Nonadherent Cell Population of Human Marrow Culture Is a Complementary Source of Mesenchymal Stem Cells (MSCs)

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ABSTRACT: To obtain enough quantity of osteogenic cells is a challenge for successful cell therapy in bone defect treatment, and cell numbers were usually achieved by culturing bone marrow cells in a relatively long duration. This study reports a simple and cost-effective method to enhance the number of mesenchymal stem cells (MSCs) by collecting and replating the nonadherent cell population of marrow MSCs culture. Bone marrow MSCs were isolated from 11 patients, cultured at a density of $1 \times 10^5/cm^2$ to $1 \times 10^6/cm^2$ in flasks. For the first three times of media change, the floating cells were centrifuged and replated in separate flasks. The total number of cells in both the primary and replating flasks were counted at day 21. Cell proliferation rate, potentials for osteogenic, chondrognenic, and adipogenic differentiation were examined in both cell types in vitro. In vivo osteogenic potentials of the cells were also tested in mice implantation model. The results showed that MSCs derived from nonadherent cell population of marrow cell cultures have similar cell proliferation and differentiation potentials as the originally attached MSCs in vitro. When implanted with hydroxyapatite/tricalcium phosphate (HA-TCP) materials subcutaneously in serve combined immune deficiency (SCID) mice, newly formed bony tissues were found in both cell type groups with osteocalcin expression. We have obtained 36.6% (20.70%–44.97%) more MSCs in the same culture period when the nonadherent cell populations were collected. The findings confirmed that the nonadherent cell population in the bone marrow culture is a complementary source of MSCs, collecting these cells is a simple and cost-effective way to increase MSCs numbers and reduce the time required for culturing MSCs for clinical applications. Published by Wiley Periodicals, Inc. J Orthop Res 24:21–28, 2006

Keywords: mesenchymal stem cells (MSCs); replate; nonadherent; tissue engineering; cell therapy

INTRODUCTION

In clinical orthopaedics, large bone defect(s) may occur when part of the bone is lost or excised in trauma, tumor surgery, bone infections, and other conditions. Under those circumstances, enhancement of bone healing is usually required. Autologous bone graft is the current gold standard treatment for bone loss, but its application is limited due to the limited amount of autologous bone available and the associated complications.[1] Bone marrow contains a rare population of multipotent MSCs that can differentiate into mesenchymal lineages, and can fabricate bone, cartilage, adipose, tendon, muscle, and other connective tissues.[2,3] Experimental results suggest that marrow-derived MSCs together with appropriate biomaterials may be an alternative to autologous bone grafts and marrow MSCs may serve as a major cell resource for the treatment of musculoskeletal disorders.[4–8]

To obtain sufficient quantity of osteogenic MSCs is an essential aspect for successful bone tissue engineering or cell therapy approaches. Osteogenic MSCs are usually cultured from autologous bone marrow aspirates or bone tissues in a relatively long duration (4–6 weeks) before the cell number is enough for clinical use. In some circumstances, such as patients with metabolic

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bone diseases, the culture time may be much longer, which further limited the clinical applications of MSCs. The long duration of cell in culture may also increase the risk of contamination and the associated costs of maintaining cell culture.

It has been suggested that MSCs may exist in the nonadherent cell population of bone marrow cell cultures, but all the nonadherent cell populations were discarded during media change. We hypothesized that (1) the nonadherent MSCs have similar proliferation and differentiation potentials as the adherent MSCs; and (2) collecting and replating the nonadherent cells from bone marrow culture may result in more osteogenic MSCs in the same duration of cell culture.

