Effects of CD44 antibody– or RGDS peptide–immobilized magnetic beads on cell proliferation and chondrogenesis of mesenchymal stem cells

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Abstract: We evaluated the efficacy of a novel mesenchymal stem cell (MSC) delivery system using an external magnetic field for cartilage repair *in vitro*. MSCs were isolated from the bone marrow of Sprague Drawley rats and expanded in a monolayer. To use the MSC delivery system, two types of MSC-magnetic bead complexes were designed and compared. Expanded MSCs were combined with small-sized (diameter: 310 nm) carboxyl group-combined (0.01–0.04 μmol/mg) magnetic beads, Ferri Sphere 100C®, through either anti-rat CD44 mouse monoclonal antibodies or a synthetic cell adhesion factor, arginine (R)-glycine (G)-aspartic acid (D)-serine (S) (RGDS) peptide. Both cell complexes were successfully created, and were able to proliferate in monolayer culture up to at least day 7 after separation of magnetic beads from the cell surface, although the proliferation of the complexes was slower in the early period of culture than that of non-labeled rat MSCs (after 7 days of culture: proliferation of CD44 antibody-bead complexes, approximately 50%; RGDS peptide-bead complexes, 70% versus non-labeled rat MSCs, respectively). These complexes were seeded onto culture plates with or without an external magnetic force (magnetic flux density was 0.20 Tesla at a distance of 2 mm from plate base) generated by a neodymium magnet, and supplemented with chondrogenic differentiation medium. Both complexes could be attached and gathered effectively under the influence of the external magnet, and CD44-bead complexes could effectively generate chondrogenic matrix in monolayer culture. In a three-dimensional culture system, the production of a dense chondrogenic matrix and the expression of type II collagen and aggrecan mRNA were detected in both complexes, and the chondrogenic potential of these complexes was only a little less than that of rat MSCs alone. Thus, we conclude that due to the fact that MSC-RGDS peptide-bead complexes are composed using a biodegradable material, RGDS peptide, as a mediator, the RGDS peptide-bead complex is more useful for minimally invasive clinical applications using our design of magnetic MSC delivery system than CD44 antibody-beads. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 773–784, 2006

Key words: magnetic beads; magnetic force; mesenchymal stem cell; cell proliferation; chondrogenic differentiation

INTRODUCTION

Regenerative medicine for tissue repair has been the focus of many studies. In the field of orthopedics, the expectation of regenerative medicine for cartilage repair has been increasing, because articular cartilage has poor intrinsic healing capacity due to a lack of blood vessels and its isolation from the systemic regulation.[1–3] Although much work on regenerative medicine is currently focused on tissue engineering, this usually requires technically demanding procedures with some special equipment or facilities and proper scaffolds or growth factors. Therefore, intravenous or intraarticular cell transplantation without scaffolds is a more attractive option, for repair. However, one problem with the injection of isolated cells is the fact that they are likely to be easily diluted in the joint fluid, and they probably cannot reside in the injured site for the required period for tissue regeneration.

To solve the aforementioned problem, we attempted to develop a technique for cell-based cartilage repair in which cells were coupled with magnetic beads. Cells coupled with magnetic beads were in-

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jected into a joint, and external magnetic fields were used to localize the transplanted cells at the desired location.[4] For transplanted cells, bone marrow-derived mesenchymal stem cells (MSCs) were used, because it is well known that under the appropriate conditions they can differentiate into several lineages, including osteogenic, chondrogenic, or adipogenic lineages[5] and MSCs, when cultured with buffy coat and red blood cells from bone marrow after isolation without cell separation using a density gradient, express specific surface antigen molecules such as CD29 and CD44.[6] Recently, Majumdar et al. have reported that CD44 on CD105-positive bone marrow stromal cells, including MSCs showing the ability to proliferate and differentiate along the chondrogenic lineage, is expressed at ~98% of the level of freshly isolated cells up to at least passage five.[7] Based on this information, we hypothesized that CD44-positive bone marrow-derived MSCs cultured by Kotobuki's method (which is easier than Majumdar's isolation method) would have the ability to proliferate and differentiate along the chondrogenic lineage. To verify this hypothesis and use our magnetic cell delivery system for cartilage repair, anti-CD44 antibodies were chosen as a mediator to couple cells with magnetic beads. Following a successful pilot study using CD44-antibody-immobilized magnetic bead-conjugated MSCs developed by us (MSC-CD44 antibody-magnetic-bead complex),[4] we report here on further experiments using this complex.

In addition, most cells including MSCs express integrin proteins in the cell membrane, and attach to extracellular matrix proteins, such as fibronectin, through the cell adhesion factor, arginine (R)-glycine (G)-aspartic acid (D) (RGD) amino acid sequence.[8–10] MSCs also express integrin αv, which is one of the ligands of RGD peptides,[11] and are known to be adhesive cells.[5–7] Subsequent to our design of the MSC-CD44 antibody-magnetic-bead complex, we wished to assemble an MSC-magnetic bead complex, using a more biodegradable material than antibodies as the mediator, because antibodies are thought to be difficult to degrade in the body as binding between the antigen and the antibody is strong. We, therefore, also combined MSCs with a synthetic cell adhesion factor, RGD-serine (S), to generate (RGDS) peptide-immobilized magnetic beads (MSC-RGDS peptide-magnetic-bead complex).

To use our magnetic cell delivery system for cartilage repair, the present study set out to investigate the effects of the two materials (CD44 antibody- or RGDS peptide-immobilized magnetic beads) on cell proliferation, the delivery to the desired location by external magnetic field, and the chondrogenic potential of MSCs, and to verify *in vitro* which material is most useful for our magnetic cell delivery system.

MATERIALS AND METHODS

Isolation and expansion of mesenchymal stem cells (MSCs)

Experimental rats were kept in the research facilities for laboratory animal science at our university. The research protocol of this experiment was reviewed and approved by the university ethical committee. The method of isolation and *in vitro* expansion of bone marrow-derived MSCs, retaining the phenotype, is well known and has been previously described.[6] A modification of Kotobuki's culture method was used. Briefly, bone marrow from Sprague Dawley (SD) rats (12-weeks-old) was flushed out of the marrow cavities, using a pressurized culture medium consisting of high glucose-Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corp., Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS, Sigma–Aldrich Corp., St. Louis, MO) and penicillin-streptomycin-fungizone (Bio-Whittaker, ML). The cells including buffy coat and red blood cells were seeded onto 100-mm culture dishes (Falcon, BD Bioscience, Franklin Lakes, NJ) in culture medium, and incubated in a humidified atmosphere of 5% CO_2–95% air at 37°C. The medium remained unchanged for the first 7 days, and was subsequently changed every 2–3 days. Two to 3 weeks after seeding, the cells had proliferated and reached confluence. The cells were then harvested by treating with 0.25% trypsin and 0.02% EDTA, and rinsed twice with culture medium. To expand the MSCs, 2–3 × 10^5 of the harvested cells were seeded on 100-mm culture dishes. On reaching confluence again, the cells were reseeded under the same conditions.

Expression of the cell surface antigen, CD44, in MSCs (immunohistochemical staining)

To examine whether rat MSCs expanded in monolayer culture express the surface antigen, CD44, immunohistochemical staining was performed. About 3 × 10^4 MSCs were attached to a glass slide by centrifugation, dried, and fixed with ethanol for 30 min. To avoid a non-specific reaction, the prepared specimens were treated with Histofine® blocking reagent (Nichirei Co., Tokyo, Japan). The specimens were then treated with anti-rat CD44 mouse monoclonal antibodies (Chemicon International Inc, Temecula, CA) at a final concentration of 1 μg/mL in 3% bovine serum albumin (BSA, Sigma) in phosphate buffered saline without calcium and magnesium (PBS(−)) at 4°C overnight in a humidified atmosphere. Biotin-labeled anti-mouse secondary antibody (Nichirei) was applied for 10 min at 25°C, followed by alkaline phosphatase-labeled streptavidin (Nichirei) treatment for 10 min. Finally, the specimens were incubated with alkaline phosphatase substrate solution (color development: fast red, Nichirei) for 20 min at 25°C, and the nuclei were counterstained with Haematoxylin. The specimens were examined with a Nicon light microscope. Controls were treated following the same procedure, except that the primary antibody was omitted.

