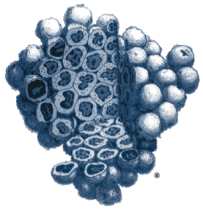

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IMT504, the Prototype of the Immunostimulatory Oligonucleotides of the PyNTTTTGT Class, Increases the Number of Progenitors of Mesenchymal Stem Cells Both In Vitro and In Vivo: Potential Use in Tissue Repair Therapy

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Stem Cells 2007;25;1047-1054
DOI: 10.1634/stemcells.2006-0479

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IMT504, the Prototype of the Immunostimulatory Oligonucleotides of the PyNTTTTGT Class, Increases the Number of Progenitors of Mesenchymal Stem Cells Both In Vitro and In Vivo: Potential Use in Tissue Repair Therapy

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Key Words. Mesenchymal stem cell • Oligodeoxynucleotide IMT504 • Bone repair

ABSTRACT

Bone marrow (BM)-derived adult mesenchymal stem cells (MSCs) have the capacity to differentiate in vitro into different cell lines. This makes them a likely source for application in tissue repair therapies. Here, we report evidence indicating that, both in vivo and in vitro, IMT504, the prototype of the PyNTTTTGT class of immunostimulatory oligonucleotides, significantly increases the number of fibroblast colony-forming units (CFU-Fs) that originate MSCs. When rat BM cells were cultured with IMT504, the mean number of CFU-Fs increased about three times as compared with untreated controls (CFU-F: 19 ± 6.3 vs. 6.8 ± 2.0/2 × 10^6 seeded BM cells, $p = .03$). Furthermore, rats inoculated with IMT504 had a significantly higher number of CFU-Fs both in BM (CFU-F: 124 ± 33 vs. 38 ± 17/femur, $p = .04$) and in peripheral blood (animals with detectable CFU-Fs in circulation 8/12 vs. 2/12, $p = .04$) as compared with untreated animals. On the other hand, BM-derived adherent cells either treated in vitro with IMT504 or obtained from animals injected with IMT504 possess the capacity to differentiate to the osteogenic and adipogenic cell lineages as regular MSCs. Finally, we found that repair of a bone defect was accelerated in rats injected with IMT504 as compared with control animals (area with consolidated bone: 80% ± 6.4% vs. 49% ± 3.5%, $p = .03$, $n = 10$ rats per group). Importantly, when two human BM were cultured in the presence of IMT504, the mean number of fibroblastic adherent colonies also increased as compared with controls. These results suggest the possibility of clinical use of IMT504 in bone, and presumably other, tissue repair therapies. STEM CELLS *2007;25:1047–1054*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Bone marrow (BM)-derived adult mesenchymal stem cells (MSCs) have the capacity to differentiate in vitro into osteocytes, chondrocytes, adipocytes, myocytes, hepatocytes, endothelial cells, and neurons [1–13]. This capacity makes them a likely cellular source for clinical application in tissue repair therapies. Preclinical studies performed in different animal models of tissue damage have given support to this hypothesis [14–25]. As a consequence, clinical trials using MSCs as tissue repair medicines have been initiated [26–28].

Immunostimulatory oligodeoxynucleotides (ODNs) are synthetic molecules that stimulate different kinds of cells of the immune systems of animals that have been assayed as adjuvant in vaccines and as medicines in the therapy of cancer and allergy [29]. ODNs that are active on human cells are grouped into two major classes: (a) CpG ODNs, characterized by the presence of at least one active site bearing an unmethylated CpG in a given context [29], and (b) PyNTTTTGT ODNs, which have at least one active site bearing the sequence PyNTTTTGT, in which Py is C or T and N is A, T, C, or G [30]. In vitro, both kinds of ODNs act on B cells and plasmacytoid dendritic cells, causing activation, proliferation, immunoglobulin secretion, and expression of costimulatory molecules. However, phosphorothioate CpG ODNs induce the secretion of interferon (IFN)α [29], whereas phosphorothioate PyNTTTTGT ODNs do not [30]. On the other hand, in the presence of interleukin (IL)2, PyNTTTTGT ODNs induce the secretion of granulocyte macrophage-colony stimulating factor (GM-CSF) acting on human peripheral mononuclear blood cells, whereas CpG ODNs do not (unpublished observations).

Unlike CpG ODNs, which are active in a widespread spectrum of animals [31], PyNTTTTGT ODNs are mainly restricted to primates [30] and rats [32]. We have now discovered that, both in vitro and in vivo, the PyNTTTTGT prototype IMT504 increases the number of fibroblast colony-forming units (CFU-Fs) that originate cells with multipotent differentiation capacity. In contrast, CpG ODNs or nonimmunostimulatory ODNs were not effective. On the other hand, when subcutaneously injected, IMT504 accelerates the

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reparation of experimental defects in the tibia of rats. These results clearly show the potential of IMT504 as a prospective medicine in tissue repair therapy.

Materials and Methods

Animals

Male Sprague-Dawley 8–12-week-old rats (weighing approximately 300 g) were obtained from FUCAL Laboratories (Buenos Aires, Argentina) and housed in a facility at the School of Natural Sciences, University of Buenos Aires, Buenos Aires, Argentina. Animal care and use were according to international guidelines (Guide to the Care and Use of Experimental Animals, Canadian Council on Animal Care, 1998).