MATERIALS AND METHODS

Human Bone Marrow Cell Culture

Mononuclear cells were isolated by density gradient centrifugation from bone marrow washouts of the bone samples obtained from 11 patients who had undergone total hip replacement, plated at a density of $1 \times 10^5/cm^2$ to $1 \times 10^6/cm^2$ in T-75 flask with basal culture media containing Delbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2.5 μg/mL fungizone (Gibco), and cultured in a humidified atmosphere at 37°C with 5% CO_2. In multipotential differentiation studies, the basal culture media was replaced by specific induction culture conditions. The osteogenic induction conditions (OIC) were composed of basal culture media with additional supplements including 50 μg/mL L-ascorbic acid, 10 m*M* β-glycerophosphate, $10^{-8}M$ dexamethasone (Sigma Diagnostics Inc.). The adipogenic induction conditions (AIC) contained basal culture media with 0.5 m*M* IBMX, 1 μ*M* dexamethasone, and 50 μ*M* indomethacin (Sigma Diagnostics Inc.). The chondrogenic induction conditions (CIC) were serum-free medium supplemented with 10 ng/mL transforming growth factor β1 (TGF-β1) (PeproTech EC Ltd., London, UK), Premix ITS+ (BD Biosciences), and 1 m*M* pyruvate (Sigma Diagnostics Inc.). The culture media were replenished every 3 days for total 21 days.

Replating Nonadherent Cell Population

The first media change was made at day 7 and every 3 days thereafter. For the first three times of media change, the removed media were centrifuged at 250 *g* for 10 min and replated in a separate T-75 (first change) and T-25 flasks (second and third changes). Thus, one T-75 flask and two T-25 flasks of nonadherent cells resulted from one original T-75 flask.

Cell Expansion, Number Counting, and Cell Proliferation Assay

In primary and nonadherent cell population cultures, the cells in all flasks were harvested at 21 days from the day when the original flask was set up. The total number of cells in all replating flasks and original flasks were counted separately using the hematocytometer, and the percentage of increased cell number by the replating cultures to the total cell number was calculated. Cell viability was assessed for each experiment performed by trypan blue exclusion and was always more than 99%.

Rate of cell proliferation with or without osteogenic induction medium were examined by MTT method for both cell types using cells from passage 1. Cells were seeded at the density of 1×10^3 per well in 96-well plates in basal culture media. After 24 h, fresh media was changed, and medium was changed every 3 days thereafter. One plate was used for proliferation test by MTT assay from day 1 till day 12. For each selected plate, 150 μL of stock MTT (0.5 mg/mL) was added to each well, and the cells were further incubated at 37°C for 4 h. The supernatant was removed and 150 μL DMSO was added to each well. An ELISA reader was used to measure the absorbance at a wavelength of 492 nm.

Characterization of Nonadherent MSCs

Alkaline Phosphatase (ALP) Staining

At day 21, OIC treated cells were stained with a Sigma alkaline phosphatase kit (kit 86-R, Sigma Chemical) according to the manufacturer's instructions.

Mineralized Nodules and Von Kossa Staining

On days 35 to 42, mineralized nodules in the OIC-treated cultures were examined. Cells were fixed with 95% ethanol, then freshly prepared 2.5% silver nitrate solution was added to 6-well plates (3 mL/well), which were incubated in the dark for 10 min, then exposed under sunlight or ultraviolet (UV) light for 45 min. The reaction was terminated by rinsing thoroughly with distilled water, and treated with 3% thiosulphate for 5 min, then washed in distilled water.

Alcian Blue Staining

At day 21, nodulelike structures appeared in the CIC-treated cultures. They were fixed with 95% ethanol and stained with 1% alcian blue in 3% glacial acetic acid solution.

Oil-Red-O Staining

Following 14–21 days in AIC media, cells were washed with PBS, fixed with 10% neutral buffered formalin for 15 min, then stained with 0.5% oil-red-O in isopropyl alcohol: distilled water (60:40) for 30 min,

counterstained with hematoxyline, rinsed in tap water, air-dried and mounted on a cover slip with aqueous mounting gel.

In Vivo Implantation

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act (1986) at the Queen's University Belfast Biomedical Research facility. Approximately 1.5×10^6 of primary MSCs and replate-derived MSCs (all at third passage) were seeded onto hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic blocks with random porosity ($4 \times 4 \times 2$ mm) (BD Bioscience) or mixed with 40 mg of HA/TCP powder (Bicomposites), and incubated in a humidified atmosphere at 37° with 5% CO_2 for 2 h to allow the cell attachment. Then, MSCs–HA/TCP ceramic block complexes were transplanted and MSCs–HA/TCP powder slurry was injected subcutaneously into the dorsal side of 12-week-old male SCID (serve combined immune deficiency) mice (Harland).