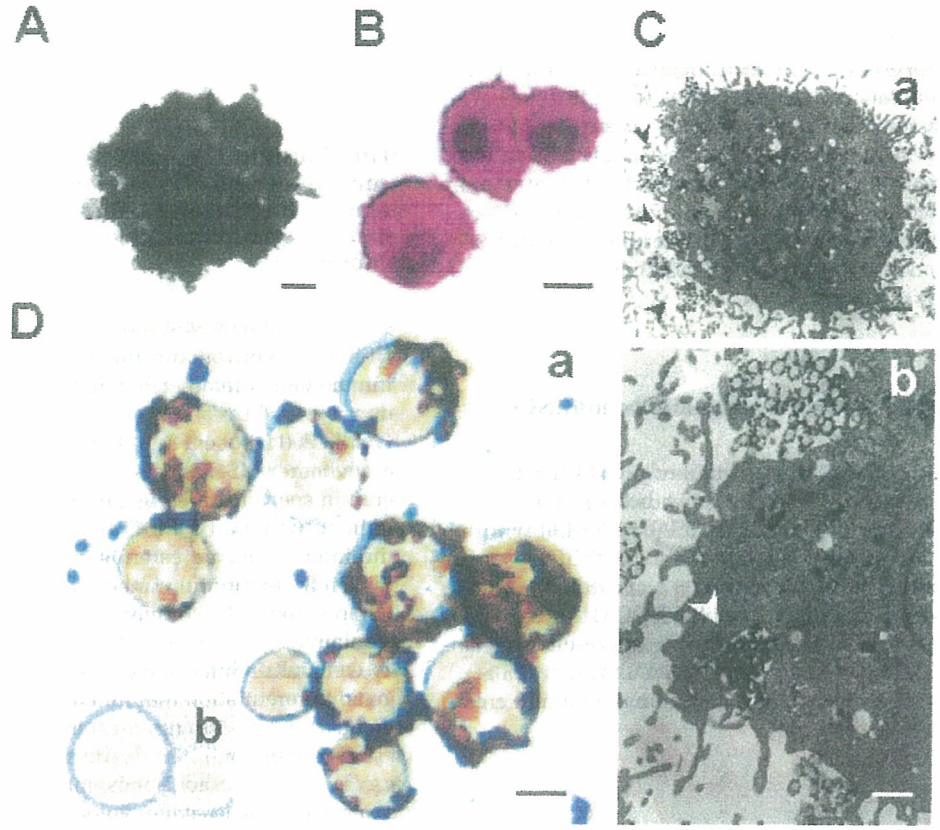


Figure 1. MSC-CD44 antibody-magnetic bead complex. A: Electron microscopic view of a magnetic bead, Ferri Sphere 100C®. Magnification, ×100,000. The scale bar indicates 50 nm. B: Expression of CD44 antigens in rat MSCs. Rat MSCs at passage four were stained immunohistochemically, which showed that the CD44 antigens were exhibited on the surface of rat MSCs up to at least passage four. Magnification, ×400. The scale bar indicates 5 μm. C: Electron microscopic views of MSC-CD44 antibody-magnetic bead complex. (a) Magnification ×2500. The scale bar indicates 2 μm. (b) Magnification, ×5000. The scale bar indicates 1 μm. The arrows indicate CD44 antibody-immobilized magnetic beads attached to the surface of an MSC. D: Light microscopic views of MSC-CD44 antibody-magnetic bead complex (a) and normal non-labeled rat MSCs (b). Magnification, ×400. The scale bar indicates 5 μm. The data shown are typical of two independent experiments ($n = 3$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Assembly of MSC-magnetic beads complex

Two types of MSC-magnetic bead complex were composed using MSCs and a mediator: CD44 antibodies (Chemicon) or RGDS peptides (Peptide institute, Osaka, Japan) with mono-sized and carboxyl group-combined magnetic beads. To assemble the MSC-magnetic bead complex, first a mediator was immobilized to the magnetic beads, and then the prepared magnetic beads were combined with expanded MSCs.

Carbodiimide-mediated immobilization of CD44 antibody or RGDS peptide to the magnetic beads by amide bond formation

We used uniform, mono-sized magnetic beads composed of styrene-acryl polymers as a core, coated with magnetic ferrite thin film [Fig. 1(A)] (diameter: 310 nm; density: 1.8 g/cm^3; the amount of magnetization: 27 emu/g; carboxyl groups are introduced on the surface: 0.01–0.04 μmol/mg; Ferri Sphere 100C®, Nippon Paint, Tokyo, Japan). The coupling procedure involved the formation of an amide bond between a primary amino group of the CD44 antibody or RGDS peptide and the carboxyl groups on the surface of the magnetic beads, mediated by carbodiimide activation. Because the intermediate product of the reaction between the carboxylic acid and the carbodiimide is very labile and would hydrolyze quickly, a less labile intermediate (NHS: *N*-hydroxy succinimide) was used. Briefly, after 3 mg of magnetic beads were washed twice with 500 μL 0.01 *N* NaOH for 10 min with thorough mixing, they were washed three times with de-ionised water in the same manner to remove excess liquid. Then, 50 μL of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) solution (50 mg/mL in 25 m*M* MES (2-[*N*-morpholino]ethane sulfonic acid), pH 5) and 50 μL NHS solution (50 mg/mL in 25 m*M* MES, pH 5) were added, mixed well, and incubated with slow tilt rotation at room temperature for 30 min. After incubation, the tube containing the magnetic beads was placed on a magnet for 4 min and the supernatant was removed. The beads were washed twice with 25 m*M* MES, pH 5. Twenty micrograms of CD44 antibody or RGDS pep-

tide dissolved in 25 m*M* MES-buffer, pH 5, were added to the activated beads, making a total volume of 500 μL. The mixture was vortexed and then incubated for 3 h at 25°C with slow tilt rotation. After incubation, the tubes were placed on a magnet for 4 min and the supernatant was removed. 0.05 *M* ethanolamine in PBS(−), pH 8, was added and the magnetic beads were incubated for 1 h at room temperature with slow tilt rotation to quench non-reacted groups. Finally, the beads were washed four times with 0.5% BSA in PBS(−) and suspended in 0.5% BSA in PBS(−) at the concentration of 3 mg beads/mL.

Complexing of prepared magnetic beads to MSCs

Forty five microliters of CD44 antibody- or RGDS peptide-immobilized magnetic beads (225 μg beads) and 1×10^6 MSCs were mixed in 355 μL of 0.5% BSA in PBS(−) with slow tilting and rotation for 1 h at 4°C or at 37°C, respectively. The tubes were then placed on a magnet for 4 min to collect the complexes. The assembled MSC-CD44 antibody-bead complexes and the MSC–RGDS peptide-bead complexes were washed 4 times with 0.5% BSA in PBS(−), respectively, and resuspended in culture medium at a concentration of 5×10^6 cells/mL.

Preparation of specimens of MSC-magnetic beads complexes for electron microscopy

MSC-magnetic bead complexes were centrifuged and supernatant was discarded. Precipitated complexes were fixed with 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) for 2 h at 4°C, then post-fixed with 1% OsO_4 in 0.1 *M* phosphate buffer (pH 7.4) for 2 h at 4°C. Specimens were then dehydrated in an ascending series of ethanols, replaced with propylene oxide, and embedded in epoxy resin. Semithin sections (~1 μm) were cut and stained with 0.5% toluidine blue in 0.5% borate for light microscopy. Thin sections (0.1 μm) were cut, stained with 3% uranyl acetate and lead citrate, and observed with a transmission electron microscope, JEM-1200 (JEOL, Tokyo, Japan).

Cell proliferation assay

Cell proliferation was assayed by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) procedures basically as previously described.[12] The assay was performed using a commercial kit, Cell Counting Kit-8 (Dojindo laboratories, Kumamoto, Japan). About 1×10^4 cells of the MSC-magnetic bead complex or control rat MSCs were seeded into each well of a 24-well plate. At various times after seeding (0, 1, 3, 5, and 7 days of culture), the working solution containing WST-8 and 1-Methoxy PMS (5 and 0.2 m*M*, respectively, as the final concentration) was added to each well at 1:10 volume of culture medium. After incubation for 4 h, the absorbance of each well was measured at 450 nm, with a microplate spectrophotometer, SpectraMax 190 (Molecular Devices, Sunnyvale, CA).

Distribution of prepared MSC-magnetic bead complexes under the influence of an external magnetic force, and monolayer culture for generation of chondrogenic matrix by the complexes

Before seeding MSC-bead complexes, magnetic flux density in the vertical and horizontal directions of the neodymium magnet (diameter: 5 mm; height: 5 mm; magnetic flux density: 0.43 Tesla (T)) was measured using a gauss meter, HGM-8200 (Probe: FS-3, ADS Co., Tokyo, Japan). To evaluate whether MSC-magnetic bead complexes could be gathered in a specific area by an external magnetic force, distribution of prepared MSC-magnetic bead complexes under the influence of an external magnetic force was observed. The cell distribution method used was modified from Hirao's method.[13] Briefly, 40 μL of MSC-bead complexes (containing 2×10^5 cells) were seeded onto the center of 24-well culture plates, which contained 300 μL of chondrogenic differentiation medium: High-glucose DMEM supplemented with 10 ng/mL transforming growth factor (TGF)-β3 (Sigma), 10^{-8} *M* dexamethasone (Sigma), 50 μg/mL ascorbic acid-2-phosphate (Sigma), 40 μg/mL L-proline (Nacalai tesque, Kyoto, Japan), ITS-A supplement (Invitrogen, 10 μg/mL insulin, 6.7 ng/mL sodium selenite, 5.5 μg/mL transferrin, 110 μg/mL sodium pyruvate), and 1.25 mg/mL BSA (Sigma). In the experimental group (magnet group), a neodymium magnet was placed beneath the center of the plate (magnetic flux density was 0.20 T at a distance of 2 mm from the plate base). No magnet was used in the control group. After incubation for 1–2 h at 37°C to allow for cell attachment, 700 μL of chondrogenic differentiation medium was added to each well. Sixteen hours after adding the differentiation medium, the magnet was removed from underneath the plate. Cell number was counted in the center of the plate (area M) and in four peripheral areas, each 3 mm in diameter [areas C1–C4; Fig. 5(A)]. The cell number in each area in six different plates was counted using a light microscope (100-fold) and the mean was calculated.

After culturing the MSC-bead complexes for 21 days with chondrogenic medium, the plates were washed three times and stained with toluidine blue. The plates were then assessed macroscopically and histologically.

Pellet culture for chondrogenesis of MSC-magnetic bead complexes

To assess the chondrogenic potential of the MSC-magnetic bead complexes or of control rat MSCs in standard three-dimensional culture conditions, a modification of Johnstone's pellet culture system[14] was used. About 2×10^5 MSCs, alone or as an MSC-magnetic bead complex, suspended in the earlier mentioned chondrogenic differentiation medium, were placed in a 15-mL polypropylene tube

(Greiner Bio One, Frickenhausen, Germany), and centrifuged to form a pellet. The pellet was cultured for 21 days at 37°C with 5% CO_2 and 95% air in 1 mL of the chondrogenic differentiation medium. In the control group, culture medium without chondrogenic differentiation factors (TGF-β3 and dexamethasone) was used.

