Undifferentiated Human Adipose Tissue–Derived Stromal Cells Induce Mandibular Bone Healing in Rats

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Objective: To test the osteo-regenerative potential of adipose tissue–derived stromal cells (ATSCs), an attractive human source for tissue engineering, in a rat model of mandibular defect. Human dermal fibroblasts (HDFs) were used as a differentiated cellular control in the study.

Design: The ATSCs and HDFs were isolated from human lipoaspirate and skin biopsy specimens, respectively. Cells were characterized in vitro and then adsorbed on an osteo-conductive scaffold to be transplanted in a mandibular defect of immunosuppressed rats. Naked unseeded scaffold was used as a negative control.

Main Outcome Measures: Bone healing was studied by computerized tomography and histologic analysis after 4, 8, and 12 weeks.

Results: Computed tomography showed that undifferentiated ATSCs induced successful bone healing of the mandible defect when transplanted in animals, compared with HDFs and negative controls. Histologic analysis demonstrated that the newly formed tissue in the surgical defect retained the features of compact bone.

Conclusion: Undifferentiated human ATSCs are suitable for cell-based treatment of mandibular defects, even in the absence of previous osteogenic induction in vitro.

mal cells, were used as cell controls in this study.7 Previous studies13,15 have approached the cell-based therapy of bone defects by focusing on different issues, such as the impact of different cell sources, scaffolds, growth factors, and gene transfer techniques for producing genetically modified cells. Local activation of distinct signaling cascades is required for fracture repair and bone regeneration.16 Based on this rationale, the experimental hypothesis of this study was intended to verify the osteoinductive properties exerted by the disected bone microenvironment on undifferentiated ATSCs implanted at the site of bone defect. For this purpose, a rat model of mandibular defect was chosen because previous studies4,15 have demonstrated the pertinent use of such a model for the preclinical evaluation of alternative osteoinductive treatments.

**METHODS**

**PATIENTS AND SPECIMENS**

Adipose tissue-derived stromal cells were isolated from liposapirates of 3 patients aged 23 to 30 years from both sexes. Human dermal fibroblasts, which served as a “differentiated cell” control, were isolated from a 0.2-cm-diameter biopsy specimen of shaved skin obtained from the retroauricular region of healthy donors from both sexes aged 30 to 43 years. All the procedures used in this study were approved by the ethical committee of the Catholic University of Rome.

**CELL ISOLATION, CULTURE, AND CHARACTERIZATION**

Cell culture media, sera, and supplements were purchased from Lonza (Basel, Switzerland) unless otherwise specified. All the chemicals were purchased from Sigma-Aldrich Corp (St Louis, Missouri).

**Adipose Tissue–Derived Stromal Cells**

Adipose tissue specimens were extensively washed with a phosphate-buffered saline (PBS) solution and then were digested with 0.1% collagenase type VIII for 30 minutes at 37°C under gentle agitation. Enzymatic digestion was blocked by adding 10% fetal bovine serum, and the solution was filtered through 100-µm mesh to remove all residual tissue. The cell suspension was centrifuged at 2000 rpm for 5 minutes, and then pellet cells were seeded into a T75 tissue culture flask using Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and fibroblast growth factor β, 2 ng/mL. The next day, nonadherent cells were removed, and ATSCs were cultured up to 7 passages. The “mesenchymal” immunophenotype was confirmed by cytometry as previously described.4

**Human Dermal Fibroblasts**

Skin biopsy specimens were washed with a sterile PBS solution and then were mechanically mashed into small fragments. Each skin fragment was placed in a 35-mm culture dish and was incubated for 10 minutes at 37°C with 5% carbon dioxide and 85% humidity to increase the adhesion of the dermal surface to plastic. Complete culture medium (Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 5% horse serum, penicillin [1 U/mL], and streptomycin [1 µg/mL]) was carefully added. Tissue cultures were incubated until confluent fibroblast layers were obtained; the tissue fragments were removed, and cells were expanded in T75 flasks.