Histology and Immunohistochemistry Examinations

At 8 weeks postimplantation, the implants were harvested and put into phosphate-buffered saline (PBS, pH 7.4) immediately. The surface characteristics, including the extent of blood-vessel invasion of the implants, were examined and documented under a dissecting light microscopy. Then, the implants were fixed with 10% neutral buffered formalin, decalcified with buffered 20% formic acid (pH 6.0) for 2–3 weeks, and embedded in paraffin. Five-micrometer sections were cut, deparaffinized, and processed for hematoxylin/eosin (HE) staining. Sections were also subjects for Alcian blue and Sirus red staining. Briefly, sections were rehydrated through graded alcohols, stained with Erlich's hematoxylin after being differentiated in 1% acid alcohol and blued in 2% sodium carbohydrogen, then stained with 0.5% Alcian blue in 1% acetic acid, 1% molybdophoric acid, and 0.1% Sirius red in saturated picric acid.

For immunostaining, cells on chamber slides were rinsed in PBS, fixed with 95% ethanol for 20 min, permeabilized with 0.1% Triton X-100/PBS for 1 min, then treated with 3% H_2O_2 for 5 min, and blocked in 10% normal swan serum, 0.1% bovine serum albumin (BSA) in PBS for 20 min. The cells were incubated sequentially for 1 h with or without the primary antibody diluted 1:100, including antibodies of collagen type I, osteocalcin, bone sialoprotein, and collagen type II (Santa Cruz Biotechnology, Inc.), the biotinylated secondary antibody diluted 1:100, and a Vectastain Elite ABC–HRP reagent. The bound ABC–HRP complex was detected using a Vector-AEC substrate kit for peroxidase, and counterstained with hematoxylene. Tissue sections were deparaffinized, rehydrated, and subject to immunohistochemistry staining with primary monoclonal anti-human osteocalcin antibody in a similar fashion.

Statistical Analysis

Cell proliferation data was analyzed by three-way analysis of variance (ANOVA) using statistical package SPSS11.0, and the significant level was set at $p < 0.05$.

RESULTS

Cell Proliferation Assay

Mesenchymal stem cells (MSCs) derived from the nonadherent cell population of human bone marrow cell cultures had similar cell proliferation rates in vitro when compared with the MSCs derived from the primarily adherent cell population. Adherent, spindle-shaped colonies were formed at day 7 in the replating nonadherent cell culture and they became confluent at day 21. When the nonadherent cells were collected and cultured accumulatively, the total number of MSCs increased to an average of 36.6% (20.70%–46.97%) in the same culture period, compared to the number of cells obtained from the primarily adherent cell cultures without nonadherent cell collections (Table 1). During 11-day period, the rates of cell proliferation were similar in both cell types when cultured in basal and OIC meida ($p > 0.05$, Fig. 1).

In Vitro Cell Differentiation Studies

MSCs derived from the nonadherent cell population of human bone marrow cell cultures had similar cell differentiation potentials in vitro. Under OIC, cells expressed osteoblastic markers including ALP (Fig. 2A), Von Kossa for mineralization (Fig. 2B), collagen type I (Fig. 3D), bone sialoprotein (BSP) (Fig. 2E), and osteocalcin (Fig. 2F). When cells underwent chondrogenic differentiation in CIC, they were positive for Alcian blue staining for the synthesis of proteoglycans (Fig. 2G) and collagen type II (Fig. 2H). Oil-red-O–positive lipid droplets appeared under AIC, suggesting adipocytic differentiation (Fig. 2I). No staining was seen in the methodological negative control (Fig. 2C).

In Vivo Implantation Study

After 8 weeks subcutaneous implantation in SCID mice, more new blood vessels were formed and grew into the MSCs/HA-TCP implants (Fig. 3A), compared with the cell free implants (Fig. 3B).

