp Med 189:729–734.

Niyibizi C, Bonadio J, Byers PH, Eyre DR. 1992. Incorporation of type I collagen molecules that contain a mutant (2(I) chain ($Gly^{580}\rightarrow$ Asp) into bone matrix in a lethal case of Osteogenesis Imperfecta. J Biol Chem 267:3108–23112.

Oyama M, Tatlock A, Fukuta S, Kavalkovich K, Nishimura K, Johnstone B, Robbins PD, Evans CH, Niyibizi C. 1999. Retrovirally transduced bone marrow stromal cells isolated from a mouse model of human Osteogenesis Imperfecta (oim) persist in bone and retain the ability to form cartilage and bone after extended passaging. Gene Ther 6:321–329.

Pereira RF, Halford KW, O'hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockkop DJ. 1995. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage and lung in irradiated mice. Proc Natl Acad Sci USA 92:4857–4861.

Pereira RF, O'Hara MD, Laptev AV, Halford KW, Pollard MD, Class R, Simon D, Livesey K, Prockkop DJ. 1998. Marrow stromal cells as a source of progenitor cells for non hematopoietic tissues in transgenic mice with a phenotype of Osteogenesis Imperfecta. Proc Natl Acad Sci USA 95:1142–1147.

Phillips CL, Lever LW, Pinnell SR, Quarles LD, Wenstrup RJ. 1991. Construction of a full-length murine proα2(I) collagen cDNA by the polymerase chain reaction. J Invest Dermatol 97:980–984.

Phillips CL, Morgan AL, Lever LW, Wenstrup RJ. 1992. Sequence analysis of a full-length cDNA for the murine proα2(I) collagen chain: comparison of the derived primary structure with human proα2(I) collagen. Genomics 13:1345–1346.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Prockop DJ. 1990. Mutations in type I procollagen genes: an explanation for brittle bones and a paradigm for other diseases of connective tissue. Annu Rheum Dis 49:440–444.

Prockop DJ. 1997. Marrow stromal cells as stem cells for non-hematopoietic tissues. Science 276:71–74.

Prockop DJ, Kivirikko KI. 1999. Collagens: molecular biology, diseases, and potentials for therapy. Annu Rev Biochem 64:403–434.

Prockop DJ, Colige A, Helminen H, Khillan JS, Pereira R, Vandenberg P. 1993. Mutations in type 1 procollagen that cause Osteogenesis Imperfecta: effects of the mutations on the assembly of collagen into fibrils, the basis of phenotypic variations, and potential antisense therapies. J Bone Miner Res 2(S4):89–92.

Prockop DJ, Kuivaniemi H, Tromp G. 1994. Molecular basis of Osteogenesis Imperfecta and related disorders of bone. Clin Plast Surg 21:407–413.

Rowe DW, Shapiro JR, Poirier M, Schlessinger S. 1985. Diminished Type I collagen synthesis and reduced α(I) collagen mRNA in cultured fibroblasts from patients with dominantly inherited Osteogenesis Imperfecta. J Clin Invest 6:604–611.

Stewart FM, Temeles D, Lowry P, thrives T, Grosh WW, Quesenberry PJ. 1993. Long-term engraftment of normal and post-5-fluorouracil murine marrow into normal nonmyeleablated mice. Blood 81:2566–2571.

Tilstra DJ, Byers PH. 1994. Molecular basis of hereditary disorders of connective tissue. Annu Rev Med 45:149–163.

Wang Q, Marini JC. 1996. Antisense oligodeoxynucleotides selectively suppress expression of the mutant α1(I) collagen allele in Type IV Osteogenesis Imperfecta fibroblasts. A molecular approach to therapeutics of dominant negative disorders. J Clin Invest 97:448–454.

Woolf TM, Jennings CGB, Rebagliati M, Melton DA. 1990. The stability, toxicity and effectiveness of unmodified and phosphorothioate antisense oligodeoxynucleotides in Xenopus oocytes and embryos. Nucleic Acids Res 18: 1763–1769.

Yeh P, Perricaudet M. 1997. Advances in adenoviral vectors: from genetic engineering to their biology. FASEB J 11:615–623.