Toluidine blue staining and expression of type II collagen in MSC-magnetic bead complexes

After culturing the pelleted MSC-magnetic bead complexes or control rat MSCs for 21 days, they were fixed with 4% paraformaldehyde and embedded in paraffin. After deparaffinization, sections (5 μm) were stained with toluidine blue solution. To assess expression of type II collagen, the sections were permeabilized with proteinase K (Dako, Japan) in 0.1% Tween-20 in PBS(−) for 5 min at room temperature. The expression of type II collagen was assessed using the same immunohistochemical method as used for CD44 expression described earlier, with anti-rat type II collagen goat polyclonal antibodies (Santa Cruz Biotechnology Inc., CA) at a final concentration of 1 μg/mL.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from the pellets of MSC-magnetic bead complexes or rat MSCs using the RNeasy micro kit (Qiagen, Tokyo, Japan). Prepared RNA was converted to cDNA using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. PCR was performed in a Minicycler (PTC-150, Bio-Rad, Hercules, CA). PCR amplification conditions for rat integrin αv, aggrecan, type II collagen, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 56°C (or for GAPDH: 58°C) for 30 s, and 68°C for 1 min. The reaction products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. The GAPDH primers were designed ourselves, the integrin αv primers were as described[11] and the aggrecan and type II collagen primers were as described.[15] The primers were as follows:

integrin αv (forward): 5′-CCTGCTTTCTTCAGGACGGC-AC -3′,
integrin αv (reverse): 5′-CCCATCGGAACCTCGGTC-CATGA-3′,
aggrecan (forward): 5′-TAGAGAAGAAGAGGGGTTAGG-3′,
aggrecan (reverse): 5′-AGCAGTAGGAGCCAGGGTTAT-3′,
type II collagen (forward): 5′-GAAGCACATCTGGTTTG-GAG-3′,
type II collagen (reverse): 5′-TTGGGGTTGAGGGTTTT-ACA-3′,
GAPDH (forward): 5′-GCCAAAAGGGTCATCATCTC-3′,
GAPDH (reverse): 5′-GCCTGCTTCACCACCTTCTT-3′.

Statistical analysis

To compare the cell number between each area (area M and areas C1–C4) in the magnet or control group, one-way analysis of variance was used. If a statistical difference existed, Scheffe's post-hoc test was used. The Mann–Whitney *U*-test was used to compare the cell number between the magnetic group and control group in each area (M and C1–C4). A P value of less than 0.05 was regarded as statistically significant.

RESULTS

Assembly of MSC-magnetic bead complexes

Immunohistochemical staining for CD44 antigens revealed that CD44 antigens are expressed in ~90% of expanded MSCs up to at least passage four [Fig. 1(B)]. We decided to use MSCs which were expanded up to passage four for assembling MSC-CD44 magnetic bead complexes. Next, CD44 antibody-immobilized magnetic beads were combined with expanded MSCs, and subsequently, MSC-CD44 antibody-magnetic bead complexes were observed morphologically by electron and light microscopy. This showed that some massive bead conglomerates were attached on the surface of MSCs [Fig. 1(Ca,Cb,Da)]; in contrast no massive bead conglomerates were attached to non-labeled rat MSCs [Fig. 1(Db)]. MSCs could be combined with small-sized magnetic beads through rat CD44 antibodies [Fig. 1(Ea)]. For MSC-RGDS peptide-bead complexes assembled using MSCs expanded up to passage four, the same result was obtained by interaction through the RGDS peptide [Fig. 2(A, Ba,Bb,C). Moreover, cell counts performed with a light scope showed that the assembly ratio of complex was ~80–90% after antigen–antibody reactions or interaction between integrins on MSCs and RGDS peptide. Thus, MSCs could be coupled with small-sized magnetic beads through CD44 antibodies or RGDS peptides.

Proliferation of MSC-magnetic beads complexes

MSC-CD44 antibody-bead complexes did not proliferate up to 3 days of culture although they were attached to the bottom of the well. However, we then observed microscopically that CD44 antibody-immobilized magnetic beads became separated from the surface of MSCs in the well, and the cells then proliferated. After 7 days of culture, cell proliferation of MSC-CD44 antibody-bead complexes was ~50% that of rat MSCs (Fig. 3). MSC–RGDS peptide-bead complexes also attached to the bottom of the well, and gradually proliferated in culture. After 2 days in culture, RGDS peptide-immobilized magnetic beads became separated from the surface of MSCs, and cell

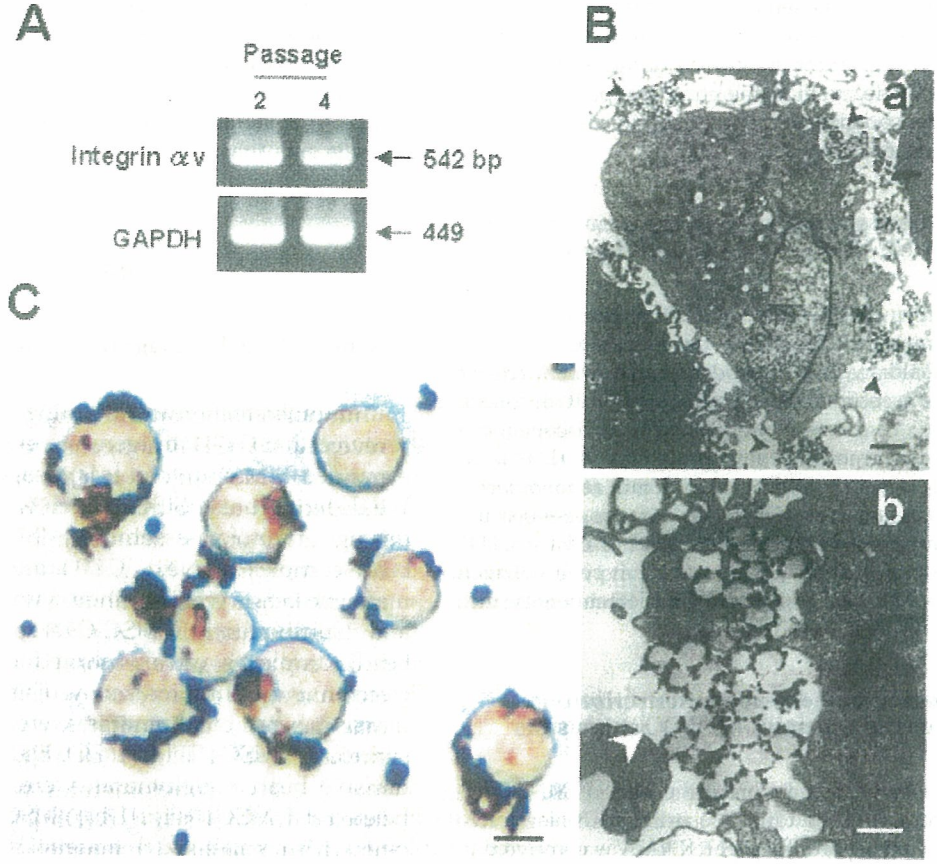


Figure 2. MSC-arginine (R)-glycine (G)-aspartic acid (D)-serine (S), RGDS peptide-magnetic bead complex. A: Expression of integrin αv and GAPDH mRNA in rat MSCs at passage 2 and 4. B: Electron microscopic views. (a) Magnification ×2000. The scale bar indicates 2 μm. (b) Magnification ×12,000. The scale bar indicates 500 nm. The arrows indicate RGDS peptide-immobilized magnetic beads attached to the surface of an MSC. C: Light microscopic view. Magnification, ×400. The scale bar indicates 5 μm. The data shown are typical of two independent experiments (n = 3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proliferation of MSC–RGDS peptide-bead complexes was then approximately 70% that of rat MSCs at 7 days of culture (Fig. 3). We found that our assembled MSC-magnetic bead complexes could proliferate by separating from the mediator-immobilized magnetic beads, although the proliferation of the complexes was slower during the early period of culture than that of non-labeled rat MSCs.

Distribution of prepared MSC-magnetic bead complexes under the influence of an external magnetic force, and monolayer culture for generation of chondrogenic matrix by the complexes

In a vertical direction from the magnet, magnetic flux density was 0.44 T at a distance of 0.3 mm from the magnet, and reduced as the height from the magnet increased [Fig. 4(A)]. In a horizontal direction at 1.7 mm above the magnet, magnetic flux density was 0.25 T at 0 mm from the magnet, and reduced as the distance from the center of the magnet increased [Fig. 4(B)]. To evaluate whether MSC-magnetic bead complexes could be gathered in a specific area by an external magnetic force, the complexes were seeded with or without the influence of a magnetic force. In the magnet group (a neodymium magnet was placed beneath the plate at the center: area M), seeded MSC-bead complexes were attached and gathered mainly in area M [Fig. 5(A,B), M+]. By statistical analysis, there was a significant difference in cell number between area M and areas C1–C4 [Fig. 5(B), M+]. In contrast, in the control group (no magnet), the complexes were scattered mainly in the peripheral area of the plate [Fig. 5(B), M−] and the cell number in area M was statistically lower than those in other areas [Fig. 5(B), M−]. The distributional effects of the magnet were the same for both the two complexes we designed.