Table 1. Comparison of Cell Number of Primary and Replating (Puff Off) Marrow MSCs

Patient	Gender	Age	Pull Off ($\times 10^6$)	Primary ($\times 10^6$)	Total ($\times 10^6$)	Increase (%)
1	F	69	1.20	4.60	5.80	20.70
2	M	60	2.20	4.92	7.12	30.90
3	F	60	3.00	5.20	8.20	36.60
4	M	84	2.08	3.16	5.24	39.70
5	F	69	2.40	3.20	5.60	42.90
6	M	70	1.68	2.05	3.73	44.97
7	M	39	1.61	2.02	3.63	44.35
8	F	80	1.29	2.25	3.54	36.44
9	F	56	1.86	2.10	3.96	46.97
10	M	21	1.00	2.40	3.40	29.41
11	F	78	1.00	2.75	3.75	26.67

Newly formed bony tissues were seen in both the HA-TCP block (Fig. 3C) and HA-TCP powder (Fig. 3D) seeded with the replating derived MSCs. Bone matrix formation in implants with either cell type was evident by Alcian blue and Sirius red staining (Fig. 3E,F). Immunostaining showed osteocalcin positive in the extracellular matrices of the newly formed bony tissues in the HA-TCP powders seeded with replating-derived MSCs (Fig. 3G), whereas no positive osteocalcin staining was seen in the negative methodological control for immunostaining (Fig. 3H).

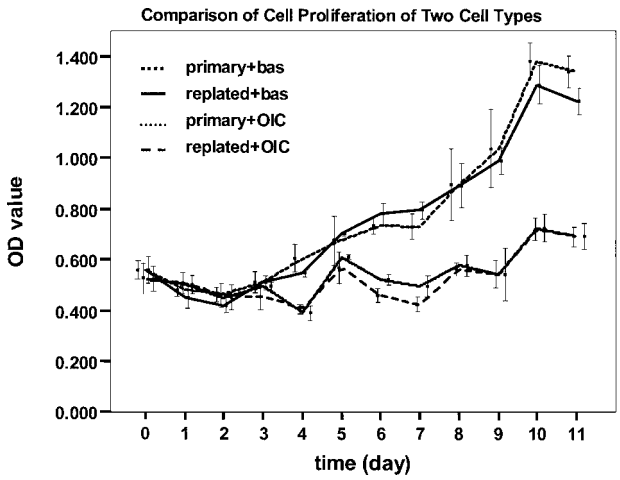

Figure 1. Comparison of cell proliferation rates of two cell types during an 11-day period. The replating-derived MSCs cultured under basal culture media and OIC (osteogenic induction condition) media had similar rates of cell proliferation at all time points, when compared to the MSCs derived from the primarily adherent cultures ($p > 0.05$, t-test).

DISCUSSION

Since Friedenstein first described bone marrow stromal cells three decades ago, the purification, culture expansion, differentiation, and implantation of marrow-derived MSCs have been widely explored.[9–11] MSCs can differentiate into many mesodermal-derived lineages, including osteoblasts, chondrocytes, adipocytes, hematopoietic supportive stroma cells, skeletal muscle cells, and others.[2,3,11] Besides bone marrow, MSCs have also been isolated and identified in many other tissues such as periosteum,[12] trabecula bone,[13] skeletal muscle,[14,15] adipose tissue,[16,17] dermis,[18] adult peripheral blood,[19] umbilical cord blood,[20] amniotic fluid,[21] and spleen.[22] To date, bone marrow is considered the most reliable source of MSCs.

