Table 2. mRNA expression of cell surface antigen markers for stem cells and odontoblast differentiation markers by real-time reverse transcription-polymerase chain reaction analysis in porcine pulp SP cells and non-SP cells compared with tooth germ

Marker	SP/tooth germ	Non-SP/tooth germ	SP/non-SP
CD31	9.0	1.0	9.0
CD34	0.9	0.1	9.0
CD45	0.1	1.3	0.1
CD90	0.5	2.2	0.2
CD105	9.5	1.1	8.6
CD146	10.0	3.0	3.3
CD150	36.0	0.1	360.0
Vegfr2/Flk-1	9.2	0.3	30.7
α-Smooth muscle actin	0.3	5.0	0.1
NG2	1.2	1.8	0.67
Desmin	0.8	3.5	0.2
Alkaline phosphatase	0.04	0.5	0.08
Colα1(I)	0.05	0.09	0.56
Colα1(III)	0.2	1.0	0.2
Dspp	0.0	0.01	0.0
Enamelysin	0.0	0.01	0.0

The experiment was repeated five times, and one representative experiment is presented.
Abbreviations: Dspp, dentin sialophosphoprotein; SP, side population; Vegfr2, vascular endothelial growth factor receptor 2.

secondary antibody. The stained sections were observed under a fluorescence microscope IX 71 (Olympus, Tokyo, Japan, http://www.olympus.co.jp) after being counterstained with Hoechst 33342.

Differentiation of Pulp SP Cells into Odontoblast Lineage in Three-Dimensional Pellet Culture

Pulp SP cells were cultured in a three-dimensional pellet (cellular aggregates) supplemented with and without recombinant human BMP2 (kindly provided by Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan, http://informagen.com) at a final concentration of 50 ng/ml, 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), and 50 μg/ml L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan, http://www.wako-chem.co.jp/english), and penicillin-streptomycin. The medium was changed twice a week. To determine the effect on proliferation, the pellets were digested by trypsin and total cell numbers were calculated on days 0, 1, 3, 6, 10, and 14. On days 1, 3, 7, 10, and 14, the pellets was sonicated in 100 μl of 10 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 at 20 kHz for 5 minutes. Alkaline phosphatase activity was assayed by the method of Lowry et al. [33]. Pellets were fixed on days 14 and 35, and the paraffin-embedded sections were stained in Masson trichrome for extracellular matrix formation and alizarin red for calcification, respectively. Total cellular RNA was isolated at each point on days 10, 14, and 28, and real-time RT-PCR was performed using porcine Dspp, enamelysin/MMP20, dentin matrix protein 1 (Dmp1), and α1(I) collagen as differentiation markers of odontoblasts.

Induced Chondrogenic, Adipogenic, and Neurogenic Differentiation of Pulp SP Cells

The differentiation of pulp SP cells into adipogenic, chondrogenic, or neurogenic cells was determined by the method of Pittenger et al. [34]. For chondrogenic differentiation, 2.5×10^5 porcine pulp SP cells, non-SP cells, and primary pulp cells at the third passage were centrifuged in 15-ml conical polypropylene tube (Asahi Techno Glass) at 1,000 rpm for 5 minutes. The pellets were maintained in DMEM supplemented with 10% heat-inactivated bovine calf serum, 10 μg/ml insulin-transferin-selenite X (Invitrogen), 5.35 μg/ml linoleic acid (Sigma-Aldrich), 1.25 μg/ml bovine serum albumin (Sigma-Aldrich), 1.0 μg/ml dexamethason, 10 μg/ml L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries), and 10 ng/ml transforming growth factor β3 (Peprotech, London, http://www.peprotechec.com). Medium was changed every 3 days, and cultures were maintained for 28 days. Pellets were fixed in 4% paraformaldehyde overnight, and the paraffin-embedded sections (4–5 μm thick) were stained by Alcian Blue. The total cellular RNA of the pellets was isolated on day 28 for analysis of α1(II) collagen and aggrecan (Table 1) by RT-PCR.

