AUTOLOGOUS HUMAN-DERIVED BONE MARROW CELLS EXPOSED TO A NOVEL TGF-131 FUSION PROTEIN FOR THE TREATMENT OF CRITICALLY SIZED TIBIAL DEFECT

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We report the first clinical case of transplantatlon of autologous bone marrow-derived cells In vitro exposed to a novel recombinant human transforming growth factor (rhTGF)-β1 fusion protein bearing a collagen-binding domain (rhTGF-β1-F2), dexamethasone (DEX) and β-glycerophosphate (15-GP). When such culture-expanded cells were loaded into porous ceramic scaffolds and transplanted into the bone defect of a 69-year-old man, they differentiated into bone tissue. Marrow cells were obtained from the iliac crest and cultured In collagen gels impregnated with rhTGF-p1-F2. Cells were selected under serum restricted conditions in rhTGF-β1-F2-contalnlng medium for 10 days. expanded in 20% serum for 22 days and osteoinduced for 3 additional days in DEX/β-GP-supplemented medium. We found that the cell number harvested from rhTGF-P1-F2-treated cultures was significantly higher (2.3· to 3-fold) than that from untreated cultures. rhTGf·P,·F2 treatment also significantly increased alkaline phosphatase activity (2.2· to 5-fold) and osteocalcin synthesis, while calcium was only detected in rhTGF-p1-F2-treated cells. Eight weeks after transplantatlon, most of the scaffold pores were filled with bone and marrow tissue. When we tested the same human cells treated in vitro in a rat model using diffusion chambers, there was subsequent development of cartilage and bone following the subcutaneous transplantatlon of rhTGF-p1-F2-treated cells. This supports the suggestion that such cells were marrow-derived cells. with chondrogenic and osteogenic potential, whereas the untreated cells were not under the same conditions. The ability for differentiation into cartilage and bone tissues, combined with an extensive proliferation capacity, makes such a marrow-derived stem cell population valuable to Induce bone regeneration at skeletal defect sites.

Keywords: BMP, bone marrow, bone repair, chondro-osteogenesis, hydroxyapatite, mesenchymal stem cell, non-union fracture, TGF-β, tissue engineering

Introduction

Adult stem cells from human marrow stroma, operationally termed mesenchymal stem cells or marrow stromal cells (MSCs) [1,2], are now being considered for use in a wide range of tissue engineering technologies, and cell or gene therapy strategies, because of their high capacity for self-renewal [3–5], their multipotentiality for differentiation [5–7] and their demonstrable contributions to somatic tissue restoration [8–11]. With respect to the potential for clinical benefit in skeletal disorders, the possibility of using MSCs for bone tissue engineering has been suggested as an alternative strategy and a promising option, since the requirement for new bone in cases of bone loss caused by trauma, age and metabolic or genetic bone diseases is a major clinical and socioeconomic need [11–15].

To generate bone, MSCs need to undergo differentiation into the osteogenic lineage. Although the factors that regulate their ex vivo expansion and promote their osteogenic maturation remain poorly defined, it is now well established that members of the transforming growth factor (TGF)-β family play a prominent role in the development, growth and maintenance of the vertebrate skeleton [16,17]. The effect of TGF- β1 on the proliferation and osteoblastic differentiation of MSCs in vitro – causing an increase in total cell number, alkaline phosphatase activity (ALP), and osteocalcin (OC) production – is well documented [18–20]. Therefore, controlled administration of TGF-β1 could represent an emerging tissue engineering technology that may modulate cellular responses to encourage bone regeneration at a skeletal defect site [21].

In animal models of osteogenesis, TGF-β1 administration has been shown to stimulate osteoblast activity, causing the formation of new woven bone [22–24], and promoting the healing of fractures and skeletal defects [25,26]. It is important to note, however, that discrepant effects of TGF-β on proliferation and differentiation of MSC populations with osteogenic potential and bone formation have been reported [27–30]. Overall, the data emphasize that the physiological effects of TGF-β on cells of the osteoblast lineage appear to be highly complex and are influenced by the state of commitment and differentiation of the target cells, the cytokine milieu of the microenvironment, and the conditions of culture [16,28–30]. Thus, the effect of this growth factor is context-dependent and the apparent discrepant effects of TGF-β on proliferation and differentiation of cells with enhanced osteochondrogenic potential as well as on bone formation are probably owing to the manner in which it exerts its effects.

We have successfully developed a 3D collagen gel culture system in which chondrogenic and osteogenic stem cells are selected and expanded from rat marrow aspirates in the presence of a recombinant human TGF-β1 bearing a collagen-binding domain (rhTGF-β1-F2) which prolongs its biological half-life [31–34]. Previous results from our group suggest that rhTGF-β1-F2, when applied to a bovine collagen matrix as a vehicle and delivery system, could be of advantage in promoting survival, proliferation, differentiation and colony mineralization of the osteogenic precursor cell population. This system allows us to test the concept that MSCs could be selected and expanded by virtue of their intrinsic physiological responses to TGF-β1. We report the isolation, expansion and growth factor responsiveness of human marrow-derived cells in this culture system and evaluate the capacity of these cells to undergo differentiation into bone and/or cartilage tissue when introduced into porous hydroxyapatite ceramics and transplanted into the tibial defect of an elderly patient, as well as in an in vivo diffusion chamber assay.