BM Extraction

Animals were anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) and euthanized for bone marrow harvesting. After removing epiphyses and gaining access to the marrow cavities, whole BM plugs were flushed out from femoral bones using a 1-ml syringe with α-minimal essential medium (MEM) (Gibco, Grand Island, NY, http://www.invitrogen.com) supplemented with 100 IU/ml gentamicin and 2.5 μg/ml amphotericin. The cell suspension (15 ml) was centrifuged at 400*g* for 10 minutes, and the pellet was suspended in fresh medium. This procedure was repeated twice. Cell concentration was evaluated by microscopic cell counting using a Neubauer hemocytometer using samples treated with 3% acetic acid. Cellular viability was determined using the trypan blue staining method.

Blood Extraction

Animals were anesthetized and blood samples obtained by cardiac puncture. Peripheral blood mononuclear cells (PBMC) were isolated by gradient density (Histopaque-1083; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). Total blood volume was estimated as 8% of body weight.

Donors

Human sample donors gave consent to participate in these studies, which were performed in accordance with the principles of the Declaration of Helsinki. BM aspirates from two healthy donors (a 32-year-old female and a 30-year-old male) were kindly provided by Dr. Feldman from Fundación Favaloro, Buenos Aires, Argentina.

BM samples were collected under local anesthesia from the posterior iliac crest into heparinized saline without preservatives (25 U/ml; Gibco). BM mononuclear cells (MC) were isolated from the cell suspension by gradient density (Histopaque-1077; Sigma-Aldrich) and suspended in fresh α-MEM. Cell concentration was evaluated by microscopic cell counting using a Neubauer hemocytometer and cell viability was determined by trypan blue dye exclusion. MC obtained after gradient isolation were: 7.9×10^6 cells per milliliter aspirate for BM number 1 and 7.6×10^6 cells per milliliter aspirate for BM number 2.

Oligonucleotides

Oligonucleotides having phosphorothioate internucleotide linkages were purchased from Oligos Etc. (Wilsonville, OR, http://www.oligosetc.com) and purified by high-pressure liquid chromatography (HPLC). ODNs were suspended in depyrogenated water, assayed for lipopolysaccharide (LPS) contamination using the limulus test, and kept at −20°C until used. Purity was assessed by HPLC and polyacrylamide gel electrophoresis assays. ODN preparations were used if LPS levels were undetectable. ODNs 5′–3′ sequences are as follows: IMT504: TCATCATTTTGTCATTTTGTCATT; 2006: TCGTCGTTTTGTCGTTTTGTCGTT; 2216: GGG*G*G*A*C*G*A*T*C*G*T*C*G*GGGGGG (asterisks indicate phosphodiester backbone).

IMT504 Treatment

For in vivo assays, rats were subcutaneously (s.c.) injected daily either with saline or with 250 μg per dose of the ODN IMT504 dissolved in 1 ml of saline solution for 5 consecutive days. Two days after this, the injected animals were anesthetized and blood and BM samples extracted as described above. IMT504 doses used in vivo were chosen because our pharmacodynamic studies (unpublished data) demonstrated that, using these doses, B lymphocytes in the blood of injected animals are activated as well as in the in vitro experiments previously reported [30].

CFU-F Assay in Rat BM

The number of adherent BM MSC progenitors was evaluated by the biological functional unit CFU-F assay. BM cells (2×10^6) were seeded in 25-cm^2 tissue culture flasks (Nunc, Rochester, NY, http://www.nuncbrand.com or Orange Scientific, Braine-l'Alleud, Belgium, http://www.orangesci.com) containing α-MEM supplemented with 100 IU/ml gentamicin (Larjan, Buenos Aires, Argentina, http://www.veinfar.com.ar), 2.5 μg/ml amphotericin B (PAA Laboratories, Linz, Austria, http://www.paa.at), 2 mM L-glutamine (Sigma-Aldrich), and 20% fetal calf serum (FCS) (Gibco) and incubated at 37°C under 5% CO_2 atmosphere. After a 7-day culture, nonadherent cells were removed by washing adherent cells twice with phosphate-buffered saline (PBS). After this, fresh medium was added and incubation proceeded for 7 more days under the same conditions. At day 14, cells were washed twice with PBS, fixed with methanol 100% for 15 minutes, and stained using Giemsa's azur eosin methylene blue solution (Merck & Co., Whitehouse Station, NY, http://www.merck.com). Cells and colonies were observed and counted using an optical microscope. A CFU-F was here defined as a group of at least 50 cells with an approximately circular disposition. Results were expressed as total number of CFU-Fs per BM 2×10^6 cells seeded for the in vitro analysis, or CFU-Fs per femur for the in vivo analysis.

Total nucleated cells from both femoral bones were combined and samples from each animal were seeded separately as indicated above (2×10^6 cells per 25-cm^2 flask). For the in vitro assays (n = 6 animals), cultures were treated with a single 1 μM dose of the indicated ODN or PBS (control group) and incubated for the first 7 days of culture. For the in vivo assays (n = 10 animals per group), BM cells or PBMC from animals s.c. inoculated with IMT504 or saline were seeded and incubated without further stimuli. To compare the size of the colonies and the cell morphology in CFU-F assays, digital photographs were taken at 40× and 100× magnification and analyzed by the Image-Pro Plus v 4.5 software. To measure the size, 10 colonies were randomly chosen from each sample (n = 6 animals for in vitro assays; n = 10 animals per group for in vivo assays). To analyze the cell morphology, 10 cells were randomly chosen from each colony and the largest and shortest axes measured. Statistical differences were analyzed with a paired or unpaired Student's t test as stated.