For adipogenic differentiation, cells were seeded at 2×10^5cells per milliliter in 35-mm collagen type I-coated dishes in DMEM high-glucose supplemented with 10% heat-inactivated fetal calf serum. The third-passage culture of the SP cells was grown to confluence in DMEM low-glucose supplemented with 10% heat-inactivated fetal calf serum and 10 μg/ml L-glutamine (Invitrogen). Thereafter, adipogenic differentiation was induced by subjecting confluent monolayers to three rounds of adipogenic treatment. Each round had two steps: incubation with induction medium of Poietics hMSC Differentiation Media Bullet Kit-Adipogenic (Cambrex, Walkersville, MD, http://www.cambrex.com) for 3 days and incubation with maintenance medium for 3 days. Cells were cultured for another 7 days in maintenance medium. As a control, cells were cultured only in maintenance medium. The cell layers were stained with oil red O, and the mRNA levels of adaptor protein 2 (aP2) and peroxisome proliferator-activated receptor γ (PPARγ) (Table 1) were analyzed by RT-PCR.

For neurogenic differentiation, pulp SP cells were cultured in noncoated 35-mm dishes in neurosphere medium (Neurobasal A; Invitrogen) containing B27 supplement (Invitrogen), penicillin-streptomycin, L-glutamine, endothelial growth factor (EGF) (Invitrogen), and bFGF (Invitrogen) for 15 days, designated for neurosphere formation. The neurospheres were triturated using polished glass pipettes, and the obtained single-cell suspension was cultured in gelatin-coated 35-mm dishes in neurodifferentiation medium one (Neurobasal A) containing 1 μg/ml laminin (Invitrogen), 5 μg/ml fibronectin (Nitta Gelatin, Osaka, Japan, http://nitta-gelatin.co.jp), 2 mM L-glutamine, 10 μg/ml N_2 supplement (Invitrogen), 20 ng/ml bFGF, and 40 ng/ml EGF. The medium was changed to neurodifferentiation medium 2 (Neurobasal A) containing 1 μg/ml laminin, 5 μg/ml fibronectin, 2 mM L-glutamine, 10 μg/ml N_2 supplement, 20 ng/ml neurotrophin-3 (Peprotech) after 24 hours of cultivation. The medium was changed every 3 days: Immunocytochemical analysis was performed 28 days after cultivation. The cells were fixed for 30 minutes in cold 4% paraformaldehyde in PBS, treated with 0.1% Triton-X for optimal penetration of cell membrane, and incubated at room temperature in a blocking solution (protein block; Dako, Glostrup, Denmark, http://www.dako.com) for 30 minutes. After an overnight incubation at 4°C with a primary antibody anti-neuromodulin (GAP-43) (Clone91E12) (1:100; Roche Diagnostics), the cells were incubated for 30 minutes at

room temperature with a secondary antibody (Envision+; Dako) and stained with diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). The mRNA expression of neuromodulin and neurofilament (Table 1) was analyzed by RT-PCR.