Clinical use of culture-expanded marrow-derived MSCs in the fields of tissue engineering, cell therapy, and gene therapy has become popular and widely accepted. Preclinical studies have shown that marrow-derived MSCs could improve bone regeneration in large segmental bone defect models.[4,23] However, the safe, effective, and standardized full-scale approaches using MSCs for clinical orthopaedics have not yet been established. The first important step in MSCs-based cell therapy is to obtain sufficient quantity of cells in a clinically permitted period. Usually, this was achieved by culturing bone marrow or bone-derived cells in a relatively long period. MSCs can be isolated by density gradient centrifugation and expanded by their adherence characteristics, purified with certain cell surface markers.[24–27] Other methods have been employed to increase the proliferation of marrow-derived MSCs, including

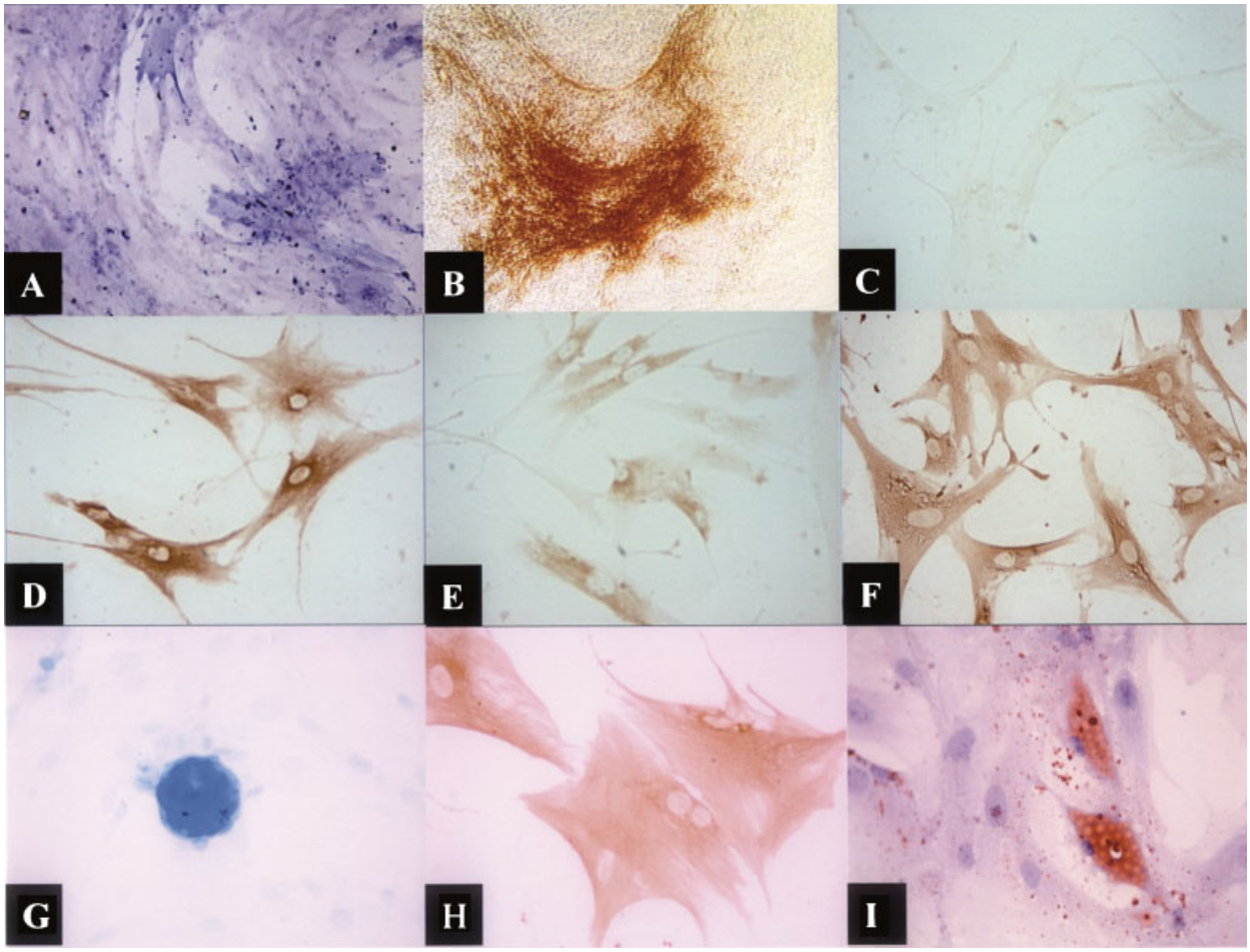

Figure 2. The replating-derived MSCs possessed multidifferentiation potentials when cultured in specific induction media. Under OIC, these cells expressed osteoblastic markers including ALP (A; original magnification, 100×), Von Kossa staining for mineralization (B; original magnification, 100×), collagen type I (D; original magnification, 200×), bone sialoprotein (E; original magnification, 200×), and osteocalcin (F; original magnification, 200×). The cells underwent chondrogenic differentiation in CIC, with Alcian blue–positive staining for the synthesis of proteoglycans (G; original magnification, 200×) and collagen type II expression (H; original magnification, 200×). Oil-red-O–positive lipid droplets appeared under AIC, suggesting adipocytic differentiation (I; original magnification, 200×). No positive staining was seen in the nonprimary antibody negative control (C; original magnification, 200×). [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

conditioned medium,[28] autologous serum or plasma,[29,30] and combinations of growth factors.[31] All these methods need additional manipulations of cells, which may affect their differentiation potentials as well as increase the risk of contamination.

We confirmed in this study that MSCs existed in the nonadherent cell population of primary bone marrow cell culture; when the nonadherent cells were collected and cultured accumulatively, the total number of MSCs increased to an average of 36.6% (20.70%–44.97%), compared with the number of cells obtained only from the primary bone marrow cultures in the same time frame. This is a simple and cost-effective method to increase cell numbers and shorten the cell culture time, hence reducing the risks of contamination. MSCs derived from the nonadherent cell population of human bone marrow cell cultures showed similar cell proliferation and differentiation potentials in vitro when compared with the MSCs derived from the primarily adherent cell populations.