Subsequently, 21 days after seeding the MSC-CD44 antibody-bead complexes, in the magnet group the complexes had formed clusters of cells in the center of the plate, and the chondrogenic matrix was strongly stained by toluidine blue [Fig. 5(C), M+]. In the con-

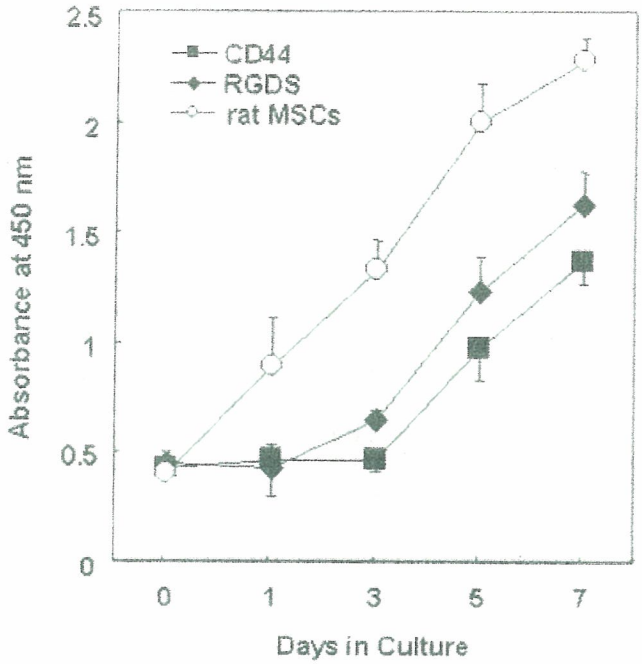


Figure 3. Proliferation of MSC-magnetic bead complexes. 1×10^4 cells, as MSC-magnetic bead complexes or control rat MSCs were seeded onto each well of a 24-well plate. At various times after seeding (0, 1, 3, 5, and 7 days), cell proliferation of the complexes or rat MSCs was measured by the WST-8 method. The data shown are typical of three independent experiments ($n = 6$). The bar represents the SD of each group.

trol group, chondrogenic matrix was observed in the peripheral area, and the cells stained strongly for toluidine blue [Fig. 5(C), M−]. Thus, MSC-CD44 antibody-bead complexes, gathered in a specific area by an external magnetic force, could generate chondrogenic matrix in monolayer culture when supplemented with chondrogenic differentiation medium.

Chondrogenesis of MSC-magnetic bead complexes in three-dimensional culture system

After 21 days in culture, MSC-CD44 antibody-bead complex-derived-MSCs were found to be surrounded by cartilage-characteristic proteoglycans that stained metachromatically with toluidine blue [Fig. 6(Aa), D+], while in the pellets cultured without chondrogenic differentiation factors, the area stained with toluidine blue was weak and sparse [Fig. 6(Ac), D−]. Immunohistochemical staining for type II collagen also revealed an increase in type II collagen expression in the pellets cultured with chondrogenic differentiation medium [Fig. 6(Ab), D+] compared with pellets cultured without chondrogenic differentiation factors [Fig. 6(Ad), D−]. This chondrogenic phenotype of MSCs cultured in chondrogenic differentiation medium was observed in ~50–60% of the total area in MSC-CD44 antibody-bead complex sections, in contrast to 80% in non-labeled MSCs sections (data not shown). In addition, almost the same results were obtained histologically and immnunohistochemically in the pellet culture system using the MSC–RGDS peptide-bead complex-derived MSCs (data not shown). Moreover, RT-PCR analysis of the pellet cultures demonstrated that type II collagen and aggrecan mRNA expression could be detected in the MSC-CD44 antibody-bead complex-derived, the MSC–RGDS peptide-bead complex-derived, and control rat MSC (non-magnetically labeled) -derived pellets cultured with chondrogenic differentiation medium [Fig. 6(B), D+], while in the pellets cultured without TGF-β3 and dexamethasone, mRNA expression of those markers was not detected [Fig. 6(B), D−]. Thus, MSC-magnetic bead complexes could also differentiate into chondrocytes in three-dimensional culture systems when supplemented with chondrogenic differentiation factors and the chondrogenic potential of the complexes shows only a slight reduction compared to that of normal rat MSCs.

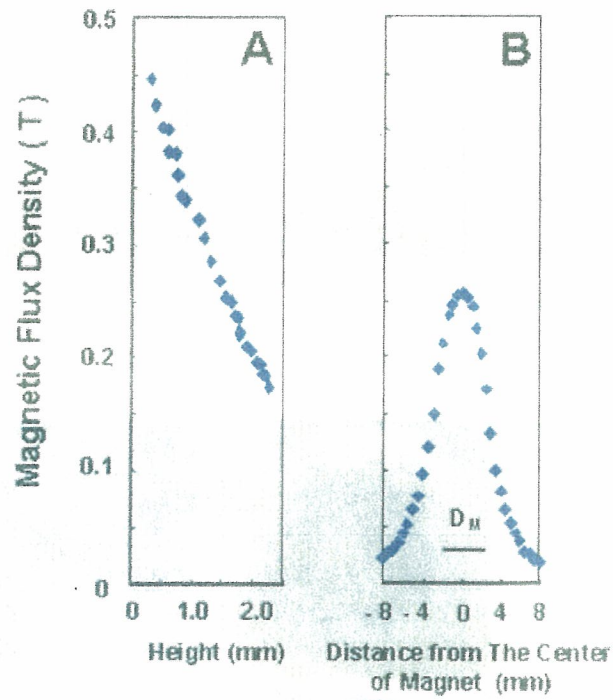
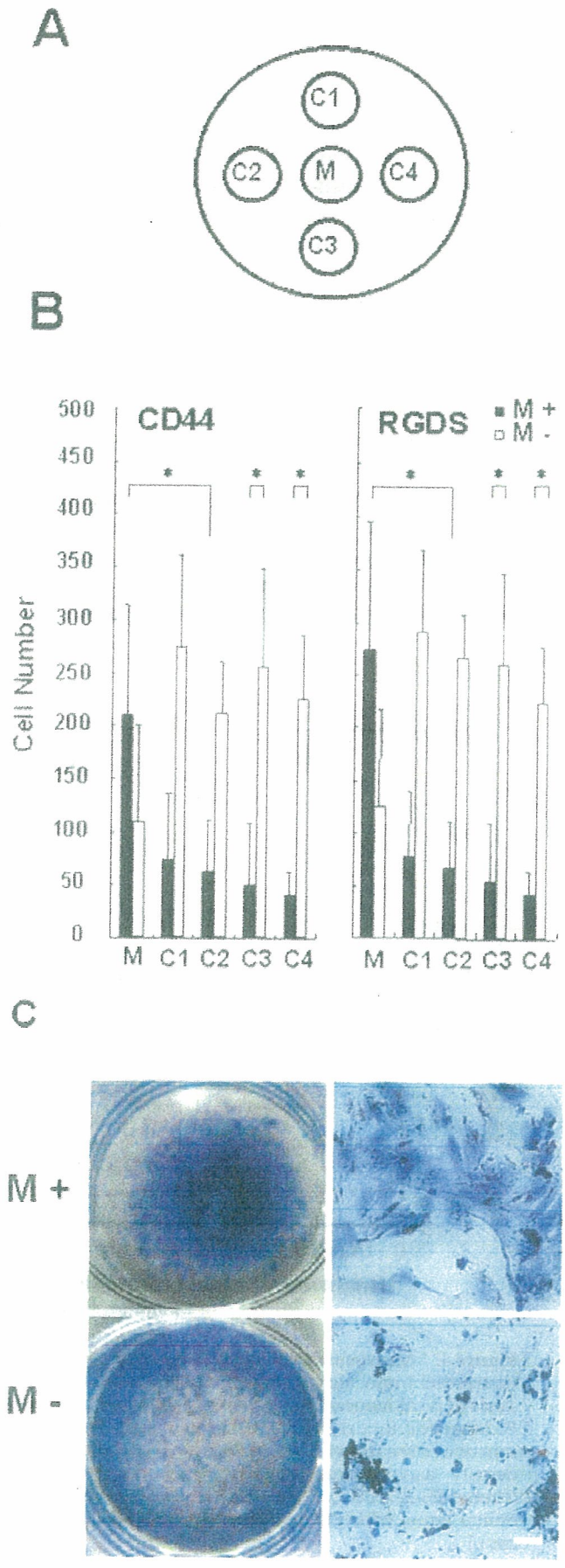


Figure 4. Magnetic flux density of the neodymium magnet (diameter: 5 mm, height: 5 mm, magnetic flux density: 0.43 Tesla [T]). A: Magnetic flux density in the vertical direction. B: Magnetic flux density in the horizontal direction at 1.7 mm above the magnet. D_M: width of magnet. Magnetic flux density was measured using a gauss meter, HGM-8200 (Probe: FS-3, ADS Co.). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



DISCUSSION

The present study clearly demonstrates that MSCs coupled to magnetic beads can proliferate, after separating from mediator-immobilized magnetic beads, and can be attached and gathered effectively in a desired area by the influence of an external magnet *in vitro*. They can also generate chondrogenic matrix effectively with the addition of chondrogenic differentiation factors after cell accumulation in the monolayer culture system. Moreover, in a three-dimensional culture, they can differentiate into the chondrogenic lineage, as proved by the production of a dense chondrogenic matrix and the expression of type II collagen and aggrecan mRNA, although the chondrogenic potential of the complexes is a little reduced compared to that of normal rat MSCs.