Materials & methods

Case summary

A 69-year-old man presented with a fracture of the left proximal tibia (Supplementary images A) treated with an open reduction and internal fixation with a stainless steel plate in a foreign country. Ten days later, the patient developed an infection with bone involvement and sequestrum. The

fracture did not consolidate and the patient was unable to bear weight or walk. Eight years thereafter, we admitted the patient with the diagnosis of supurative chronic osteomyelitis. Local skin presented atrophic in appearance, very poor vascular perfusion of the limb and muscle atrophy. As an above-knee amputation was suggested and refused by the patient, a bone bloc resection was carried out together with an osteosynthesis by using a Hoffman II external fixator (Stryker-Howmedica, USA). Ten days later, bone transportation was initiated for a 10 cm lengthening in a regime of 0.25 mm every 6 h. New bone formation was evident according to radiological studies 4 months thereafter. However, by the end of transportation, the x-rays did not show proximal consolidation of the transported bone to the proximal tibia (Figure 1A). As no changes were observed in the following weeks, amputation was the appropriate indication. Nevertheless, after informed consent of the patient, according to procedures approved by the ethics committee of the hospital, a new open procedure with bone decortication, AO titanium plate osteosynthesis and autologous bone marrow (BM) cell implantation, using hydroxyapatite (HA) particles as a carrier vehicle, was performed.

rhTGF-β1 fusion protein

The full coding region of the rhTGF-β1 fusion protein, rhTGF-β1-F2, was generously provided by ME Nimni (University of Southern California, USA), and details of the technique have been described elsewhere [31–34]. Briefly, the cDNA sequence encoding the conserved carboxy-terminal region of rhTGF-β1 was engineered to include a high-affinity collagen-binding decapeptide derived from von Willebrand factor (vWF) bracketed by strategic linkers in frame with an Nterminal 6 × His purification tag provided by an expression vector.

Bone marrow cell culture in collagen matrices

Collagen matrices were prepared using a modification of a previously described method [31]. Briefly, a collagen substrate for cell culture was prepared using a solution containing 0.85 mg/ml human Type I collagen (Sigma), 1 M NaOH, 10 × α-minimum essential medium (αMEM, GIBCO), 100 μg/ml penicillin (Sigma Chemical Co.), 50 μg/ml gentamicin (Sigma Chemical Co.), 0.3 μg/ml fungizone (GIBCO) in MilliQ water at pH 7.4. 48-well plates (Iwaki, Scitech Div., Japan) were coated with 150 μl of this solution and placed at 37°C for 30 min in order for it to solidify into a thin collagen gel matrix.

Bone marrow samples were obtained from the iliac crest of the patient after informed consent and according to procedures approved by the local ethics committee. Marrow cells were harvested by drawing the marrow into syringes fitted with an 18-gauge needle several times. Cells were recovered after centrifugation at 400 × g for 5 min, washed in Dulbecco´s phosphate buffered saline (DPBS, GIBCO), and a single cell suspension was prepared by filtration through a 20 μm pore size cell strainer (Falcon, NJ, USA) and counted with a hemocytometer. After centrifugation, all of the cells were re-suspended in αMEM and mixed with the collagen solution plus 0.5% autologous serum, in the absence (control cultures) or presence of 1 ng/ml rhTGF-β1-F2 [31,33] at a density of 2 × 106 cells/ 250 μl collagen/well in 48-well plates, in a total of six plates. The culture plates were left at 37°C for 30 min to allow the collagen to gel. Then, 250 μl/well of culture medium that consisted of RPMI-1640 (Sigma) supplemented with 10 mM of HEPES (N-[2-hydroxyethylpiperazine- N´]-2-ethansulfonic acid, GIBCO), autologous human serum (0.5 or 20%), 2 mM L-glutamine (Sigma), and the same amount of penicillin-gentamicin and fungizone as described above were added on the top of the gel, in the presence or absence (control cultures) of rhTGF-β1-F2 (1 ng/ml) and 10-8 M DEX plus 2 mM β-GP (Sigma), and cultures were maintained at 37°C in 95% air/5% CO2. Cells were incubated in culture medium containing 0.5% serum for 10 days (selection period) and expanded in culture medium containing 20% serum for 22 days (amplification period). At the end of the culture period, the cells were incubated for 3 days in culture medium (not containing TGF-β1-F2) supplemented with DEX and β-GP to help

with the osteogenic differentiation. Cultures were fed every third day with fresh medium in the presence or absence of TGF-β1-F2 and DEX/β-GP as appropriate over a period of 5 weeks.

Biochemical assays

After 10 days in culture, until the end of the culture period, quantitative measurements of cell number (expressed as DNA amount), alkaline phosphatase (ALP) activity, OC synthesis and calcium (Ca) content were determined at 3–4 day intervals using a modification of a previously described method [31]. In brief, DNA synthesis was determined by a semi-automated microfluorimetric method using Hoechst 33258 dye (Sigma). The results were expressed as micrograms of DNA per well. For analysis of ALP activity, cells were lysed with 0.15 M NaCl, 3 mM NaHCO3 and 0.1% Triton X-100 in distilled water, pH 7.4, and repeatedly frozen–thawed for three times to disrupt the cell membranes. After sonication, aliquots of cell lysates were incubated with pnitrophenolphosphate (pNPP, Sigma) for 30 min at 37°C, and the results were quantified at 405 nm using a microplate reader. The activity was normalized to unit values and the results were then expressed as units of ALP per microgram of DNA per well. OC synthesis was extracted from residues of ALP assays with 10% formic acid for 16 h at 4°C, and determined by radioimmunoassay as previously described [31]. Data were expressed as nanograms of OC per microgram of DNA per well. For determination of Ca levels, the cells were washed with Ca2+ and Mg2+ free PBS and then solubilized with 0.6 N HCl. Measurements were done by colorimetry, using the 587-A Ca assay kit (Sigma), and the colorimetric reaction was read at 570 nm. The absolute Ca concentration was determined according to a standard curve for Ca according to the manufacturer's recommendations. Values were expressed as micrograms of Ca per milligram of dry weight of tissue per well.

All assays were determined in quadruplicate for each condition and assessed for at all timepoints. The data are expressed as mean ± standard deviation (SD).