CFU-F Assay in Rat PBMC

PBMC (2×10^6) were seeded in 25-cm^2 tissue culture flasks containing α-MEM supplemented with 100 IU/ml gentamicin, 2.5 μg/ml amphotericin B, 2 mM L-glutamine, and 20% FCS and incubated under the conditions described above. PBMC from treated rats were cultured without IMT504 addition.

CFU-F Assay in Human BM

Human BM mononuclear cells (2×10^6) were seeded in 25-cm^2 tissue culture flasks with α-MEM supplemented with 100 IU/ml gentamicin, 2.5 μg/ml amphotericin B, 2 mM L-glutamine, and 20% FCS. Cultures containing either 1 μM of ODN IMT504 or saline were incubated at 37°C under 5% CO_2 atmosphere for 7 days. After this, medium was replaced and incubation proceeded for 7 more days. At day 14, culture dishes were washed twice with PBS, fixed in methanol 100%, and stained as described above. Each sample was cultured in triplicate.

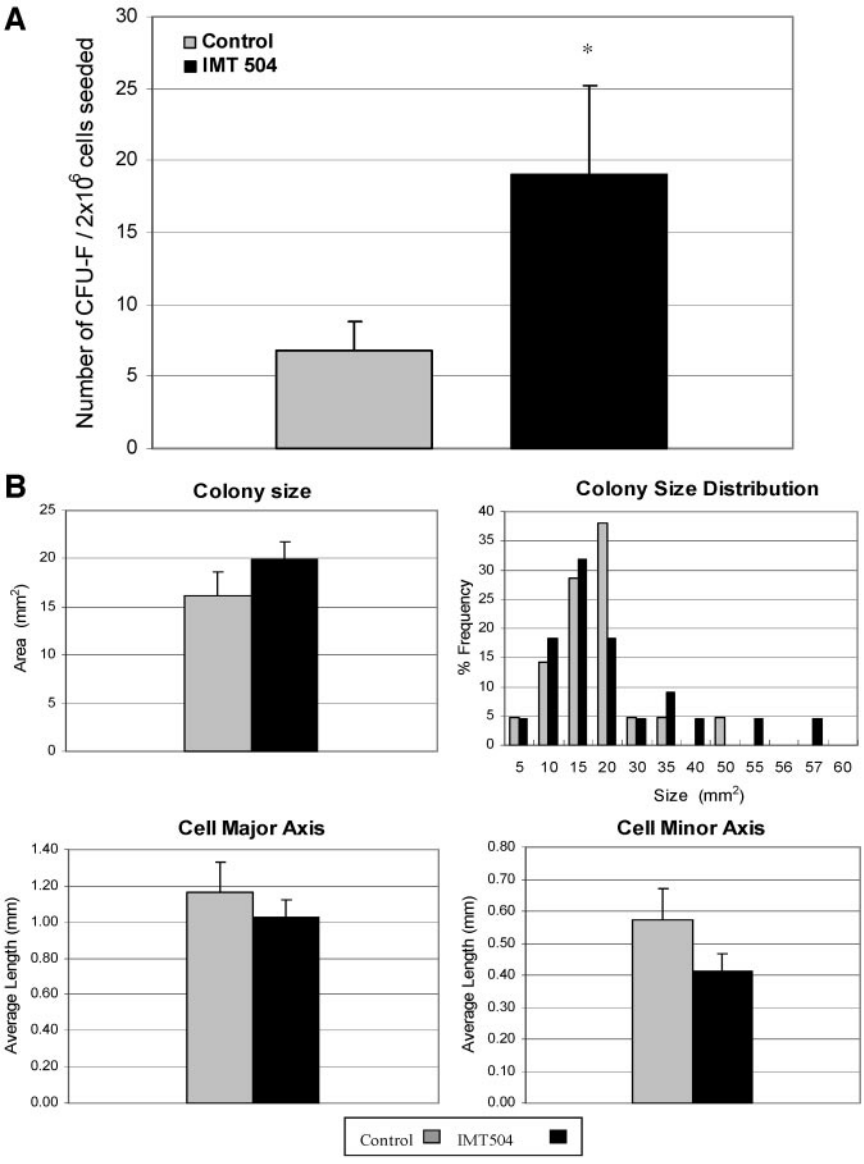

Figure 1. Effect of IMT504 on the CFU-F recovery in vitro. **(A):** Mean ± SEM of the number of CFU-Fs per 2×10^6 seeded bone marrow cells ($n = 6$ animals). **(B):** The upper row shows size and size distribution of colonies and the lower row shows the major and minor axes from fibroblastic-like cells in the colonies from cultures treated either with or without IMT504. Asterisks indicate statistically significant differences as compared with controls when $p \leq .05$ (**[A]**, Wilcoxon test; **[B]**, paired *t* test). Abbreviation: CFU-F, fibroblast colony-forming unit.