The MSC-magnetic bead complexes were assembled by antigen–antibody reactions between CD44 antigens on MSCs and CD44 antibody-immobilized magnetic beads or by interactions between integrins on MSCs and RGDS peptides. As far as can be ascertained by light and electron microscopy, the assembly of the complexes was not 100% efficient. Before using our MSC delivery system with an external magnetic force *in vivo*, optimization of the number of magnetic beads and the cell number, and modifications of the reaction method such as reaction time and temperature, are

Figure 5. Distribution of MSC-magnetic bead complexes under the influence of magnetic force and generation of chondrogenic matrix by the MSC-CD44 antibody-magnetic bead complexes in a monolayer culture system. A, B: In the experimental group (magnet group, M+), a neodymium magnet was placed beneath the center of the 24-well plate (area M). The MSC-magnetic bead complexes were seeded under the influence of the magnetic force (magnetic flux density was 0.20 T at 2 mm from plate base, [Fig. 3(Ba)]. In the control group (M−), a magnet was not used. After allowing the cells to attach, the magnet was removed from underneath the plate and cell numbers were counted in area M and in four peripheral areas (areas C1–C4) (each area was 3 mm in diameter). There was a statistically significant difference in cell number between area M and areas C1–C4 in the magnet group ($n = 6$, each area, $^*P < 0.05$). The data shown are typical of three independent experiments. The bar represents SD of each group. C: Generation of chondrogenic matrix by the MSC-CD44 antibody-magnetic bead complexes in a monolayer culture system. Images on the left show macroscopic views of the plates after 21 days culture with chondrogenic differentiation medium. In the M+ group, the MSC-CD44 antibody-bead complexes were attached and localized in area M, and the chondrogenic matrix stained by toluidine blue was observed macroscopically. In contrast, in the M− group, chondrogenic matrix was observed in the peripheral area of the plate. Images on the right show microscopic views of MSC-CD44 antibody-bead complexes in area M of each group. Magnification, ×200. The scale bar indicates 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

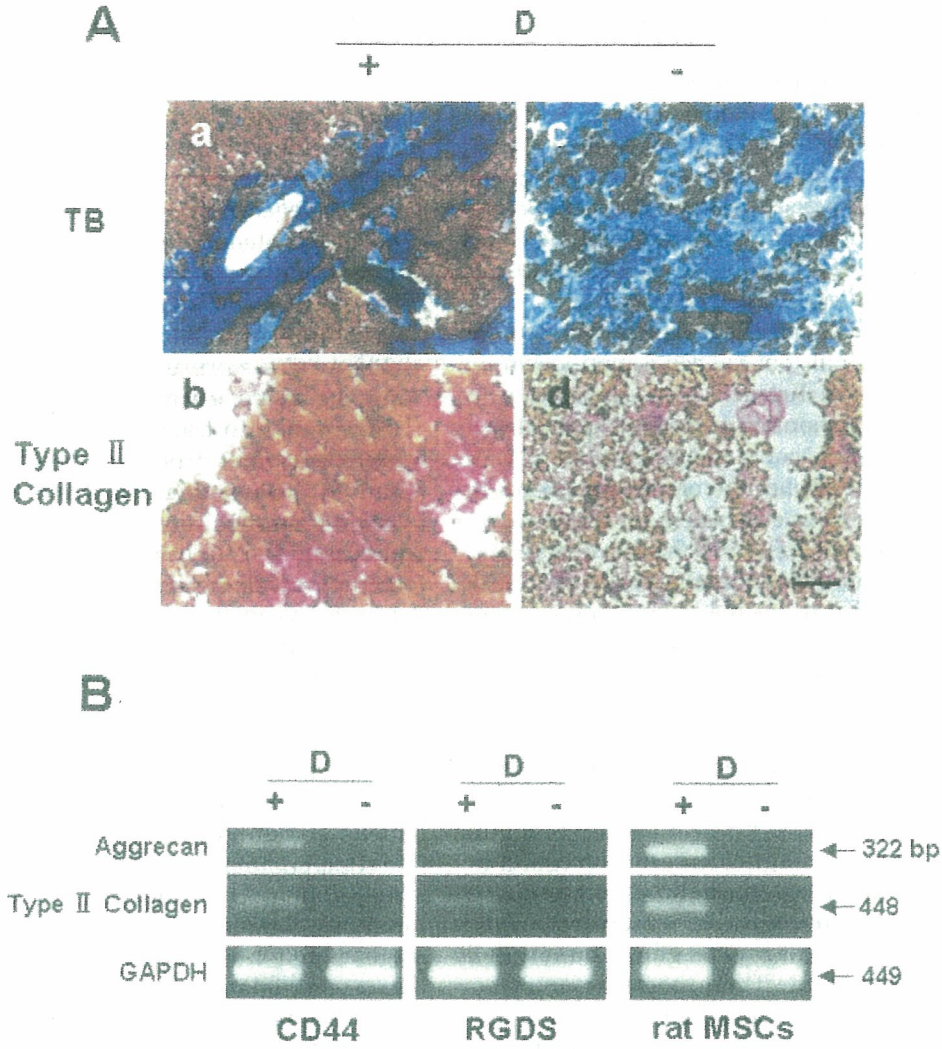


Figure 6. Chondrogenesis of the MSC-magnetic bead complexes in the pellet culture system. A: In a pellet cultured with chondrogenic differentiation medium (D+), MSC-CD44 antibody-bead complex-derived MSCs were surrounded by denser chondrogenic matrix stained by toluidine blue (TB), (a), and stronger type II collagen expression, detected by immunohistochemical stain (b) than those in the pellet cultured without chondrogenic differentiation factors (TGF-β3 and dexamethasone, (D−), (c,d). B: Reverse transcription-polymerase chain reaction (RT-PCR) analysis. The data shown are typical of three independent experiments ($n = 3$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

necessary. In addition, morphological observation of the complexes by electron and light microscopy showed that some massive bead conglomerates were attached on the surface of MSCs [Figs. 1(C,Da) and 2(B,C)]. The complexes were able to separate from the mediator-immobilized magnetic beads as the culture period increased, and the cells could proliferate up to at least 7 days of culture (Fig. 3). It has been demonstrated by other researchers that micro-sized magnetic beads are phagocytosed by some cells such as active dendritic cells[16] or CD8 positive lymphocytes.[17] These data and our results suggest that it is possible that our assembled complexes have the potential to cover an injured site, such as a cartilage defect, by proliferation of the complexes, and that mediator-immobilized magnetic beads, once released from the cell surface, may be taken up by cells such as macrophages when the complexes are applied *in vivo*. Moreover, our designed complexes could be gathered effectively under the influence of an external magnetic force [Fig. 5(B)], and were able to generate chondrogenic matrix in monolayer culture [Fig. 5(C)]. They could also differentiate into the chondrogenic lineage in a three-dimensional culture system when supplemented with chondrogenic differentiation factors [Fig. 6(A,B)], although the chondrogenic potential of the complexes was a little less than that of normal rat MSCs. To assess more clearly the difference in chondrogenic potential between the complexes and normal rat MSCs, quantitative gene expression analysis by real time PCR is required. Taken together, our results show that the coupling of CD44 antibody- or RGDS peptide-immo-

bilized magnetic beads with expanded MSCs have scarcely any effects on cell proliferation and growth or on the potential of expanded MSCs to differentiate into chondrocytes, compared to non labeled-rat MSCs, but that our system allows their effective delivery to the desired location. When comparing the component of magnetic beads, it is suggested that RGDS peptide-immobilized beads would be more useful for clinical applications than CD44 antibody- beads, because the RGDS peptide is a biodegradable material.

In previous studies, magnetic beads have been applied as a very useful tool for cell separation in biotechnology[18–21] since Kronick et al. successfully reported cancer cell separation using magnetic particles with surface markers, using the ganglioside GM1 in 1978.[22] Recently the application of magnetic beads has been expected as a magnetic system for the therapy of diseases such as liver tumors.[23] We have previously successfully demonstrated the efficacy of a drug delivery system (DDS) using magnetic liposomes containing magnetite: Fe_3O_4 (mean diameter: 10 nm) and cytokines (recombinant human bone morphogenetic protein-2, rhBMP-2 or TGF-β1) and the implantation of a permanent magnet into the target site of a bone or cartilage defect in a rabbit model.[24,25] Treatment with magnetic liposomes containing TGFβ1 together with a permanent magnet implanted near the osteochondral bone defect resulted in an increase in the concentration of TGFβ1 in the osteochondral defect and effectively promoted chondrogenesis.[25] However, in this technique, the permanent magnet had to be surgically implanted at the injured site. Thus, the next step was to use an external magnetic force, which is less invasive and more convenient than surgical implantation of the magnet because the application time and site can be changed based on clinical demands. Recently, we have successfully used >0.3 T (maximum: 0.4 T) of an external magnetic force for systemic chemotherapy with magnetic liposomes containing doxorubicin in osteosarcoma-bearing hamsters.[26] As a modification of this magnetic DDS, we considered using this magnetic system to deliver pluripotent stem cells after combining the cells with magnetic beads, and thus, we created a novel magnetic stem cell delivery system.[4] Before our assembled MSC-magnetic bead complexes can be applied to an *in vivo* study, we think it is important to obtain further experimental results as follows: (1) the external magnetic force needs to be appropriate for all kinds of injury site and carefully shaped to fit the target organ; (2) to attract MSC-bead complexes to the injured site, we have to determine the appropriate time of applying magnetic force; (3) we have to investigate whether MSC-bead complexes induce an immune response in a host, and (4) investigate any potential side effects on transplanted MSCs or host cells when the appropriate magnetic force is applied.

Moreover, for future directions, our novel approach could be used to deliver stem cells to an injury site but also could be used to deliver appropriate cytokines or signaling molecules, if stem cells are capable of coupling with small-sized magnetic liposomes containing such growth factors for enhancement of tissue repair. Tissue regeneration would be promoted with this novel simultaneous stem cell and growth factor delivery system using a magnetic force. Additionally, we believe that our novel system would also be effective in the treatment of brain or spinal cord injury and for malignant tumors, using natural killer cells instead of autologous bone marrow-derived MSCs.