Transplantation of culture-selected BM cells into the bone defect

After 35 days in culture, the collagen gels containing the cells were incubated with 0.05% type I collagenase (Worthington Biochemical Co., USA) for 10 min at 37°C in Ca2+- and Mg2+-free Hank's balanced salt solution (HBSS, GIBCO) followed by trypsinization for 5 min at 37°C using 0.25% trypsin in 1 mM ethylene-diamine tetra-acetic acid (EDTA, GIBCO). Cell suspensions were centrifuged and cell pellets were re-suspended in serum-free medium, and counted. 95 porous resorbable hydroxyapatite (HA) ceramic particles (mean pore size = 500 μm), approximately 5 mm diameter (Interpore, Irvine, CA) were used as carriers of cell transplantation. Medium containing 17 × 106 cells treated with TGF-β1-F2 was combined with the ceramic particles. To allow the cells to infiltrate the ceramic pores, loaded particles were incubated for 2 h at 37 °C in 95% air/5% CO2 prior to transplantation. For transplantation of HA–cell complexes into the patient's tibia defect, drill holes were made in both ends of the proximal tibia and were filled with HA particles loaded with cells (Figure 1B). After accurate reduction and rigid osteosynthesis the remaining HA–cell complexes were transplanted into the defect (Figure 1C). Soft x-ray photographs were taken before (Figure 1A) and after (Figure 1D) intervention. As 8 weeks after transplantation the radiographical view showed a primary post-healing period, we proceeded to extract some implants and the tissues including the transplanted HA–cell complexes were dissected from muscles, recovered from the postextraction bone, and prepared for histological and immunohistochemistry analyses.

In vivo diffusion chamber assay

To investigate whether the patient marrow-derived cells isolated and expanded in collagen gels in the presence of rhTGF-β1-F2 and osteogenic agents had an osteogenic and/or chondrogenic differentiation potential, eight diffusion chambers, 14 mm diameter × 2 mm height (Millipore

Ltd), 0.45 μm pore-sized filter, were filled with 1 × 106 TGF-β1-F2-treated cells and transplanted into subcutaneous pockets on the dorsal surface of 7-month-old male Fischer-344 rats under anesthesia induced with pentobarbital (35 mg/kg). Controls included eight diffusion chambers exposed to cells cultured without growth factor. Four rats were used in this study. To ensure internal consistency, the experimental and control transplants were inserted in contralateral sides of the same host. The rats were euthanized 4 weeks after the transplantation and the chambers were recovered for histological and immunohistochemistry analyses. All procedures were performed in accordance with specifications of institutionally approved animal protocols.

Histological analyses

The tissues, including transplanted HA–cell complexes and the retrieved chambers, were fixed in Bouin's fluid or 10% phosphate-buffered neutral formalin for 24 h at the time indicated. After decalcifying the tissues, including transplanted HA–cell complexes with 10% EDTA and dissolving the plastic rings of diffusion chambers in toluene for 10 min, fixed samples were dehydrated, embedded in paraffin, and sectioned at 7 μm. Sections were stained in Picrosirius-hematoxylin (PSH) [35], Alcian blue (AB) and Goldner´s trichrome.

Immunohistochemistry

For the detection of Type I collagen, transplants were fixed in 4% (w/v) paraformaldehyde in PBS at pH 7.4 for 12 h at 4ºC. Tissue sections previously de-paraffinized and hydrated were washed once in PBS tween (PBST) and endogenous peroxidase was blocked by immersing slides in 0.3% H2O2 in 70% methanol/PBST for 30 min. Nonspecific binding sites were blocked using 1% BSA in PBST for 30 min and sections were incubated with the rabbit polyclonal anti-collagen Type I antibody (Calbiochem-Novabiochem Co., USA) diluted at 1:60 in 3% BSA in PBST overnight. After three washes in PBST, sections were exposed to a 1:50 dilution of a goat antirabbit immunoglobulin G (IgG) secondary antibody for 45 min. After this, they were washed 3× in PBST and also incubated with a rabbit peroxidase-anti-peroxidase (rabbit PAP, Sigma) and diluted at 1:200 in PBST for 30 min. Finally, after three washes in PBST, sections were exposed to 0.1% H2O2 in 5% of diaminobenzidine (DAB, Sigma) in PBST for 10 min and washed once in water. Control sections in which no primary antibody was used at all were under identical conditions. All incubations and wash steps were done at room temperature.

Statistics

The data are reported as mean ± SD of determinations made in quadruplicate. The Mann- Whitney U-test was used to analyze for differences between the DNA content for the TGF- β1-F2-treated and control cultures. Student's ttest was used to determine significance in the ALP and OC activities as well as Ca content between the control and treated cultures. Results were considered significantly different at $p < 0.05\%$.

Results

Number of cells harvested from culture in collagen gels

A sharp decrease in cell number resulted from the serum-restricted conditions for the initial 10 days of culture either in the presence or absence of TGF-β1-F2 (Figure 2A). From day 10 onwards, once normal serum conditions were reestablished, the selected cells began to proliferate either in the presence or absence of TGF-β1-F2. The number of cells harvested from TGF-β1-F2- treated cultures, however, was consistently higher (2.3- to 3-fold, $p = 0.001$, Mann-Whitney U-test) when compared with controls.

Biochemical in vitro assay

Alkaline phosphatase is a well-known early marker for bone cell differentiation. When the cells began to proliferate in serum-containing medium there was an increase in the activity of ALP either in the presence or absence of TGF- β1-F2 (Figure 2B). Nevertheless, in lysates prepared from cultures treated with TGF-β1-F2, the ALP activity was consistently greater (2.2- to 5-fold, $p < 0.001$, Student t-test) than that observed in those prepared from cultures treated without the factor either in the absence or presence of DEX and β-GP.

OC, an osteoblast-specific protein and a well-established marker of the mature osteoblast phenotype was measured in the cell layer. No detectable OC was found in controls. Cells treated with TGF-β1-F2 showed significant levels of OC (Table 1) during the induction period (DEX and β-GP added). The amounts of Ca produced by the cultured cells were detectable only in treated cells, varying from 7.2 ± 1.7 μg/mg dry weight/well at day 18 to 16.3 ± 2.9 μg/mg dw/well at day 35 (Table 2).