Canine Pulp SP Cells and In Vivo Transplantation on Amputated Pulp

The ultimate utility of the SP cell-derived stem cells is the in vivo differentiation into dentin-forming odontoblasts. A switch was made to an experimental model of canine pulp partial removal and transplantation of SP cell-derived pulp stem cell population. Adult dogs were obtained from Narc (Chiba, Japan, http://www.narc.co.jp). The canine SP pulp cells were isolated from the pulp tissues surgically extracted from the incisors by flow cytometry as performed in porcine pulp tissue. Canine autogenous pulp pellets (cellular aggregates) were prepared from third-passage SP cells supplemented with and without recombinant human BMP2. Anesthesia for surgery was performed by intravenous administration of pentobarbital sodium. Surgical amputation was carried out in the canine teeth of the dog, and the pellets were implanted autogenously on the amputated pulp. The cavity was sealed with zinc phosphate cement and composite resin with the caution of using minimal pressure. The teeth were obtained by extraction 1 month after transplantation, fixed in 4% paraformaldehyde at 4°C overnight, and demineralized in 10% formic acid. Reparative dentin formation was evaluated by histology in serial paraffin sections. For in situ hybridization in sections, after dewaxing and inhibition of endogenous peroxidase activity by treatment with 0.3% H_2O_2 in methanol solution for 30 minutes, the procedure described above was performed. The first-strand cDNA was synthesized from 2 μg of total RNA isolated from canine primary pulp pellet cultures on day 28, and RT-PCR amplifications were performed at 95°C for 10 seconds, 58°C for 15 seconds, and 72°C for 8 seconds using canine *Dspp* primers (forward, 5′-GTCCTAGTGGGAATGGAGCA-3′; reverse, 5′-TCTTCAGGGCCATCATCTTC-3′), and *enamelysin* primers (forward, 5′-TATTCACCGTTGCTGCTCAC-3′; reverse, 5′-TACAATGCCTGGATCCCTTT-3′). RT-PCR products of *Dspp* (190 bp) and *enamelysin* (151 bp) were subcloned into pGEM-T Easy vector (Promega) and confirmed by sequencing based on published cDNA sequences. The canine Dspp and enamelysin cDNA, linearized with SpeI and NcoI, respectively, were used for making RNA probes. The animal experiments were conducted using the strict guidelines of the Kyushu University Animal Protocol Committee and DNA Safety Program.

RESULTS

Isolation of SP Cells from Dental Pulp from Various Species

Pulp cells isolated from human, bovine, canine, and porcine adult pulp tissues and stained with Hoechst 33342 identified approximately 0.2% of the population with relatively lower Hoechst 33342 fluorescence (SP cells) (Fig. 1A–1D). Although some markers (CD90 and CD117) reacted with human pulp cells (data not shown), the markers required to subfractionate pulp SP cells are not well-defined due to small yields of isolated human SP cells. Therefore, sensitivity to verapamil, an inhibitor

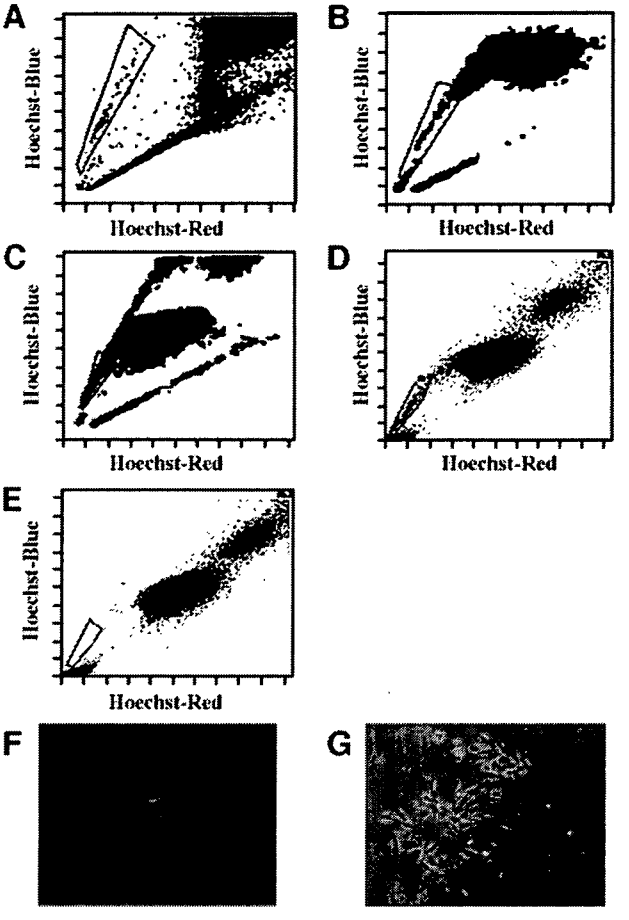

Figure 1. Isolation of side population (SP) cells from dental pulp from various species. SP cells were isolated from human **(A)**, bovine **(B)**, canine **(C)**, and porcine **(D)** dental pulp. It is noteworthy that in the presence of 50 μM verapamil, few SP cells were obtained from porcine **(E)** (compare **[D]** and **[E]**). The growth of a cell colony derived from single porcine SP cell is shown on day 1 **(F)** and on day 7 **(G)**. SP cells could form a colony at a rate of 0.2%. Scale bars = 50 μm.