Currently, to track migration of transplanted cells such as stem and progenitor cells by magnetic resonance (MR) imaging, these cells have been used after labeling magnetically for detection in myocardium[27,28] and brain.[29,30] To our knowledge, magnetic targeting therapy involving cell delivery for repair of cartilage defects has not been reported. In the present study, the door of a novel magnetic targeting therapy using magnetic force and MSC-magnetic bead complexes has been opened. Basic research has started to elucidate whether magnetically labeled MSCs are able to differentiate into several lineages. There has been controversy over whether or not magnetic labeling of MSCs inhibits chondrogenesis. Arbab et al. demonstrated that superparamagnetic iron oxide (SPIO, ferumoxide, ~80–150 nm in size)-labeled MSCs were formed through the mediation of a polycationic transfection agent, poly-L-lysine (PLL),[31] and could differentiate into several lineages including the chondrogenic lineage.[32] On the other hand, most recently, Kostura et al. reported that SPIO-labeled MSCs inhibited chondrogenesis, and that the blocking of chondrogenic activity was mediated by the SPIO, rather than by PLL.[33] This blocking of chondrogenic activity by SPIO may be due to the effect of 'intracellular' magnetic labeling of SPIO-coated PLL, because SPIO-coated PLL acts through electrostatic interactions and binds to the cell membrane, inducing membrane bending, following which the magnetic label is endocytosed.[33] In the present study, Ferri Sphere 100C® (310 nm in size), which is approximately twice the size of SPIO, was used for 'extracellular' magnetic labeling through cell surface molecules, and we found that our magnetically labeled MSCs could differentiate into the chondrogenic lineage, although the chondrogenic potential of the complexes was somewhat reduced compared to that of normal rat MSCs. In fact, as far as could be observed by electron microscopy, magnetic beads could not be observed intracellularly within MSCs [Figs. 1(C) and 2(B)]. However, before clinical application of our magnetic cell delivery system can be considered, further studies by Western blot or real time PCR analysis to rule out any inhibitory effects of magnetic beads on signal transduction molecules such

as the transcription factor, Cbfa1 or Runx 2, which could affect chondrogenic differentiation will be required.

In conclusion, our design of two types of MSC-magnetic bead complexes were able to proliferate, effectively gather at a desired location under the influence of an external magnetic field, and had chodrogenic potential. Because of the fact that MSC–RGDS peptide-bead complexes are composed with an easily biodegradable material, RGDS peptides as a mediator, the RGDS peptide-bead complex is likely to prove more useful for minimally invasive clinical applications of our designed magnetic MSC delivery system than CD44 antibody-bead complexes. Further studies are necessary to evaluate the effectiveness of our stem cell delivery system for cartilage repair *in vivo*, but we have clearly demonstrated the potential of this system *in vitro*.

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MINI REVIEW

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Physiological role of bone morphogenetic proteins in osteogenesis

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Introduction

Bone morphogenetic proteins (BMPs) are members of secreted signaling proteins that belong to the transforming growth factor-β superfamily. BMPs were originally identified as molecules that induced ectopic bone formation when implanted into rodent muscles [1,2]. In this process, mesenchymal precursor cells condense and differentiate into two distinct tissues, namely the cartilage template and the surrounding perichondrium. Subsequently, chondrocytes in the cartilage template undergo a program of proliferation, cell cycle arrest, hypertrophy, calcification, and ultimately cell death. Ossification of the calcified matrix around the hypertrophic layer occurs upon vascularization and the recruitment of osteoblasts to the site [3]. In accordance with such in vivo effects, BMPs have been shown to regulate osetoblast differentiation in vitro [4].

BMPs bind to a characteristic pair of type I and II transmembrane serine/threonine kinase receptors (Fig. 1). They first bind to the type II receptor, which phosphorylates the GS domain of the type I receptor. The activated type I receptor subsequently recruits and phosphorylates the transcription factors Smad1, Smad5, and Smad8 (R-Smads) through the GS domain. R-Smads then physically associate with Smad4 (Co-Smad), translocate into the nucleus, and activate the target genes in concert with other coactivators. Smad6 blocks BMP signaling by inhibiting the phosphorylation of the BMP-dependent R-Smads by the BMP type I receptors [5,6].

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Skeletal phenotypes of BMP knockout mice

Because BMP2, 3, 4, 5, 6, 7, and GDF5 are expressed in the developing skeletal elements [7], these BMPs may be involved in skeletogenesis. To clarify the physiological roles of BMPs, a number of BMP knockout mice were generated (Table 1). Briefly, homozygous *Bmp2*-deficient (*Bmp2*–/–) or *Bmp4*-deficient (*Bmp4*–/–) mice are embryonically lethal before the onset of skeletogenesis [8,9]. Twelve percent of heterozygous *Bmp4*+/– mice exhibit preaxial polydactyly. *Bmp3*–/– mice exhibit increased bone density [10]. *Short ear* mice, which are caused by a naturally occurring mutation in the *Bmp5* gene, have many anatomical abnormalities: reduced ear size, reduction in body size, reduction in the number of ribs, misshapen xiphoid appendix, hydronephrosis, and lung abnormalities [11]. *Bmp6*–/– mice have no skeletal patterning defects except a mild delay in sternum ossification that can be traced to the formation of mesenchymal condensation [12]. *Bmp7*–/– mice have patterning abnormalities in the hind limbs. However, histological analysis failed to detect any other abnormalities in osteoblast differentiation [13]. Brachypodism is a spontaneously occurring phenotype caused by a mutation in the *Gdf5* gene, characterized by a reduction in the length of the long bones and the replacement of two bones in most digits by a single skeletal element [14]. A generation of mice harboring mutations in two different BMP genes adds little to the knowledge of physiological bone formation (see Table 1) [15–21]. Thus, all naturally occurring or genetically engineered mice deficient in BMPs reported to date are either normal, exhibit abnormalities in skeletal patterning, or die during early embryonic development, and thus provide no information on the physiological role of endogenous BMPs in bone formation [7].

Table 1. Information from BMP knockout mice

BMP2–/–	Embryonically lethal, small allantois, lack of amnion
BMP3–/–	Increased bone density
BMP4+/–	Preaxial polydactyly of hindlimb, cystic kidney
BMP4–/–	Embryonically lethal, lack of allantois, heart defect
BMP5–/–	Short ear mice, abnormality of rib cage
BMP6–/–	Delayed sternum ossification
BMP7–/–	Abnormality of rib cage, polydactyly
BMP8a–/–	Defect in spermatogenesis
BMP8b–/–	Defect in spermatogenesis
BMP11–/–	Abnormality of A–P patterning of axial skeleton
BMP12–/–	Hydrocephalic abnormality
BMP15–/–	Defect in oogenesis
GDF5–/–	Brachypodism, shortened limbs, reduced digit bones
GDF8–/–	Skeletal muscle hypertrophy
GDF9–/–	Defect in oogenesis
BMP2+/–; BMP4+/–	Fewer primordial germ cells than BMP2+/– or BMP4+/–
BMP2+/–; BMP6–/–	Reduction in bone formation due to osteoblast dysfunction
BMP2+/–; BMP7+/–	No skeletal abnormality
BMP4+/–; BMP7+/–	Skeletal defect in rib cage, polydactyly
BMP5–/–; BMP6–/–	Almost the same as BMP5–/–
BMP5+/–; BMP7+/–	No skeletal abnormality
BMP5–/–; BMP7–/–	Embryonically lethal, defect in cardiac cushion and septation
BMP5–/–; GDF5–/–	Combination of BMP5–/– and GDF5–/–
BMP6–/–; BMP7–/–	Embryonically lethal, lack of allantois, heart defect
BMP7+/–; BMP8a–/–	More severe defects in spermatogenesis than BMP8a–/–
BMP8a+/–; BMP8b+/–	Defects in spermatogenesis and epididymis

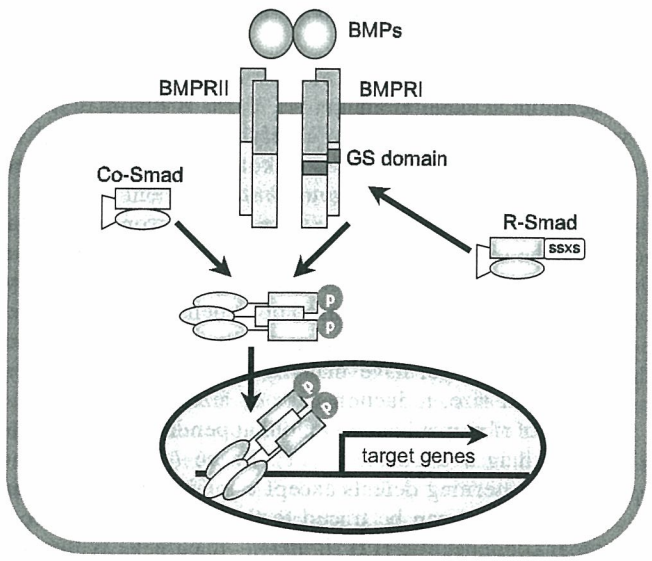

Fig. 1. BMP/Smad signaling pathway. BMPs bind to type I and type II receptors that are membrane-bound serine/threonine kinase receptors. Upon ligand binding, type II receptor phosphorylates the GS domain of the type I receptor, and type I receptor induces phosphorylation of R-Smads. Phosphorylated R-Smads form complexes with Co-Smad, and translocate into the nucleus