In vivo human osteogenesis in HA scaffolds

Histological examination of the transplants after 8 weeks showed indications of new dense tissue deposition developing at the ceramic surfaces, together with surrounding soft and well-vascularized tissue, resembling BM (Figure 3A). Subsequent picrosirius staining plus polarization microscopy revealed a highly birefringent conspicuous layer of tissue at the ceramic surfaces denoting fibrillar collagen nature (Figure 3B). Moreover, the new tissue expressed osseous phenotype as confirmed by expression of Type I collagen determined immunohistochemically (Figure 3C), and Goldner's trichrome stain showed bone matrix formation in a progressive state of maturation (Figure 3D). As shown in Figure 3E, bone was deposited on the surfaces of the ceramic particles by mature osteoblasts, and filled osteocytes lacunae can be seen surrounded by bone matrix. Old bone trabeculae located near the transplants showed new remodelling bone matrix (Figure 3F; the insert shows active osteoblast producing new bone matrix over the old one), whereas no sign of new osteogenesis was observed in bone trabeculae located far from the transplants (Figure 3G). Figure 1E corresponds to a follow-up of 90 days and shows a bone bridge together with local sclerosis.

Osteochondral potential in diffusion chamber assay

At 4 weeks after implantation of the committed cells (Figure 4A), control implants were scarcely filled with loose fibrous connective tissue adjacent to the filters (Figure 4B). By contrast, experimental chambers showed several nodules of cartilage and bone-like tissue located near the filters (Figures 4C–F). Sections stained with PSH and Goldner's trichrome showed strong posivity at the fibrous perichondral layer surrounding cartilage, and the bone-like areas between cartilage and the adjacent filters (Figure 4C & D). Chondrocytes were clearly identifiable along the cartilage nodules and occupying their lacunae embedded in their cartilage matrix (Figure 4C &D). Immunohistochemical localization of bone- and cartilage-specific matrix proteins indicated the presence of Type I collagen at the bone-like and perichondral areas (Figure 4E) and Type II collagen at the cartilage nodules (data not shown). As shown in Figure 4F, cartilage nodules were positively stained for AB, indicating accumulation of a cartilaginous matrix.

Discussion

Members of the TGF-β superfamily regulate the fate of multipotential stem cells by selectively regulating the expression of required growth factors and their receptors [36]. The effect of TGF-β in bone-forming cells depends on the stage of differentiation of the target cells, the culture conditions and the presence or absence of other growth factors. In general, TGF-β has been found to have a biphasic mitogenic effect on cultured osteoblasts and stimulates synthesis of collagen and noncollagenous extracellular matrix proteins by concentrations as low as 1 ng/ml [37]. Together with BMPs, TGF-βs are a group of structurally related proteins which have been shown

to stimulate bone formation in vivo. Since these proteins are concentrated in the organic matrix of bone and would be released during bone resorption, they are likely to have a profound effect on the remodeling bone and may provide a link between bone resorption and bone formation [38]. During skeletal development, TGF-βs have unique functions and act sequentially to modulate osteoblast differentiation [36].

In this report we demonstrate for the first time the isolation and expansion of adult human autologous marrow-derived cells in a collagen gel culture system exposed to a novel recombinant human TGF-β1 fusion protein, bearing a collagen-binding domain (rhTGF-β1-F2). Previous preliminary studies of our group performed in rats, using ceramic implants that were loaded with cells treated with TGF-β1 or TGF-β1-F2 have shown significant evidence of greater effectiveness of the fused proteins in terms of bone formation (Unpublished Data). We evaluate the capacity of the human cells to differentiate into bone tissue when transplanted into the bone defect of an elderly patient, as well as in a diffusion chamber assay in rats.

In vitro results

Characterization of TGF-β1-F2-responsive accumulation cell populations using different approaches (DNA content, ALP activity, OC expression, Ca accumulation and in vivo osteogenesis) revealed marked differences when compared with those receiving no treatment or treatment with DEX and β-GP alone. TGF-β1- F2-treated cells were found to proliferate more rapidly and to express higher levels of ALP activity. Based on ALP expression, this effect on proliferation appeared to be selective for cells of the osteoblast lineage. Although at present there is not a definitive marker for cells of such lineage, high expression of ALP, bone cell-specific protein OC, and production of a mineralized matrix are three widely accepted characteristics of bone cells. In our study only rhTGF-β1-F2-treated cultures exhibited synthesis of OC. As far as Ca is concerned, rhTGF-β1-F2, but not control cells, were able to induce Ca precipitation during the last days of the cultures, consistent with the mineralization of colonies formed in the presence of the recombinant growth factor. Conversely, the stimulatory effect of TGF-β1 on proliferation of cells of the osteoblast lineage and their expression of ALP observed in this investigation is in marked contrast to the results of previous investigations using BM-derived cells of both human [30] and rodent origin [29], in which inhibitory effects were observed. We reasoned that these surprising discrepancies are most likely attributable to differences in the species and skeletal site of origin of the cells under investigation as well as to their extent of maturation. Interestingly, inhibitory effects of TGF-β1 on the expression of ALP have been most frequently observed when cells have been cultured in the presence of the long-acting ascorbate analog (Asc-2-P) [29,30].

In vivo results

The results of histological preparation analysis and immunohistochemical localization of bonespecific matrix proteins suggest that cells exposed to TGF-β1-F2 and osteoinduced with DEX/β-GP in vitro (although not as an absolute requirement) were observed to differentiate into bone-like tissue in the interstices of the porous ceramics when transplanted into the patient's bone defect. In addition, our data provide evidence for new remodeling of bone matrix in old bone trabeculae located near the transplants, probably due to close influence of the transplanted cells, which might be working as templates of new osteogenesis (AI Caplan, Pers. Comm.), whereas no sign of new osteogenesis was observed in bone trabeculae located far from the transplants. Because the cells that were isolated and expanded in this study had not been marked prior to transplantation into the bone defect, we regard these findings as only suggesting evidence for osteogenic potential of cells impregnated in the ceramic particles. Nevertheless, in the diffusion chamber assay, positive controls using TGF- β1-F2-treated cells consistently generated nodules of cartilage and bone-like tissue, whereas negative controls of untreated cells were consistently devoid of bone and cartilage and differentiated to form fibrous tissue. This result demonstrates

that the cells selected by serum restriction in the presence of TGF-β1-F2, in cultures of human adult BM-derived cells, were a population of precursor cells with osteogenic and chondrogenic potential, as has been reported previously [33].