BM-Adherent Cell Differentiation

The differentiation potential of rat BM-adherent cells was assayed as previously described [33] with slight modifications. Initially, BM-adherent cells were expanded by culturing 10×10^6 cells extracted from femoral bones in 25-cm^2 tissue culture flasks containing 10 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 100 IU/ml gentamicin, 2.5 μg/ml amphotericin, 2 mM L-glutamine, and 20% FCS. To study the consequences of the in vitro incubation of the BM-adherent cells with IMT504, the culture medium was supplemented with this ODN at 1 μM concentration for the first 7 days of culture. On the other hand, to study the consequences of the in vivo treatment with IMT504, cells extracted from treated animals were incubated in the absence of the ODN. After 24 hours of culture, nonadherent cells were discarded and cultures continued until confluence, renewing the medium every 7 days. At this time, cells were harvested with trypsin-EDTA (0.05%–0.02%) (passage 1) and cultured at 10^4 cells per cm^2. When the cells reached confluence, they were trypsinized, seeded at 240 cells per cm^2, and cultured for 12 more days (passage 2). At this time, cells were harvested and incubated in differentiation medium as described below.

The osteogenic differentiation capacity of BM-derived adherent cells was determined as described [34]. In brief, cells from the second passage were trypsinized and seeded in a 9.6-cm^2 culture dish (Nunc) at 3,000 cells per cm^2 containing the differentiating medium. This medium consisted of DMEM (Gibco) supplemented with 10^{-8} M dexamethasone, 0.2 mM ascorbic acid, 10 mM β-glycerophosphate (Sigma-Aldrich), and 10% FCS. The medium was renewed every 3 days. After 21 days, adherent cells were washed twice with PBS, fixed with methanol, and stained using the alizarin red S staining (Sigma-Aldrich) to visualize calcium deposits.

The adipogenic differentiation capacity of BM-derived adherent cells was evaluated as previously described [35]. Briefly, cells from the second passage were trypsinized and cultured at 3,000 cells per cm^2 in a 9.6-cm^2 culture dish containing DMEM supplemented with glutamine, gentamicin, amphotericin B, and 20% FCS. Dishes were incubated at 37°C in a humidified atmosphere of 5% CO_2 until the cultures reached confluence (5–13 days). Medium was completely replaced every 7 days. Then, three cycles of induction/maintenance were performed in order to stimulate optimal adipogenic differentiation. Each cycle consists of feeding the cells with supplemented adipogenic induction medium (according to the Cambrex user's manual; Cambrex, Walkersville, MD, http://www.cambrex.com) and culture for 3 days at 37°C, 5% CO_2 followed by 1–3 days of culture in supplemented adipogenic maintenance medium. After this, cells were cultured for 7 more days in supplemented adipogenic maintenance medium, replacing the medium every 2–3 days. To document the adipogenic differentiation, cultures were rinsed

with PBS, fixed with methanol, and stained first with Giemsa's azur eosin methylene blue solution and then with oil red O staining (Sigma-Aldrich).

Control cultures without adipogenic or osteogenic differentiation medium were always run in parallel. On the other hand, control differentiation medium for the in vitro untreated cells or the in vivo untreated animals were also run in parallel. The percentage of differentiated stained cells per culture was calculated using the Image Pro-Plus software.

Osteogenic Effect of IMT504 In Vivo

The osteogenic potential of IMT504 in vivo was assayed using a rat model of bone defect. Briefly, rats were anesthetized i.p. with a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg). After this, the hind limb was shaved and disinfected. A 1.5-cm longitudinal incision was performed in the tibia forefront zone. To generate a bone defect consisting in a hole penetrating only one cortical up to the bone marrow, a low-speed dental drill attached to a round diamond saw under saline irrigation was used. A site free of muscular insertions was selected 15 mm above the ankle. After suture, rats ($n = 10$ per group) were daily s.c. injected either with saline or with 250 μg per dose of ODN IMT504 for 5 consecutive days. Fracture callus formation was radiographically evaluated on days 0 and 14. On day 14, animals were euthanized under ether atmosphere and the tibias were removed, fixed in 10% formol solution, decalcified in 10% EDTA solution, and embedded in paraffin. Transversal sections of each tibia at the site of the defect were cut, stained with hematoxylin and eosin, and examined under a light microscope.

In order to measure the effect of bone regeneration, a circular window of 0.5 mm^2 was superimposed to the central part of the defect and the consolidated bone area measured using the Image Pro-Plus software. Results are expressed in percentages of consolidated bone.

Statistics

Results are presented as the mean ± SEM. Statistical significance was evaluated by parametric and nonparametric tests as stated.

RESULTS

In Vitro Incubation with IMT504 Augments the Recovery of CFU-Fs from Rat BM Cultures

To study the effect of incubation of rat BM cells with IMT504 on CFU-F recovery after in vitro culture, BM cells were extracted from femurs and immediately seeded in plastic tissue culture flasks for cultivation in either the presence or absence of IMT504. After 14 days, culture-adhered CFU-Fs were counted in both control cultures and IMT504-treated cultures. Figure 1A shows that incubation in the presence of IMT504 increased about three times the mean number of fibroblastic colonies when compared with controls (CFU-F: 19 ± 6.3 vs. 6.8 ± 2.0/2 × 10^6 seeded BM cells, $p = .03$). On the other hand, there were no significant differences in the size of the colonies or in the cell morphology between cultures containing IMT504 and control cultures (Fig. 1B).