The proliferation rate of both cell populations was measured using the trypan blue exclusion assay to compare the growth kinetics, as previously described.7 The differentiation potential of both cell types was assessed to compare the plasticity of the 2 cell types before in vivo transplantation. Both ATSCs and HDFs were induced toward the osteogenic lineage in vitro as previously described.10 Acquisition of the correct phenotype was analyzed morphologically using specific histologic staining, chemically by measuring the activity of alkaline phosphatase, and molecularly by analyzing the expression of osteospecific genes. The methods for the histologic and molecular procedures have been described elsewhere.4,10 Alkaline phosphatase activity was evaluated in HDFs and ATSCs after 1, 2, and 3 weeks of osteogenic induction using cells cultured in growth medium as controls. The osteogenic medium was discarded, and cells were washed with PBS and then lysed with 1% Triton X-100 (Sigma-Aldrich Corp). The cell lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C, and the supernatant was stored at −80°C for further analysis. Alkaline phosphatase activity was determined by measuring the conversion of p-nitrophenyl phosphate to p-nitrophenol using a commercially available kit (Olympus OSR6103; Olympus Life and Material Sciences, Hamburg, Germany) following the manufacturer’s suggested procedures. The quantity of p-nitrophenol liberated from the substrate was determined by comparison with a standard curve. The absorbance was read at 405 nm using a microplate reader (Bio-Rad, Hercules, California). Enzyme activity was evaluated for 60 minutes and is expressed as maximum velocity. Reported values were normalized against the protein concentration determined in total cell lysate.

**Animal Treatments**

Undifferentiated ATSCs and HDFs were alternatively implanted into the mandibular bone defects created in recipient immunosuppressed rats to compare their bone regenerative capabilities in vivo.

**Cell Preparation**

To facilitate the delivery of cells, a nanocomposite osteoconductive scaffold composed of 20% hydroxyapatite and 80% collagen (HA/COL) was prepared and seeded with ATSCs and HDFs at the third culture passage suspended in sterile PBS, as previously described.4 The distribution and viability of living cells adsorbed on the HA/COL composite have been demonstrated in a previous study15: enhanced green fluorescence protein–expressing fibroblasts adsorbed on the same scaffold were homogeneously seeded and expressed the transgene 1 month after implantation in the rat mandible.

**Surgical Procedure**

Twelve Wistar rats (250-300 g) were anesthetized via an intramuscular injection of ketamine hydrochloride (50 mg/kg) and xylazine (6 mg/kg). A 5×5-mm full-thickness defect was created in the mandible behind the root of the incisor, as described elsewhere.4,15 The resulting defect was filled with the cell-scaffold compound, and the incisions were closed using polyglactin 910 (Vicryl; Ethicon Inc, Somerville, New Jersey). Six animals were treated with ATSCs, 6 received HDFs, and 6 received a mock treatment (a naked scaffold wetted with PBS solution) and served as controls. All the rats were immunosuppressed with daily subcutaneous administration of cyclosporine, 10 mg/kg/d, starting from the day of surgery and up to the tested time point. All the animals were fed a liquid diet during the first 4 weeks after surgery. Two animals per group...
were humanely killed 4, 8, and 12 weeks after the surgical procedures by lethal injection of pentobarbital sodium, 150 mg/kg intraperitoneally. Mandibular bone regeneration was assessed by computed tomography with tridimensional reconstruction (3D-CT). The extent of bone formation was measured in each animal by comparing the surface areas of the mandibular defect and of the bone tissue formed inside the defect based on the lateral view of 3D-CT images. Assuming the defect section to be approximately ellipsoidal, the horizontal and vertical diameters of the mandibular defect area were measured. The newly formed bone area was calculated by dividing the irregular-shaped surface into regular polygonal shapes. The surface areas of all the polygons in the area were then summed to obtain the total area of newly formed bone. Therefore, the “defect-filling” ratio was calculated as follows to obtain a percentage of bone defect filling:

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\text{defect-filling ratio} = \frac{\text{newly formed bone area}}{\text{ellipsoidal defect area}} \times 100\%
\]

Defect-filling ratios were calculated in each animal to quantify the extent of newly formed bone. The rat mandibles were then processed for histologic analysis performed using hematoxylin-eosin staining, as previously described.4,15

All the animal-handling procedures and treatments were performed according to the guidelines for animal experimentation of the ethical committee of the Catholic University of Rome, which approved this study.

CELL CHARACTERISTICS

The characteristics of ATSCs and HDFs were analyzed in vitro before transplantation to clearly define their phenotypes. The growth characteristics of the 2 cell populations were quite different, as ATSCs showed a lower mean doubling time than did HDFs after 4 culture passages (Figure 1A). This divergence increased with culture passages, as HDFs progressively slowed down after the 15th passage. A drastic arrest in cell growth occurred after the 25th culture passage in ATSCs and the 20th in HDFs. These data agree with previous studies7,17 demonstrating the higher proliferative rate of ATSCs compared with mesenchymal stromal cells isolated from different sources.