Induction of bone formation by hypertrophic chondrocytes

The vertebrate skeleton is derived from three distinct embryonic lineages: neural crest cells, paraxial mesoderm, and lateral plate mesoderm. Cells of these lineages proliferate and migrate into distinct mesenchymal condensations at the sites of future elements. Subsequently, they follow two distinct ways of bone formation: intramembranous and endochondral [3]. During intramembranous bone formation, condensed mesenchymal cells differentiate directly into osteoblasts. Most craniofacial bones and clavicles are formed through this process. Most of the bones are formed through the latter process, which is characterized by the replacement of a cartilage mold by bone and bone marrow [22]. In endochondral bone formation, there are several pieces of evidence that show that hypertrophic chondrocytes induce bone formation in adjacent tissues. First, bone formation always occurs in locations adjacent to the hypertrophic chondrocytes [23]. The primary spongiosa and bone collar are formed around the hypertrophic chondrocytes. This rule applies to all endochondral bones, large or small, without exception. Second, in mice lacking the *parathyroid hormone (Pth)-related peptode (Pthrp)* gene or its receptor, the *Pth/Pthrp receptor* (*Ppr*) gene, the regulation of hypertrophy is disturbed, and ectopic chondrocyte hypertrophy and ectopic bone formation appear [24–26]. Third, both the hypertrophy of chondrocytes and the formation of bone collar and primary spongiosa are suppressed in transgenic mice expressing the constitutively active *Ppr* under the control of the chondrocyte-specific type II collagen promoter (Col II *Ppr* transgenic mice) [27]. Furthermore, by the introduction of the Col II *Ppr* transgene, both the ectopic hypertrophy and the ectopic bone formation seen in *Pthrp*–/– mice are reversed [23]. Thus, the chondrocyte-specific manipulation of PTHrP signaling influences the appearance of ectopic bone formation. Fourth, in the growth plate of *Ppr*–/–/WT chimeric mice, ectopic bone collars are formed near clusters of ectopic prehypertrophic/hypertrophic chondrocytes [23]. Thus, during endochondral bone formation, hypertrophic chondrocytes in the growth plate link chondrogenesis to

osteogenesis by inducing osteogenesis in adjacent perichondrium and primary spongiosa.

Hypertrophic chondrocytes express a number of growth factors, cytokines, and matrix proteins. Among them, Indian hedgehog (Ihh) has been proven to be indispensable for osteogenesis by hypertrophic chondrocytes [23,28]. However, Ihh alone cannot induce bone formation [29], suggesting that other factors secreted from hypertrophic chondrocytes may also be necessary for osteogenesis. Because some BMPs can induce ectopic bone formation when implanted into rodent muscles and promote osteoblast differentiation in vitro, they are strong candidates for osteogenic factors secreted from hypertrophic chondrocytes.

BMP subtypes expressed by hypertrophic chondrocytes

Because hypertrophic chondrocytes induce bone formation in the primary spongiosa and the perichondrium during endochondral bone development, BMPs that are important for physiological bone formation are likely to be expressed by hypertrophic chondrocytes. BMP2, 3, 4, 5, 6, 7, and GDF5 are known to be expressed in the growth plate [7,12]. BMP2 is expressed in the hypertrophic chondrocytes, the perichondrium, and the developing joints. Expression of BMP3 is detected in the perochondrium along the shaft, but not surrounding the epiphyses. BMP4 transcripts are detected in the cells of the perichondrium and the transition zone adjacent to the mature hypertrophic chondrocytes. BMP5 is expressed in the perichondrium. BMP6 expression is localized to the hypertrophic chondrocytes and joints. Expression of BMP7 is detected in the proliferative chondrocytes and the inner perichondrium. These reports indicate that the main BMP subtypes expressed by the hypertrophic chondrocytes are BMP2 and BMP6 [12,30].

Bmp2+/–; Bmp6–/– mice exhibited growth retardation with proportional growth plates

As is the case with the other BMPs, however, there is no direct evidence that BMP2 and BMP6 are essential for physiological bone formation, because *Bmp2–/–* mice die at an early embryonic stage [8], and *Bmp6–/–* mice show no skeletal abnormality except for a slight delay in the ossification of the sternum [12]. Because there may be genetic redundancy between BMP2 and BMP6 in the regulation of bone formation, compound knockout mice lacking one allele of the *Bmp2* gene and both alleles of the *Bmp6* gene (*Bmp2+/–*; *Bmp6–/–*) were generated and the effect on bone formation was analyzed [30].

Although BMPs are known to play important roles in skeletal patterning during embryonic development, there was no apparent abnormality of skeletal patterning in *Bmp2+/–*; *Bmp6–/–* mice. In contrast, there was growth retardation in *Bmp2+/–*; *Bmp6–/–* mice. The size of the *Bmp2+/–*; *Bmp6–/–* growth plate was smaller than that of the WT, but the proportions of the distinct layers of growth plate chondrocytes (periarticular proliferative, columnar proliferative, and hypertrophic) showed no significant differences. Although several lines of evidence indicate that BMPs promote chondrocyte hypertrophy, *Bmp2+/–*; *Bmp6–/–* mice did not show a significant difference in chondrocyte differentiation.

Impaired bone formation due to osteoblast dysfunction in *Bmp2+/–; Bmp6–/–* mice

As for bone formation, the trabecular bone volume and cortical thickness were significantly reduced in *Bmp2+/–*; *Bmp6–/–* mice. Bone histomorphometric analysis showed that bone volume was decreased by 31% compared with that of the WT. Regarding the parameters of bone formation, there was a significant reduction in mineral apposition rate and bone formation rate per bone surface, but little difference in osteoblast number. On the other hand, the parameters of bone resorption were normal. These data suggest that bone loss in *Bmp2+/–*; *Bmp6–/–* mice was caused by the inhibition of bone formation due to an impairment of osteoblast function. Urinary deoxypyridinoline, a marker for bone resorption, showed little difference between the two groups.

Fracture healing recapitulates some aspects of the intramembranous and endochondral bone development [31], and the abnormalities of such development are sometimes exaggerated in fracture healing. The endochondral, not the intramembranous, bone formation was defective in *Bmp2+/–*; *Bmp6–/–* mice. In *Bmp2+/–*; *Bmp6–/–* mice, a massive cartilaginous callus persisted, which contained hypertrophic chondrocytes. The hypertrophic differentiation of chondrocytes was similar between WT and *Bmp2+/–*; *Bmp6–/–* mice, suggesting that the bone induction ability of the hypertrophic chondrocytes was impaired in *Bmp2+/–*; *Bmp6–/–* mice.

Comparison of *Bmp2+/–; Bmp6–/–* mice and mice deficient in the BMP signaling cascade

Recently, transgenic mice expressing a dominant-negative form of *Bmp receptor 1b* (*Bmpr-1b*) under the control of the type I collagen promoter were generated, and they closely resembled *Bmp2+/–*; *Bmp6–/–* mice [32]. They were smaller than the WT mice and showed impairment of postnatal bone formation with the number of osteoblasts and the parameters of bone resorption unchanged, suggesting that osteoblast function was impaired. In addition, transgenic mice lacking the *Bmp receptor 1a* (*Bmpr-1a*) specifically in osteoblasts using the Cre/loxP system under the control of the osteocalcin 2 promoter also exhibited low bone mass due to impaired osteoblast function [33]. Noggin

is a secreted glycoprotein and acts as a BMP antagonist. Noggin binds to BMP2, 4, 5, 6, and 7 and inhibits their binding to the BMP receptors. Transgenic mice expressing *Noggin* under the control of the osteocalcin promoter exhibited a reduction in the trabecular bone volume and the bone formation rate, but not in the number of osteoblasts [34,35]. Smurf1 is a HECT domain ubiquitin E3 ligase and inhibits BMP signaling by inducing the degradation of R-Smads and BMP receptors. A deficiency of *Smurf1* in mice showed an increase in bone mass due to the enhanced activities of osteoblasts [36], and transgenic mice expressing *Smurf1* under the control of the type I collagen promoter exhibited osteopenia [37]. The bone phenotypes of these genetically manipulated mice, in which the osteoblasts could not transduce normal BMP signaling, were similar to those of *Bmp2*+/–; *Bmp6*–/– mice. These data support our hypothesis that BMP2 and BMP6 act on osteoblasts to induce normal bone formation.

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SPECIAL REPORT

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Prevalence and clinical features of Paget's disease of bone in Japan

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Abstract The present study aimed to evaluate the prevalence and clinical presentation of Paget's disease of bone (PDB) in Japan. As PDB is a very rare disease in Japan, a nationwide mail survey was conducted targeting doctors in the specialty most frequently diagnosing and treating PDB patients in Japan. First, the literature for all case reports in Japan published between January 1990 and December 2002 was reviewed to determine who was diagnosing and treating PDB in Japan. This literature review for all case reports in Japan revealed that 72.1% of cases in Japan were reported from departments of orthopedic surgery. A nationwide two-phase mail survey was conducted for the departments of orthopedic surgery of 2320 general hospitals accredited by the Japanese Orthopaedic Association. Phase 1 involved determining how many patients with PDB were followed at each hospital. If the answer was one or more, phase 2 of the survey gathered information on the clinical presentation of current patients. The mail survey yielded a final response rate of 75.4% for phase 1 and 87.6% for phase 2. Phase 1 indicated that the prevalence of PDB in Japan is about 2.8 cases per million capita. Phase 2 revealed a slight female predominance, lower frequency of familial clustering, higher frequency of femoral fracture in the affected femur, and a higher ratio of symptomatic PDB in Japan compared with findings in countries displaying a higher prevalence of PDB. The present epidemiological study revealed that the disorder is extremely rare in Japanese individuals, and that some differences exist with regard to the clinical features of PDB between Japanese patients and patients from high-prevalence countries.