CpG ODNs Fail to Augment In Vitro Recovery of CFU-Fs from Rat BM Cultures

IMT504 is the prototype of a class of immunostimulatory ODNs characterized for the presence of the subsequence PyNTTTTGT as the active motif. The other major class of immunostimulatory ODNs described is characterized by the presence of a CpG dinucleotide in a given context as the active motif. In order to investigate the potential of these CpG ODNs to act as signals for CFU-F expansion, rat BM cells were incubated with two well known CpG ODNs (CpG-2006 and CpG-2216). Figure 2 shows typical CFU-Fs culture flasks incubated with these CpG ODNs in comparison with those incubated with IMT504. As it can be seen, none of the CpGs ODNs augmented the number of CFU-Fs. The recovery of colonies (mean ± SEM) was as follows: control, 6.8 ± 1.9; IMT504, 16.5 ± 2.6; CpG-2006, 8.6 ± 2.3; CpG-2216, 4.0 ± 1.4). One-way analysis of variance (Bonferroni post test for multiple comparisons) showed strong statistically significant differences only for IMT504 versus control ($p = .008$). These data are in agreement with those reported by Cho et al. [35], who found that CpG-ODN 2006 or CpG-ODN 2216 decrease human adipose tissue stromal cell proliferation and osteogenic differentiation. On the other hand, in our experiments, an increase in CFU-F in vitro was observed when BM cells were incubated with several immunostimulatory ODNs belonging to the PyNTTTTGT class (different from the prototype IMT504) and was not observed when BM cells were incubated with several nonimmunostimulatory ODNs (not shown).

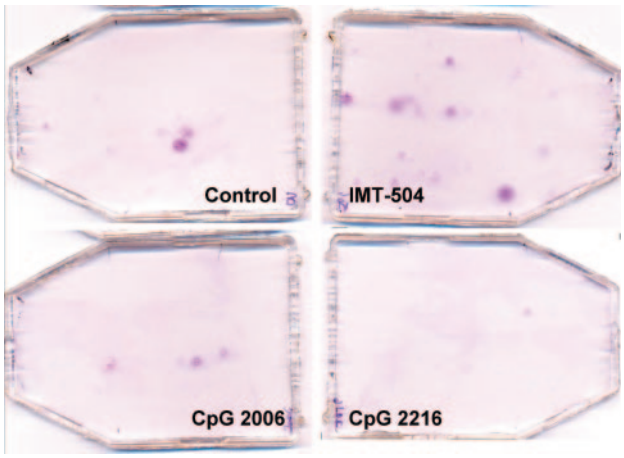

Figure 2. Giemsa stain of fibroblast colony-forming units from a representative bone marrow cultured in 25-cm^2 flasks with the in vitro addition of 1 μM of the indicated oligodeoxynucleotide: IMT504, TCATCATTTTGTCATTTTGTCATT; CpG 2006, TCGTCGTTTTGTCGTTTTGTCGTT; CpG 2216, GGG*G*G*A*C*G*A*T*C*G*T*C*G*GGGGGG (unmarked nucleotides indicate phosphothioate backbone, whereas asterisks indicate phosphodiester). Mean ± SEM as follows: control, 6.8 ± 2.0; IMT504, 16.5 ± 2.6; CpG 2006, 8.6 ± 2.3; CpG 2216, 4.0 ± 1.4). Differences were statistically significant (analysis of variance, Bonferroni post test for multiple comparisons, $p = .008$).

In Vivo Treatment with IMT504 Augments the Recovery of CFU-Fs from BM Cultures

To investigate whether IMT504 was able to augment the BM content of CFU-Fs in vivo, a group of rats was s.c. injected with IMT504 and the number of CFU-Fs generated by the BM cells of treated rats was compared with the number of colonies generated by the BM cells of rats s.c. injected with saline (untreated control). The average of total nucleated cells extracted from rats was 42 ± 4.3 × 10^3 in the control group and 36 ± 3.3 × 10^3 in the IMT504-treated group (not statistically significant according to the Student's t test, $p = .238$). Therefore, the total number of cells was not affected by the ODN treatment. In contrast, Figure 3A shows that BM of rats inoculated with ODN IMT504 presented about three times more CFU-Fs per femur as compared with rats inoculated with saline. No significant differences in the size of the colonies or in the cell morphology were observed between IMT504-treated animals and controls (Fig. 3B). This result