The osteogenic potential of ATSCs was confirmed after 3 weeks of in vitro osteogenic induction: the extent of the osteogenic differentiation of ATSCs and HDFs was confirmed by the increase in alkaline phosphatase activity after 1 week of culture in osteogenic medium (Figure 1B). Alizarin red staining confirmed a higher increase in deposition of mineralized matrix in ATSCs (C) compared with in HDFs (D). Scale bars equal 10 µm.

Figure 1. Cell growth and differentiation characteristics. A, Results of the trypan blue exclusion assay performed to measure the cell growth kinetics of the 2 cell types. Population doubling times (PDTs) of adipose tissue–derived stromal cells (ATSCs) and human dermal fibroblasts (HDFs) were calculated as means of replicate experiments based on the number of viable cells counted, as specified in the “Methods” section; a higher rate of cell duplication over time was observed for ATSCs than for HDFs at early culture passages, and at later culture passages, the differences in growth kinetics between the 2 cell populations were more evident. Error bars represent SD. B, Alkaline phosphatase–specific activity in ATSCs and HDFs during in vitro osteogenic differentiation. Enzymatic activity was statistically significantly increased after 2 weeks of culture under appropriate conditions. Data represent the mean (SEM) of 3 independent experiments (P < .05). Vmax indicates maximum velocity. Alizarin red staining of ATSCs and HDFs cultured for 3 weeks under osteoinductive conditions showed a higher increase in deposition of mineralized matrix in ATSCs (C) compared with in HDFs (D). Scale bars equal 10 µm.
osteogenic differentiation capacity in vitro of ATSCs (Figure 1C) compared with HDFs (Figure 1D). Finally, messenger RNA levels of bone-related genes were assessed by quantitative reverse transcription–polymerase chain reaction. Gene expression levels of the early osteogenic markers RUNX2 and BMP2 were transitorily increased in ATSCs at day 7 of in vitro culture with osteogenic medium; the expression of RUNX2 underwent more than 3-fold upregulation in HDFs after 3 weeks of osteogenic induction. No significant change in either early gene was observed in HDFs at the first tested time points (Figure 2A and B). Expression of the late-stage osteogenic gene ALP was dramatically upregulated in both cell types after 1 week of induction and remained elevated during differentiation, with higher levels in ATSCs compared with in HDFs at all the tested time points (Figure 2C).

ANALYSIS OF BONE REGENERATION IN VIVO

A stable mandibular bone defect was created in all the rats without interrupting the bone continuity, as previously described. The ATSCs and HDFs were alternatively adsorbed on the HA/COL osteoconductive scaffold and were implanted in a mandibular defect of recipient rats to comparatively evaluate their in vivo osteogenic potential. Rats treated with a naked scaffold served as negative controls. Both ATSCs and HDFs seeded on the HA/COL were vital and able to grow and proliferate 4 days after adsorption on the scaffold (data not shown). Moreover, a previous study demonstrated that implanted cells were vital after the HA/COL–cell compound implantation in the rat mandible.

The chewing activity of rats treated with either ATSCs or HDFs was near normal as soon as 4 weeks after surgery, whereas control rats treated with a naked scaffold displayed a clear decrease in chewing ability at all the tested time points after surgery.

The occurrence of bone formation at the site of implantation was evaluated in 2 animals per group on humane killing 4, 8, and 12 weeks after surgery by 3D-CT and histologic analysis. None of the animals displayed evidence of systemic or local toxic effects related to either the implantation procedure or the immunosuppressive treatment. The entire mandible remained in its original position in all the rats, and fracture of the mandible arch occurred in 2 control animals, as described in the “Morphologic Analysis” subsection.

3D-CT ANALYSIS

The 3D-CT imaging revealed efficient repair of the mandibular defects implanted with ATSCs adsorbed on the HA/COL nanocomposite scaffold in a time-dependent manner compared with controls (Figure 3). To quantify the extent of new bone formation in the mandibular defect, a defect-filling ratio was calculated for each animal and is expressed as a percentage, as described in the “Methods” section (Figure 4). In particular, 26.6% (averaged defect-filling ratio between the 2 replicates) of the bone defect produced in the mandible was partially filled with newly mineralized tissue as soon as 4 weeks after the implantation of ATSCs (Figures 3A and 4). The amount of new bone increased up to 47.8% and 61.3% (average between replicates in each experimental group) 8 and 12 weeks after surgery, respectively (Figures 3B-D and 4). In rats treated with HDFs adsorbed on the scaffold, some extent of bone regeneration was observed, as the percentage of newly formed bone in the mandible was partially filled with newly mineralized tissue as soon as 4 weeks after the implantation of ATSCs (Figures 3A and 4). The amount of new bone increased up to 47.8% and 61.3% (average between replicates in each experimental group) 8 and 12 weeks after surgery, respectively (Figures 3B-D and 4). In rats treated with HDFs adsorbed on the scaffold, some extent of bone regeneration was observed, as the percentage of newly formed bone in the mandible defect was below 17% up to 12 weeks after surgery (Figures 3E and F and 4). Nonsignificant amounts of new bone were observed in animals treated with HDF at the earlier time points (Figure 4). Modest and inefficient amounts of bone formation were observed in control animals treated with a naked HA/COL nanocomposite scaffold with sterile PBS. In particular, 2 control animals hu-
manely killed at 8 and 12 weeks showed a broken mandible branch as a result of partial bone resorption at the site of implantation (Figure 3G and H). In the remaining control animals, the mandible remained intact and the percentage of new bone surface in the surgical hole created in the mandible was 4% or less at all tested time points (Figure 4).