Human implantation results

Numerous studies have reported on the in vivo formation of bone within porous calcium phosphate ceramics in orthotopic sites [39–41]. However, these materials generally lack osteoinductive properties required to support bone healing in large defects [42,43]. The addition of BM cells to these ceramics has also been reported to enhance bone formation in ectopic sites [44–46], indicating the osteoinductive potential of ceramic/BM cell constructs.

Regarding the transplant of TGF-β1-F2- treated cells into the patient's bone defect, it is conceivable that the presence of these competent responsive cells in a physiological bone-forming environment, in concert with resident cells and with other osteogenic factors present in the area, may have stimulated their further differentiation into bone tissue and promoted remodelling of the bone matrix in old bone trabeculae, adjacent to the transplants. Most significantly in this regard, long-term complications such as diabetes, aging and bone nonunion do not prevent the ability of BM cells to proliferate, respond to TGF-β1 and differentiate toward osteoblastic or chondrogenic phenotypes either in vitro, or more significantly, upon in vivo transplantation. Consistent with this possibility, it has been found that the number and proliferative capacity of osteogenic stem cells are maintained during aging and in patients with osteoporosis, and that other mechanisms must be responsible for the defective osteoblast functions observed in these conditions [47]. It is important to note that the amount of newly formed bone in the transplants harvested from the postextraction bone after an 8-week period was consistently smaller than that previously reported for BM cells of rodent and human origin [48,49], and with values of OC synthesis and Ca content obtained. In spite of everything, we are concerned about the total number of cells used in this strategy since we believe that a greater number of rhTFG-β1-F2-treated cells may form the largest amounts of bone in these conditions of transplant, suggesting that subsequent applications or infusions at regular intervals could offer beneficial cellular therapy for the repair of diseased bone [50,51].

The present data suggest that adult human BM-derived osteogenic cells can be easily obtained from BM aspirates and readily separated from hematopoietic and differentiated stromal cells by virtue of their responsiveness to rhTGF-β1-F2, when cultured on 3D collagen scaffolds. Under appropriate conditions, these cells can be propagated and differentiated in vitro, indicating the feasibility of this strategy as adjuvant in the treatment of bone injuries, including use in elderly patients, and perhaps other MSC disorders as well. Finally, it may also have potentially important implications in terms of developing strategic biomatrices for wound applications and ex vivo gene therapy using targeted cells wherein MSCs are the desired targets for the treatment of skeletal disorders [52–54].

Acknowledgements

The authors wish to thank Prof. ME Nimni for his generous gift of the cDNA for the rhTGF-β1-F2, Prof. AI Caplan for his constructive criticisms and Dr Jesús A Santamaría for editorial assistance. The authors are grateful for the financial support of the Ministerio de Ciencia y Tecnología (Mat2003–07729-C03–00), FIS (PI021758), PAI (CVI/217), IMABIS and Fundación Progreso y Salud (TC 201.1.2 / 04), Spain.