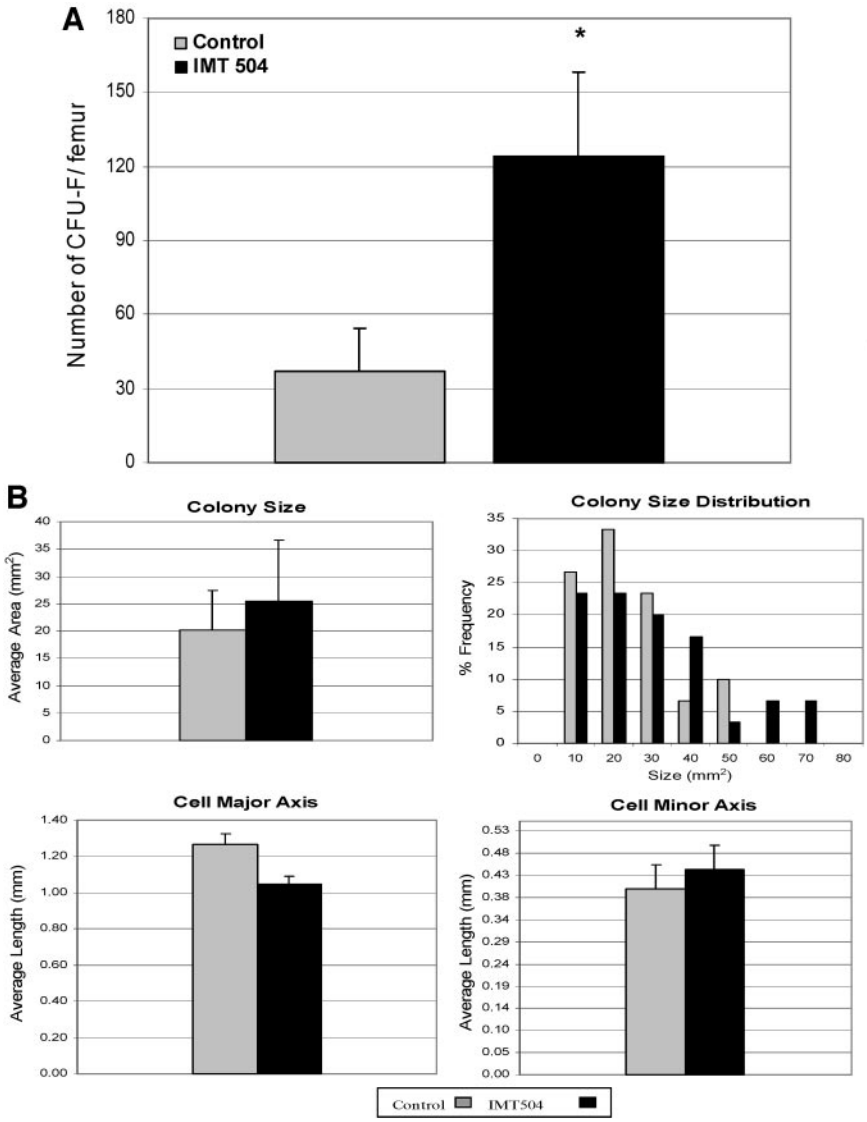

Figure 3. Effect of IMT504 on the CFU-F recovery in vivo. **(A):** Mean ± SEM of number of CFU-Fs per femur generated by rats injected with IMT504 as compared with controls ($n = 10$ animals per group). **(B):** The upper row shows size and size distribution of colonies and the lower row shows the major and minor axis from fibroblastic-like cells in the colonies of cultures corresponding to bone marrow from animals injected with either IMT504 or saline. Asterisks indicate statistically significant differences as compared with controls when $p \leq .05$ (unpaired t test). Abbreviation: CFU-F, fibroblast colony-forming unit.

indicates that the increase in BM CFU-Fs induced by the IMT504 treatment also occurs in vivo.

Differentiation Capacity of Rat BM-Derived Adherent Cells After In Vitro or In Vivo Treatment with IMT504

As shown above, when 2×10^6 cells were seeded in 25-cm^2 culture flasks, the recovery of CFU-Fs from untreated BM control cultures was 6.8 ± 2.0 for the in vitro assays and 1.8 ± 0.7 for the in vivo saline-treated animals (Mann-Whitney U test, $p = .07$). When IMT504 was present in culture flasks (in vitro) or was inoculated in animals (in vivo), the recovery of CFU-Fs was, in both cases, about three times higher than its respective control. This suggests that the effect of IMT504 on CFU-Fs may be similar in both cases. On the other hand, the treatment with IMT504 both in vitro and in vivo preserves the multiple differentiation potential of typical MSCs if cultured under specific conditions [33, 36]. Figure 4 shows that BM-derived adherent cells resulting from the treatment with IMT504 in vitro were able to differentiate into osteoblasts that form typical calcium deposits (Fig. 4B, 4C) or adipocytes showing the typical oil inclusions (Fig. 4E, 4F). Similar results were obtained with BM-derived adherent cells resulting from the treatment with IMT504 in vivo (not shown). Moreover, in both cases, untreated BM-derived adherent cells differentiated at the same rate as IMT504-treated cells. These results indicate that BM-derived adherent cells after in vitro or in vivo treatment with IMT504 have not only the morphological characteristics of regular MSC derived from BM precursors but also their typical capacity to differentiate into different cell lineages.

In Vivo Treatment with IMT504 Augments the Recovery of CFU-Fs from Rat Peripheral Blood

MSC progenitors in circulation are a likely natural source of cells for rapid repair of damaged tissues. Therefore, it was of interest to investigate whether CFU-Fs were increased in circulation upon subcutaneous injection of IMT504 in rats. Table 1 shows that the number of animals with detectable CFU-Fs was 8/12 if injected with IMT504 and 2/12 if injected with saline, a result highly statistically significant (Fisher's exact test, $p = .04$). When the recovery of CFU-Fs in these animals was considered, the IMT504-treated group reached 3.1 ± 1.6 per milliliter of peripheral blood, whereas the control group reached 0.3 ± 0.2 per milliliter of peripheral blood ($n = 12$ per group). On the other hand, and considering only the animals with detectable CFU-Fs, the estimated total number of CFU-Fs in