MORPHOLOGIC ANALYSIS

The morphologic features of the rats were then analyzed at the macroscopic and microscopic levels. The bone defect site was visible in both cell treatment groups as a smooth depression with a recognizable osseous callus in HDF- and ATSC-treated rats (Figure 5A and B). The mandibles of control animals treated with a naked scaffold allowed visualization of the full-thickness bone defect. The mandible arch was even fractured in 2 control rats (Figure 5C). Histologic analysis confirmed that the compact tissue formed in the defect displayed acidophilic affinity and uncontroversial aspects of mineralized compact bone (Figure 5D, E, G, H, J, and K). Particularly, in ATSC-treated mandibles, the newly formed bone tissue was evenly distributed, covering the defect size almost completely and reflecting the CT results. Connective tissue and traces of the reticular web of the HA/COL scaffold were visible in control mandibles (Figure 5F, I, and L).

COMMENT

Adipose tissue is commonly used for structural fat grafting in craniofacial and plastic surgery, where it has been proved to improve tissue regeneration rather than being simply a filler. Although the biological mechanisms underlying the regenerative capabilities of adipose tissue are still unclear, a role of undifferentiated multipotent mesenchymal cells (ATSCs) in the stroma of fat grafts has been proposed. In particular, ATSCs are known to efficiently differentiate toward the osteogenic lineage and increase bone regeneration on specific chemical induction or transfection with certain bone morphogenetic proteins. Other researchers have used ATSCs to induce bone healing in different animal models of cranial bone defects. These studies were based on cells induced toward the bone lineage in vitro using dexamethasone, which is known to exert systemic and local toxic effects, thus being inadequate for clinical application. Treatments with osteogenic factors or cell-transfection/transduction procedures are commonly used as alternative methods for ATSC differentiation for bone-healing purposes. Although efficacious and effective, cell transduction and production of transgenic growth factors require non–clinically adaptable procedures. In particular, the use of either plasmid or viral vectors is burdened with toxic effects and immunologic issues that must be considered before translating such experimental procedures to the bedside. The present study showed that human ATSCs, which were neither differentiated nor engineered before transplantation, implanted on a collagen-hydroxyapatite–based scaffold induced partial bone healing in an experimental model of rat mandibular defect. Although the bone defect was not completely healed, the bone regeneration allowed the functional recovery of treated rats. This finding could suggest that the microenvironment at the site of bone defect could induce the osteogenic commitment of ATSCs, promoting efficient bone healing in vivo. In fact, it has been demon-
Extrapolated that during the early stages of bone healing, a variety of inflammatory cells infiltrate the injured site and stimulate the repair process. Interleukin 1β is required to promote the proliferation of osteoblasts and the production of mineralized bone matrix, and bone morphogenetic proteins lead the molecular networking. Inside the cell, wingless-type MMTV integration site signaling is a key player in promoting bone morphogenetic protein 2–mediated osteoblastic differentiation of mesenchymal progenitors during embryo development and bone healing.

Therefore, in the experimental model described herein it could be hypothesized that the osteogenic commitment and subsequent differentiation of ATSCs in vivo occurred as a result of direct induction by locally produced growth factors and cytokines, which are physiologically implicated in the recruitment of local cells for new bone formation. Further study to detect the expression profile of growth factors secreted at the site of bone defects could clarify this aspect.

We previously demonstrated that cells adsorbed on the HA/COL composite and implanted in the rat mandible were still viable and homogenously distributed on the weblike structure of the scaffold 1 month after surgery. Together, these data could allow us to postulate that the implanted cells, rather than host surrounding cells, initiated the bone regeneration process. However, a significant contribution by the host mesenchymal stromal cells and preosteoblasts, proliferating at the site of bone defect and colonizing the scaffold, could not be excluded.