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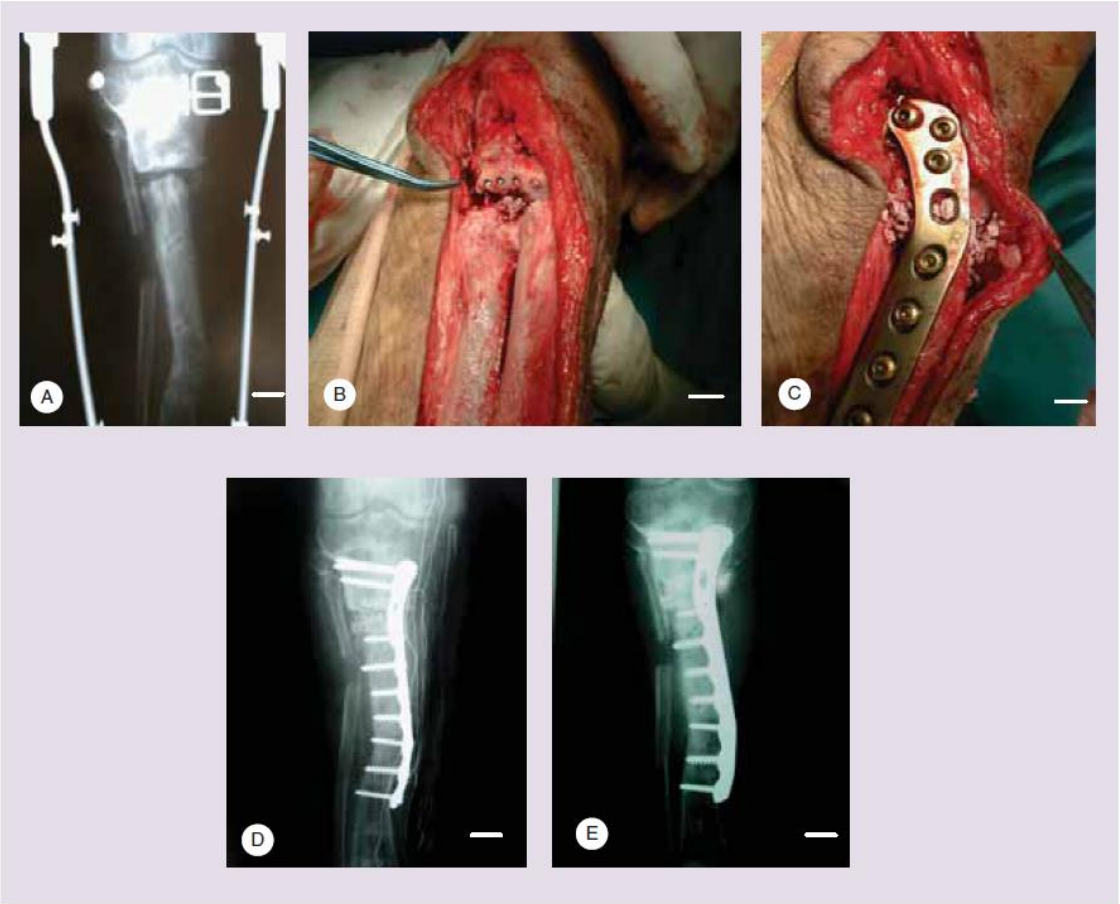
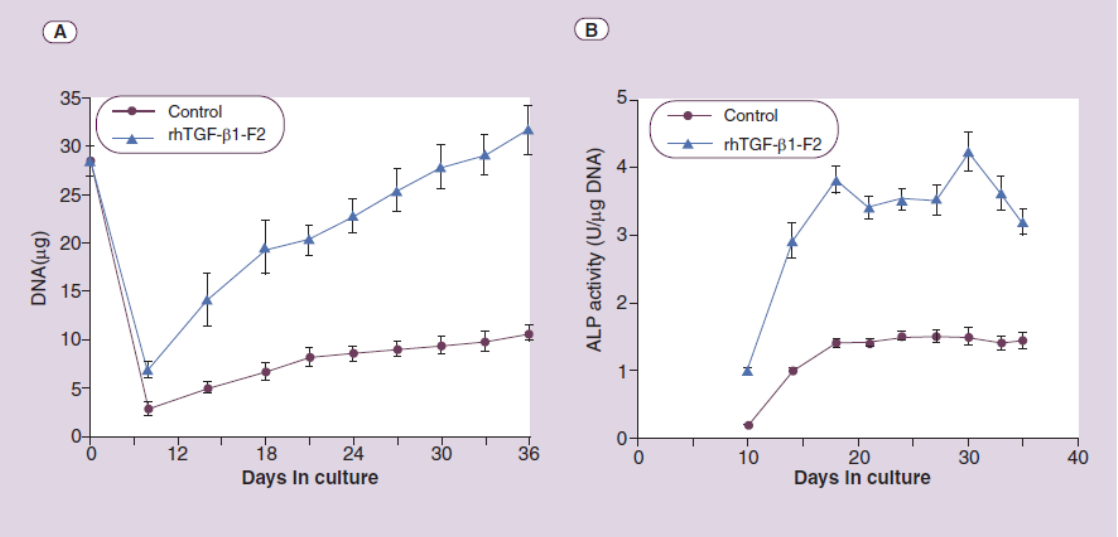
Figure 1. Soft x-ray examination of the tibial bone defect.



Soft x-ray examination of the tibial bone defect before **(A)** surgical intervention and after **(D)** transplantation of hydroxyapatite ceramic particles loaded with cells and osteosynthesis. **(B)** and **(C)** show details of the implantation technique. **(E)** corresponds to a follow-up of 90 days and shows a bone bridge among the ceramic fragments, together with local bone hyperdensity. Culture-selected autologous marrow-derived cells obtained from the iliac crest of a 69-year-old male patient and exposed to rhTGF-β1-F2, DEX and β-GP in vitro were used in this procedure. Bar = 1 cm.

β-GP: β-glycerophosphate; DEX: Dexamethasone; rhTGF-β1-F2: Recombinant human transforming growth factor β1 fusion protein.

Figure 2. Quantification of DNA content (A) and ALP activity (B) in cultures derived from bone marrow cells of a male patient, aged 69 years, at 3–4 day intervals over a period of 35 days.



Cells were cultured in collagen gels at the same densities (2 x 106/250 µl collagen/well in 48-well plates) in the absence (controls) or presence of 1 ng/ml TGF-β1-F2 up to day 32. DEX (10-8]M) and β-GP (2 mM) were added during the last 3 days of culture – at the last media change. Cells were removed from culture at indicated days and assayed for DNA content **(A)** and ALP activity **(B)** as described in Materials & methods. The sharp decrease in cell number resulted from the serum-poor conditions (< 1%) for the initial 10 days of culture, both with or without TGF-β1-F2; thereafter, the selected cells began to proliferate in serum-containing medium. The data reported as means (± SD) were obtained from four samples. Significant differences in DNA content ($p = 0.001$, Mann-Whitney U-test) and ALP activities ($p < 0.001$, t-test) are compared with the untreated controls in all cases.

β-GP: β-glycerophosphate; DEX: Dexamethasone; SD: Standard deviation; TGF-β1-F2: Transforming growth factor β1 fusion protein.

Table 1. Effects of culture conditions on OC synthesis (ng/µg DNA).

	Days in culture	
	18	***35***
Controls	No data	No data
rhTGF-β1-F2	0.62 ± 0.02	1.13 ± 0.04

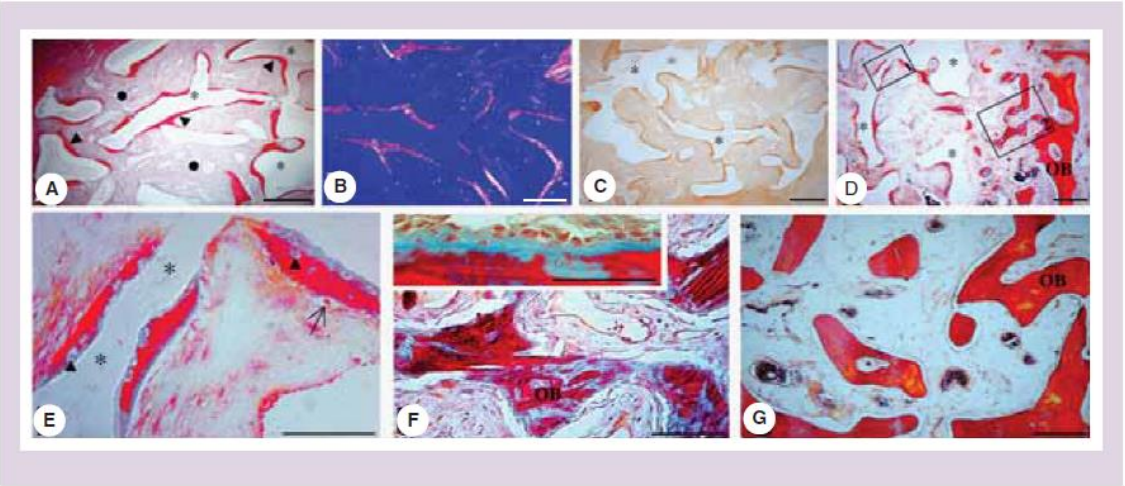
OC: Osteocalcin; rhTGF-β1-F2: Recombinant human transforming growth factor β1 fusion protein.