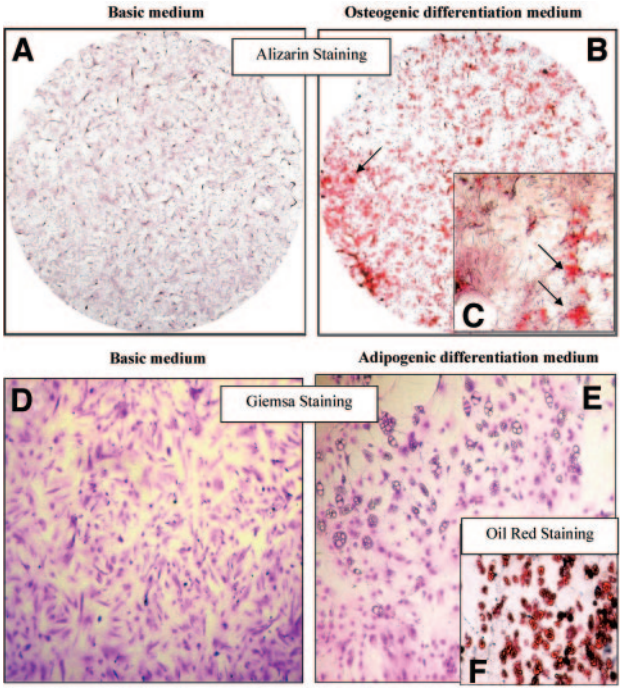

Figure 4. Differentiation of bone-marrow-adherent cells expanded in vitro in the presence of IMT504 under specific culture conditions. Right column shows differentiation medium compared with basic medium on the left column. Osteogenic differentiation **(A, B)** and Petri dishes stained with Alizarin Red **(C)**, ×100 magnification zone; arrows indicate characteristic calcium deposits. Adipogenic differentiation stained with Giemsa **(D, E)** or oil red O **(F)**; arrows indicate characteristic oil inclusions. Shown at a magnification of ×100.

circulation per positive animal was 74.8 ± 38.0 for the group of rats treated with IMT504 and 7.6 ± 6.6 for the untreated group. However, given the low number of positive controls, this last result could not be statistically analyzed. These results indicate that the treatment in vivo with IMT504 greatly increases the availability of CFU-Fs in circulation.

Injection of IMT504 Accelerates the Healing of Experimental Bone Defects in the Rat Tibia

Taking into account the demonstrated osteogenic activity of MSCs injected in the blood of animals [37] and the results here reported, we decided to investigate the osteogenic potential of the IMT504 treatment in vivo. According to the analysis of the consolidated bone surface at the site of a defect introduced in the tibia of rats 2 weeks after the injury, we found that the defect was more rapidly repaired in animals treated with IMT504 than in animals injected with saline. Percentage of consolidated bone was 79.8 ± 6.41 in IMT504-treated animals and 49.1 ± 3.59 in untreated animals (statistically significant, $p = .03$). Figure 5 illustrates the radiological and histological analysis of one control and one animal treated with IMT504. Acceleration of the ossification process in animals treated with IMT504 is apparent not only by more consolidated bone, but also because a well defined marrow space was observed as a consequence of the final steps in the bone remodelling process. These results strongly suggest that MSC precursors augmented in vivo by subcutaneous injection of IMT504 have a remarkable potential for tissue repair.

Table 1. Increase in the number of circulating CFU-Fs after in vivo treatment with IMT504

Animals/CFU-Fs	Control	IMT504
Total number of animals	12	12
Animals with detected CFU-Fs in circulation	2	8[a]
Total number of CFU-Fs in circulation	7.6 ± 6.6	74.8 ± 38.0
Number of CFU-Fs per milliliter of peripheral blood	0.32 ± 0.27	3.1 ± 1.6

Number of CFU-Fs corresponding to individual cultures of peripheral blood mononuclear cells of 12 animals subcutaneously injected with saline and 12 animals injected with five doses of IMT504 (250 μg per dose). Cultures were performed in triplicate without oligodeoxynucleotide additions.
[a] Differences were statistically significant, Fisher's test, $p = .04$.
Abbreviation: CFU-Fs, fibroblast colony-forming units.

Effect of IMT504 on CFU-F Recovery from Human BM Cell Cultures

Since the results obtained in rats were encouraging regarding the possibility of tissue repair therapy based on the systemic treatment with IMT504, we decided to test the stimulation capacity of IMT504 acting on human BM cells. For this, fresh samples of two human BMs were assayed in an in vitro test of CFU-Fs. In both cases, the number of CFU-Fs per 2×10^6 BM mononuclear cells seeded was more than twice in cultures treated with IMT504 as compared with those of untreated controls (34 vs. 13 for donor 1 and 69 vs. 34 for donor 2). Even though a statistical analysis was not possible because of the low number of samples analyzed, this result suggests that IMT504 can act on human BM cells as well as on rat BM cells.