Some extent of bone regeneration was observed also in animals treated with HDFs compared with negative controls receiving a naked scaffold. Recent studies dem-

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**Figure 4.** Quantification of computed tomography results. The extent of bone healing was calculated in each rat as the ratio between the newly formed bone area and the ellipsoidal area of the mandibular bone defect obtained from the computed tomographic images. A, Representative graphical explanation of the area calculation procedure. B, Defect-filling ratios obtained from all the animals included in the treatment groups. ATSC indicates adipose tissue–derived stromal cell; CTRL, control; HDF, human dermal fibroblast; and NA, not assessed.
onstrated the osteogenic capacities of genetically engi-
neered dermal fibroblasts.15,26-30 Also, a recent growing
body of evidence confirms the idea of a certain degree of
pluripotency potential in HDFs.31-34 The diverse degree
of new bone formation observed in animals treated al-
ternatively with ATSCs and HDFs seemed to mirror the
difference in cell osteogenic potentials analyzed in vitro
(Figure 1B and C).

Conversely, implantation of a naked scaffold, in the ab-
sence of seeded cells, did not induce successful bone re-
germination. This was expected considering that a stable non-
union defect model was used. Some aspects regarding the

Figure 5. Histologic analysis. The rat mandibles were enucleated after humane killing to observe the mandibular defect site and then were decalcified and
processed for histologic hematoxylin-eosin staining. The figures are representative of the morphologic appearance of the defect area in the 3 experimental groups
at the macroscopic (A-C) and microscopic (D-L) levels. Dotted circles indicate the defect site, and squares are used to mark the microscopic field analyzed by
histologic methods. A, In a human dermal fibroblast (HDF)–treated animal at 12 weeks, a smooth depression was visible on the bone surface at the site of the
defect, with partial bone filling in the central area (arrow). B, In an adipose tissue–derived stromal cell (ATSC)–treated rat at 12 weeks, the defect appeared
macroscopically as almost completely healed (mandible of the rat examined by computed tomography in Figure 3C). C, Mandible of a control (CTRL) rat at 8
weeks displaying the fracture of the area surrounding the full-thickness defect created in the arch. D, In an HDF-treated rat at 12 weeks, areas of compact bone
tissue were surrounded by the connective matrix. Scale bar equals 0.5 mm. E, In an ATSC-treated mandible at 8 weeks, evenly distributed compact bone is visible
as acidophilic tissue filling the mandibular defect. Scale bar equals 0.5 mm. F, In a CTRL animal treated with a naked 20% hydroxyapatite and 80% collagen
scaffold at 12 weeks, connective scarlike tissue is visible along the border (arrow) of the bone defect. G, Higher magnification of the same microscopic field as in
D showing compact bone structure with some haversian canals formed by the lamellar layers. Scale bar equals 100 µm. H, An ATSC-treated rat at 12 weeks. Scale
bar equals 0.2 mm. I, In a CTRL mandible at 4 weeks, the microscopic structure of the scaffold is visible as an acidophilic reticular web (right side of the image).
Scale bar=0.3 mm. J, An HDF-treated mandible at 12 weeks shows detail of the mineralized tissue formed at the defect site (distinct biological replicate from D
and G). Scale bar equals 0.5 mm. K, An ATSC-treated rat at 4 weeks shows nonlamellar bone tissue with some traces of the scaffold (arrows) with cellular
infiltrates. Scale bar equals 0.5 mm. L, In a CTRL mandible at 4 weeks, some cell infiltration and small vessels (arrows) are visible in the scaffold at higher
magnification. Scale bar equals 100 µm.
effective role of grafted seeded cells in tissue regeneration are still controversial. Other authors described increased bone healing obtained through implantation of an unseeded scaffold in the presence of exogenous growth factors. The osteogenic role of local autologous cells in the fractured site has been proposed to explain this finding, although this mechanism could be efficient only in cases of small bone defects. The experimental model used in this study represents a critical size defect, which is inherently more challenging in clinical practice. In this case, the use of an unseeded scaffold would not represent a valuable tool to induce efficient bone healing. Some authors observed that the amount of native cells from surrounding tissue might not be enough to efficiently generate bone tissue before degradation of the implanted scaffold. This may result in scar tissue formation rather than bone formation at the site of the defect.

Together, these data could encourage the development of innovative approaches based on the implantation of autologous undifferentiated stromal cells as alternative therapeutic strategies to be easily translated into clinical practice.

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