Table 2. Effects of culture conditions on calcium content (µg/mg dry weight).

	Days in culture	
	18	***35***
Controls	No data	No data
rhTGF-β1-F2	7.2 ± 1.7	16.3 ± 2.9

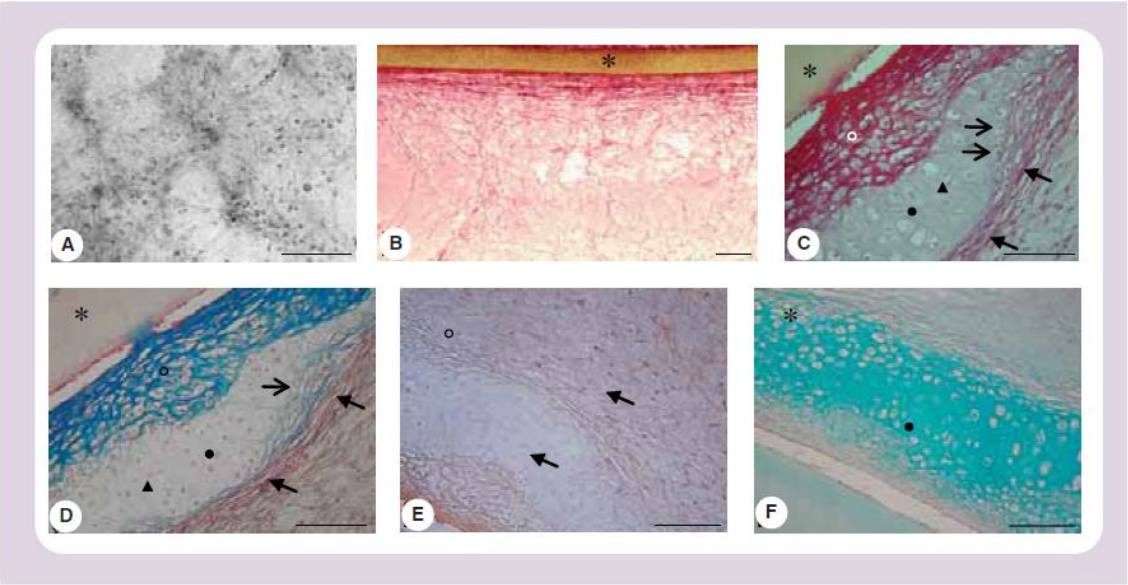
rhTGF-β1-F2: Recombinant human transforming growth factor β1 fusion protein.

Figure 3. Histological sections of hydroxyapatite ceramic particles, which were loaded with human adult autologous marrow-derived cells, transplanted into patient's tibia defect, and recovered from the postextraction bone after 8 weeks.



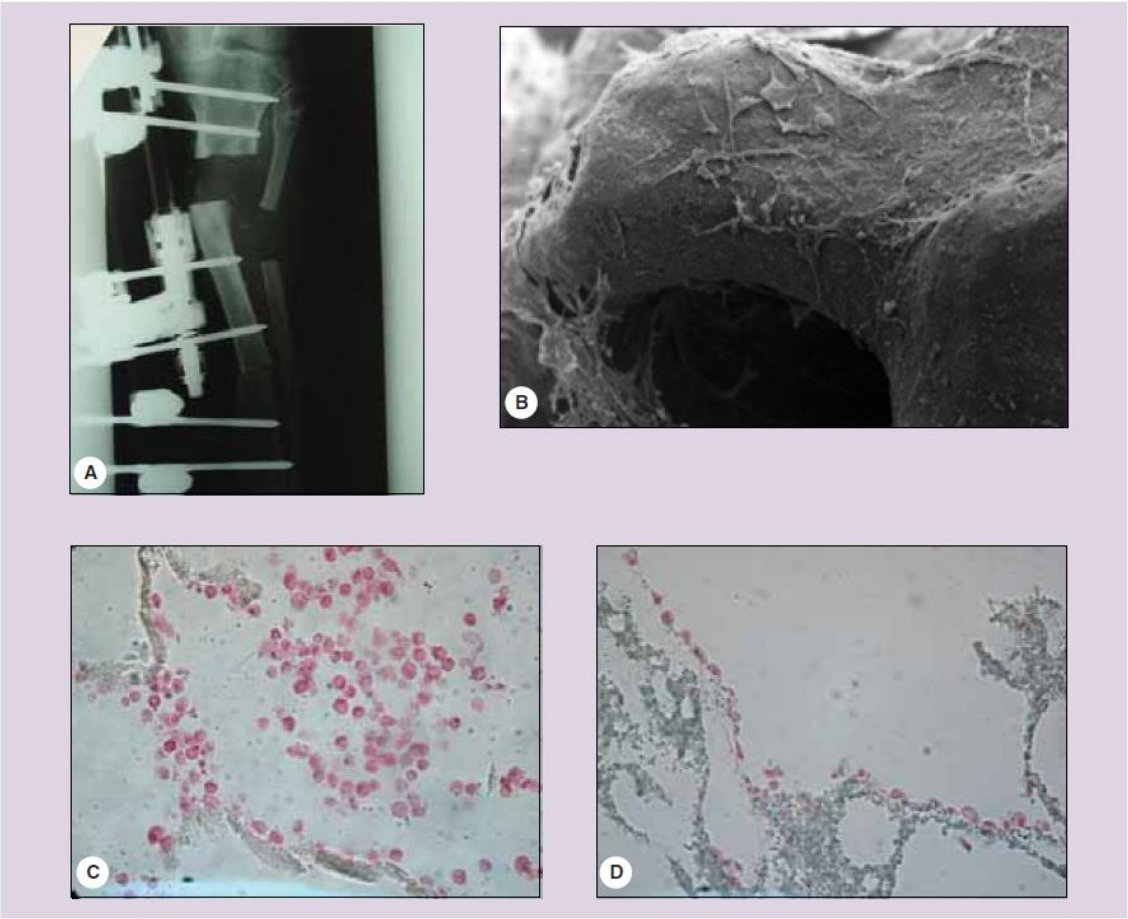
As a result of decalcification of the specimens, the ceramics (*) appear as a light gray substance between tissue-containing pores. **(A)** An overview showing intense red bands (arrowheads) deposited in the interstices of the porous ceramics, as detected by PSH staining, together with regular blood supply to the tissue (black spots), resembling a functional hematopoietic marrow. **(B)** Subsequent picrosirius staining plus polarization microscopy revealed highly birefringent bands at the ceramic surfaces denoting collagen in strong fibrilar organization compatible with bone matrix. **(C)** A contiguous section showed Type I collagen expression at bands adjacent to the walls of individual pores as detected by immunohistochemistry. **(D)** An overview showing HA–cell complexes (on the left) in close contact with old bone (OB) trabeculae (Goldner's trichrome stain). **(E)** Detail of box 1 in **(D)** shows bone-like tissue deposited on the surfaces of the ceramic particles by mature osteoblasts (arrow); osteocytes occupying their lacunae (arrowheads) were clearly seen embedded within bone matrix. **(F)** Detail of box 2 in **(D)** showing remodeling areas (blue) in old bone trabeculae located near the transplants. The insert shows active osteoblast producing new osteoid matrix (blue) over the mature bone. **(G)** OB trabeculae located far from the transplants showed no trace of remodeling. Bars: 300 µm.
HA: Hydroxyapatite; PSH: Picrosirius-hematoxylin.