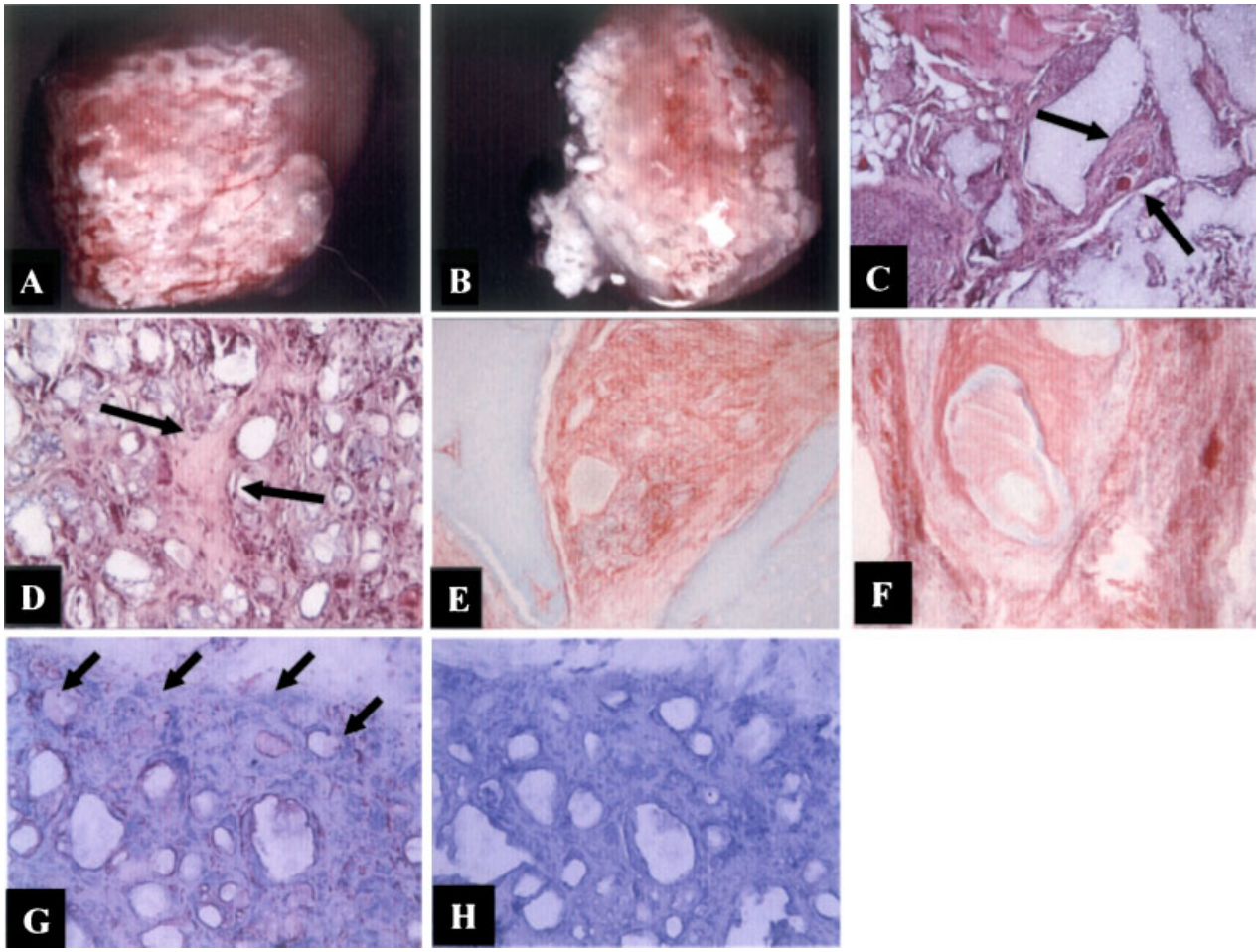

Figure 3. After 8 weeks subcutaneous implantation in SCID mice, more and larger new blood vessels formed and grew into the MSCs–HA/TCP implants (A; original magnification, 20×), compared with the cell-free implants (B; original magnification, 20×). Newly formed bony tissues (arrows) were found in both HA-TCP block implants (C; original magnification, 100×; HE staining), and HA-TCP powder implants (D; original magnification, 200×; HE staining) seeded with replating-derived MSCs. Bone matrix formation (red) was seen in the HA-TCP block implants seeded with replating-derived MSCs (E; original magnification, 200×; Sirius red staining) and primary cultured MSCs (F; original magnification, 100×; Sirius red staining). Immunostaining showed osteocalcin positive (arrows) in the extracellular matrices of the newly formed bony tissues in the HA-TCP powders seeded with replating-derived MSCs (G; original magnification, 200×), whereas no positive osteocalcin staining was seen in the negative methodological control for immunostaining (H; original magnification, 200×). [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

After 8 weeks subcutaneous implantation in SCID mice, many new blood vessels were seen the MSCs–HA/TCP implants seeded with MSCs, in contrast to the cell-free implants which have few new vessels, indicating that MSCs might produce vascular factors and improve vascularization of the implants. Newly formed bony tissue positive for osteocalcin was found in biomaterials implanted with both cell types, suggesting that both cell types