Key words Paget's disease of bone · prevalence · clinical manifestation · nationwide mail survey · Japanese population

The Japanese Committee on Clinical Guidelines of Diagnosis and Treatment of Paget's Disease of Bone of the Japan Osteoporosis Society, Japan

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Introduction

Paget's disease of bone (PDB) is very common in elderly Caucasian populations in Europe, the United States, and Australia, and is well known to display distinct geographical variation. The prevalence of PDB varies from 0.1% to 5% in individuals, depending on the country [1–9]. While PDB

is highly prevalent in countries such as the United Kingdom, France, Germany, New Zealand, and Australia, prevalence is relatively low in Northern Europe [6], and the disease is extremely rare in Asia and Africa, where data are limited to a small number of published case reports [10–14] and no prevalence data are currently available.

Recent studies in Europe, North America, and New Zealand have suggested a decline in the prevalence and an attenuation of the clinical severity of PDB [15–18]. Although a wide range of clinical manifestations is reported among patients with PDB, including bone pain, fracture, secondary osteoarthritis, hearing loss, and secondary sarcoma, the precise clinical manifestations of PDB in Asia and Africa remain unknown. Because epidemiological research in countries with an extremely low prevalence of PDB would provide valuable insights into the etiology of PDB, by comparing patients' clinical manifestations among geographical areas with clearly different prevalences, the present study sought to conduct such research in the Japanese population.

Estimates of the prevalence of PDB have primarily been derived from autopsies, bone scans, or radiographic surveys of subjects investigated in hospitals, and population-based radiological and biochemical surveys [9,19,20]. In a preliminary study, lumbar and pelvic lesions were surveyed, using abdominal radiographs of 1000 Japanese population-based subjects in a limited area, but the study failed to identify any Pagetic lesion. The present study therefore selected a nationwide mail survey to estimate the prevalence of PDB in Japan. However, contacting all Japanese medical doctors was clearly impractical. The nationwide mail survey was therefore targeted at doctors in the specialty most frequently involved in diagnosing and treating PDB patients in Japan. In order to determine which kind of doctor most frequently diagnoses and treats PDB patients in Japan, a search of the literature for case reports in Japan was conducted.

The objectives of this study were to evaluate the prevalence and clinical presentation of PDB in Japan.

Materials and methods

Review of published case reports of PDB in Japan

The literature was reviewed for all case reports in Japan published between January 1990 and December 2002, to determine who was diagnosing and treating PDB patients. A systematic search was performed using the key words "Paget's disease in bone" in electronic databases (Japana Centra Revuo Medicina). Duplication of cases was avoided by referring to the key content of each report, and cases were classified by the reporting affiliation as Department of Orthopedic Surgery, Department of Internal Medicine, or Other.

Mail survey

A mail survey was conducted from September 2002 to May 2003, proceeding in two phases. Phase 1 involved determining how many patients with PDB were followed at each hospital at the time of the mail survey. If the answer was one or more, phase 2 of the survey gathered the following information on the clinical presentation of current patients: diagnostic procedure; age; sex; familial aggregates; types of clinical symptoms; complications; pattern of bone involvement; serum alkaline phosphatase levels; therapeutic agents used; and prognosis. Surveys were mailed on three occasions to nonresponders in order to maximize the response rate.

Table 1. Sources of referral (by specialty) of 154 cases of Paget's disease in Japan (1990–2002)

Department	Frequency listed (%)
Orthopedic surgery	111 (72.1%)
Others	43 (27.9%)
Total	154

Results

Review of published case reports of PDB in Japan

Use of the key words "Paget's disease in bone" in the published databases yielded 245 hits, representing 154 cases after meticulous exclusion of duplicated data. These final 154 cases were categorized as 111 cases reported by Departments of Orthopedic Surgery (72.1%) and 43 cases reported by Others (27.9%; Table 1).

Mail surveys

Becasuse PDB was diagnosed mainly at departments of orthopedic surgery in Japan, we conducted a mail survey of the departments of orthopedic surgery at 2320 general hospitals accredited by the Japanese Orthopaedic Association. The response rate for phase 1 of the survey was 75.4%, revealing a total of 194 patients, including 1 Indian patient, being followed at departments of orthopedic surgery at 162 general hospitals. The response rate for the phase 2 survey that gathered data on the 194 PDB patients was 87.6%. Of the 194 cases identified in phase 1, phase 2 responses were gained for 170 cases (169 Japanese patients, 1 Indian patient). No duplication of cases was found among the 170 cases according to the obtained clinical data, age, sex, and initials of the name, but duplication could not be excluded from the remaining 24 cases.

Estimated prevalence of PDB in Japan

Based on 72.1% of Japanese cases of PDB being diagnosed and the patients treated at departments of orthopedic surgery, the response rate of 75.4% in the phase 1 survey, and data from the Ministry of Public Management that the Japanese population was 126008000 in 2002, the prevalence of PDB in Japan was calculated as 2.8 per million capita, using

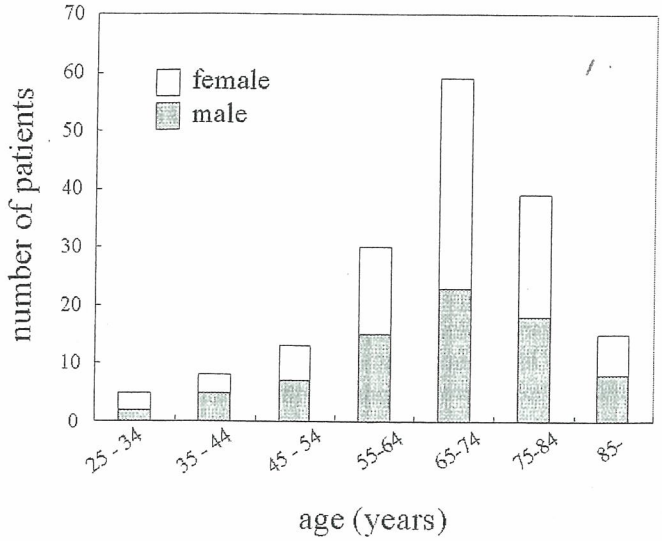

Fig. 1. Age distribution of patients with Paget's disease of bone in Japan

Table 2. The clinical features of 169 patients with PDB in Japan

Age (years) mean ± SD	64.7 ± 14.5
Sex, male/female (ratio)	78 : 91 (0.86 : 1)
Familial aggregation (n = 158)[a]	10 (6.3%)
Symptom	
Asymptomatic (%)	42 (24.9%)
Symptomatic (%)	127 (75.1%)
Monostotic/polyostotic (ratio)	87 : 82 (1.06 : 1)
Skeletal distribution (%)	
Pelvis	93 (55.0)
Spine	54 (32.0)
Femur	46 (27.2)
Skull	34 (20.1)
Tibia	25 (14.8)
Serum ALP levels (n = 164)[b]	
Normal range	17 (10.4)
≤3-fold above ULN	74 (45.1)
3- to 6-fold above ULN	49 (29.9)
≥6-folds above ULN	24 (14.6)

ULN, upper limit of normal range
[a] Data not available for 11 patients
[b] Data not available for 5 patients

the following equation: 193 cases × (100% / 72.1%) × (100% / 75.4%) × (1 000 000 / 126 008 000).

Analysis of clinical characteristics in Japanese PDB patients

Data for the 169 Japanese patients gathered in the phase 2 study were analyzed. Among these 169 patients (age range, 26 to 92 years; mean age ± SD, 64.7 ± 14.5 years; Table 2), PDB markedly increased in prevalence with increasing age up to age 74 (Fig. 1). The male/female ratio was 0.86:1, and only 6.3% of patients had familial clustering; 75.1% of patients were symptomatic, and the most frequent symptoms were pain (including lumbago, coxalgia, buttock pain, and gonalgia) followed by skeletal deformity (Tables 2 and 3). Data regarding skeletal distribution revealed that monostotic and polyostotic disease exhibited almost equal prevalence. Commonly involved skeletal sites were the pelvis, spine, and femur. Serum alkaline phosphatase levels were elevated beyond the upper limit of normal in 89.6% of patients (Table 2).

Table 3. Symptomatology of 127 patients with symptomatic PDB in Japan

Lumbago	30
Coxalgia	29
Skeletal deformity	24
Buttock pain	18
Gonalgia	11
Hearing loss	8
Dental problem	5
Others	9

The total number of patients listed is more than 127 because several patients had more than one symptom

In addition to the fundamental diagnostic procedures of radiology, bone scans, and biochemical parameters of bone metabolic activity, bone biopsy was performed for diagnosis in 55% of the patients. Medical management was limited almost exclusively to the use of calcitonin and etidronate, becaue these are the only two agents licensed for the management of PDB in Japan. However, some doctors have used other bisphosphonates, including alendronate, risedronate, and pamidronate, which are approved in countries advanced in the management of PDB. Despite the high frequency of medical management, more than half of the patients remained symptomatic. In regard to complications, 16 patients displayed fractures, 1 developed hip osteoarthritis requiring total hip replacement, 2 developed osteosarcomas, and 1 had malignant fibrohistiocytoma (MFH) in bone. The most frequent sites of fracture were the hip and pelvis (Table 4).

Discussion

PDB is known to be a very rare disease in Japan, although its prevalence and clinical features have not been clarified. Thus, the majority of cases of PDB in Japan tend to be reported in the literature as case reports. This permitted us to ascertain the sources of referral in Japanese patients with PDB through a review of published case reports. Between January 1990 and December 2002, 154 cases were reported of which 72.1% were reported from departments of orthopedic surgery in the country. We therefore performed a nationwide mail survey to the departments of orthopedic surgery of 2320 general hospitals accredited by the Japanese Orthopaedic Association; the final response rate in this mail survey was 75.4% for phase one and 87.6% for phase two. These response rates may be sufficient to delineate the prevalence of PDB in Japanese patients.

The study has revealed that the prevalence of PDB in Japan across all ages is 2.8 per million. The prevalence of PDB is reported to range from 0.1% to 5% in high-prevalence countries [1–9]; thus, the prevalence in Japan is