Figure 4. Image of a single well from a 48-well plate showing isolation and expansion of human adult marrow cells in long-term, 35-day collagen gel cultures.



Histological sections of diffusion chambers that were filled with human adult autologous marrow-derived cells cultured in vitro **(A)**, transplanted subcutaneously in rats, and recovered 4 weeks after transplantation. **(B)** Control chambers were partially filled with loose fibrous connective tissue containing collagen fibrils adjacent to the filters (PSH staining, *), with no indications of bone or cartilage formation. **(C-F)** Chambers filled with TGF-β1-F2-treated cells revealed several nodules of cartilage (•) and bone-like tissue (o) located near the filters (*). The bone-like areas, as well as the multilayered perichondral areas (arrows) surrounding cartilage were positively stained for PSH **(C)** and Goldner's trichrome **(D)**, indicative of accumulation of type I collagen. Chondroblasts (thin arrows) are easily identifiable on the leading edge along the cartilage nodule as cartilage depositon proceeds toward the center of the nodule; chondrocytes (arrowheads) occupying their lacunae can be seen embedded in cartilage matrix. **(E)** A contiguous section shows positivity at the fibers of the bone- and perichondrium-like areas (arrows) as confirmed by expression of Type I collagen determined immunohistochemically. **(F)** Alcian bluepositive nodule indicative of accumulation of a cartilaginous matrix. Bars: 300 µm.
PSH: Picrosirius-hematoxylin; TGF-β1-F2: Transforming growth factor β1 fusion protein.

Supplementary images.



(A) corresponds to an x-ray of the patient tibia at the beginning of lengthening (technique described in Materials & methods: Case summary). A non-union in the proximal gap can be seen. **(B)** a scanning electron microscopy showing that there was significant cell adhesion, as the cells spread and grew in the scaffold. The histological examination of the hydroxyapatite shows the cells just after they were seeded **(C)** and 2 h later **(D)**.

Executive summary

Genetic engineered rhTGF-β1-F2

• The recombinant human transforming growth factor (rhTGF)-β1 fusion protein bearing a collagen-binding domain (rhTGF-β1-F2) is engineered using a prokaryotic expression vector consisting of an auxiliary von Willebrand factor-derived collagen-binding domain.

• The rhTGF-β1-F2 fusion protein shows significant evidence of having an effective role in bone formation.

• The life-time in terms of biological activity as well as the time of residency in the cellular neighborhood of the rhTGF-β1-F2 fusion protein is still unresolved and need more investigation.

Capture & expansion of mesenchymal progenitor cells in collagen gels

• Hematopoietic cell lineages as well as immune cell responses, both contained in bone marrow, are profoundly inhibited in the presence of rhTGF-β1-F2 into 3D collagen gel cultures under stringent (0.5%) serum conditions.

• A primitive population of mesenchymal progenitor cells is selected and expanded by virtue of their intrinsic physiological responses to rhTGF-β1-F2 into 3D collagen gel cultures under stringent (0.5%) serum conditions.

• A small population of uniformly blastoid cells had survived in response to rhTGF-β1-F2 into 3D collagen gel cultures under stringent (0.5%) serum conditions. These TGF-β1-responsive blastoid cells formed stromal/fibroblastic colonies upon the addition of 10% serum, and osteogenic colonies upon treatment with osteoinductive agents, thus indicating their mesenchymal lineage.

• The patient marrow-derived cells isolated and expanded as described here had a chondrogenic and/or osteogenic differentiation potential demonstrated both in diffusion chambers and in hydroxyapatite scaffolds.

• The molecular and genetic characterization of the expanded mesenchymal progenitor cell population is still unresolved and requires more precise, specific markers.