have similar osteogenic potential in vivo. When high-density MSCs mixed with HA-TCP powders and incubated for 2 h, MSCs attached to the materials and MSCs/HA-TCP powder slurry formed, which could be injected subcutaneously and form new bone. This observation suggests that injectable autologous MSCs/HA-TCP powder

slurry may be used as an alternative to autologous bone grafts as previously reported.[8]

Confirmation of the existence of MSCs in the nonadherent cell population in bone marrow cell cultures indicates that some precursors of MSCs are nonadherent in nature and only become adherent when they differentiated. Although the underlining mechanisms and potential regulating factors remain unclear, several factors may involve in regulating this event: (1) the growth factors or cytokines produced by hemaotopoietic cells of bone marrow populations or proliferating MSCs may keep some of the MSCs in a nonadherent state; (2) the daughter MSCs produced during self renewing of attached MSCs may detach from the culture flasks, and this event may be controlled by local cell–cell contacts and communications.

In conclusion, this study established a simple and cost-effective method to increase the number of MSCs by replating the nonadherent cell population in the bone marrow cell cultures. The nonadherent MSCs maintained similar osteognic potentials in vitro and in vivo as the adherent MSCs do, and they may serve as a complementary source of MSCs to facilitate the clinical applications of MSCs in tissue engineering and cell therapy.

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