DISCUSSION

It is well known that synthetic ODNs bearing special subsequences are able to stimulate the immune system and may be clinically useful as adjuvants in vaccines in the treatment of allergies and cancer [38]. However, to our knowledge, this is the first report describing the capacity of selected ODNs to augment the number of MSC precursors both in vitro and in vivo. ODNs with this capacity are all grouped in the PyNTTTTGT class of immunostimulatory ODNs. Some of the human cells stimulated by ODNs of this class are lymphocytes B and plasmacytoid dendritic cells [30] and also natural killer and natural killer T cells (our unpublished data). Stimulation of these cells results in secretion of cytokines such as IL6, IL10, GM-CSF, IFNγ and tumor necrosis factor-α, secretion of immunoglobulins, expression of cell surface proteins such as CD25, CD40, CD80, and CD86, and major histocompatibility complex classes I and II. Also, incubation of lymphocytes B with PyNTTTTGT ODNs results in protection of these cells from spontaneous apoptosis and expansion of at least a subset of these cells [30, 39].

The rise in the number of CFU-F observed upon incubation of BM cells in vitro with the prototype of the immunostimulatory ODNs of the PyNTTTTGT class IMT504 may be owed either to the stimulation of the replication of this cell precursor by soluble factors secreted by cells sensitive to the ODNs present in the BM cell pool or to the direct or indirect inhibition of the apoptosis of such a precursor. Moreover, and taking into account that IMT504 in our experiments was present at the beginning of the cultures, a possible role in facilitating the

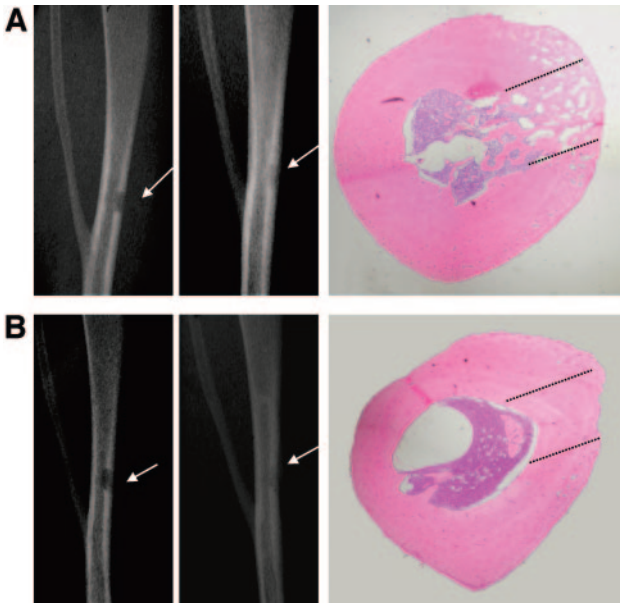

Figure 5. Osteogenesis acceleration in rats treated with IMT504. Radiography and hematoxylin-and-eosin-stained sections (×40) from rats with tibia bone injury. White arrows indicate the site of the injury both at the beginning of the treatment and 2 weeks later. **(A):** Representative animal treated subcutaneously with saline for 5 days after the injury. Stained sections show a partially invaded bone marrow space with vascular proliferation and trabecular thickness. **(B):** Representative animal treated subcutaneously with IMT504 for 5 days after the injury. Stained sections show well consolidated bone at the site where the injury was previously present (dotted line) and a normal bone marrow space, indicating the final stage in the ossification process.

attachment of the cells with the capacity to form fibroblastic colonies cannot be discarded. Regarding the in vivo treatment, it is even more difficult to anticipate the mechanism by which IMT504 augments the number of MSC progenitors in the BM and in circulation. Possible mechanisms include antiapoptotic effects and/or releasing of cytokines known to stimulate a subpopulation of CFU-Fs with more proliferation capacity such as IL17 [40] or known to augment the mobilization of CFU-Fs into peripheral blood such as granulocyte cell-stimulating factor [41].

Independently of the mechanism by which CFU-Fs are augmented by incubation with IMT504 in vitro or in vivo, the resulting colony cells possess the capacity to differentiate to the osteogenic and adipogenic cell lineages as regular MSCs. This is especially important since numerous studies involving a variety of animal models have shown that MSCs are useful in the repair or regeneration of damaged tissues [14–25]. Current protocols for tissue repair using MSC include in vitro culture of allogenic or isogenic MSCs and infusion of these cells in the circulation and local application of the cells either alone or, mostly in bone repair procedures, combined with different kinds of vehicles or supports [42]. The discovery of synthetic oligonucleotides with the capacity to increase the number of MSC active progenitors in vitro may contribute to shorten the time to obtain the necessary amount of cells for a treatment. However, clearly more important is the possibility of rapid repair of tissue damage by raising the patient's own MSCs by systemic treatment with a drug like the ODN IMT504 here described, thus avoiding ex vivo cell manipulation. The results of the bone healing study here described in a rat model seem to indicate the feasibility of this therapeutic approach. However, extension of these experiments to other animal models of tissue damage is clearly necessary in order to generalize the potential value of this approach to other tissues. Both the fact that human BM cells have an in vitro response to IMT504 similar to that of rat BM cells and the fact that, in our preclinical assays, IMT504 has demonstrated to be a very safe drug (unpublished data) warrant further studies in the route to clinical application.

Disclosure of Potential Conflicts of Interest

J.Z. and R.A.L. own stock in Immunotech S.A. (Argentina).

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IMT504, the Prototype of the Immunostimulatory Oligonucleotides of the PyNTTTTGT Class, Increases the Number of Progenitors of Mesenchymal Stem Cells Both In Vitro and In Vivo: Potential Use in Tissue Repair Therapy

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Stem Cells 2007;25;1047-1054
DOI: 10.1634/stemcells.2006-0479