of multidrug resistance (MDR) and MDR-like transporter, was used to set the SP gate (Fig. 1D, 1E). This SP subpopulation has been shown to be a more homogeneous population than primary pulp cells and non-SP cells. Some of the single SP cells plated in a 35-mm, collagen type I-coated dish formed a colony in 7 days (Fig. 1F, 1G).

Self-Renewal Capability of Pulp SP Cells

The cumulative cell number of porcine pulp SP cells was much higher, and the proliferative life span of SP cells was longer than that of pulp non-SP cells and primary pulp cells (Fig. 2A). At first, the non-SP cells and primary cells were passaged every 3–4 days from the second to the 15th passage. The rate of proliferation progressively became slower. The cells became senescent by passage 26, on day 116 and by passage 27, on day 132, respectively. Most of SP cells proliferated and maintained a spindle-like appearance until the 42nd passage. The molecular basis for the SP phenotype has been attributed to multidrug-resistance transporter (ABCG2/Bcrp 1). To characterize pulp SP cells, the expression of the markers associated with other stem cells (Bcrp1, Stat3, Bmi1, and Tert mRNA) was analyzed by the

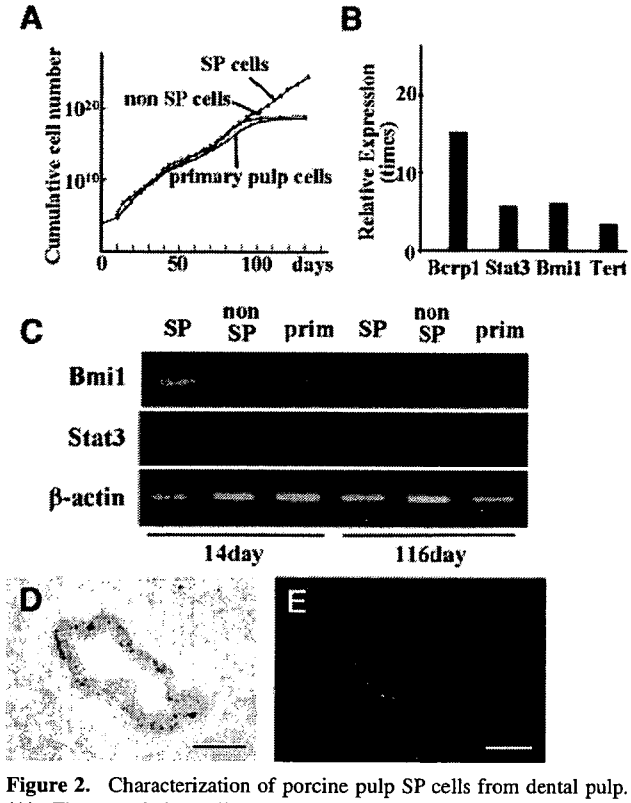

Figure 2. Characterization of porcine pulp SP cells from dental pulp. **(A):** The cumulative cell number of pulp SP cells, non-SP cells, and primary pulp cells up to 140 days. **(B):** The relative expression (fold increases) of Bcrp1, Stat3, Bmi1, and Tert mRNA in porcine SP cells compared with that of non-SP cells. **(C):** The expression of Stat3 and Bmi1 mRNA on days 14 and 116 in SP cells, non-SP cells, and primary porcine dental pulp cells. Note that SP cells demonstrate Stat3 and Bmi1 mRNA up to 116 days. **(D, E):** The in situ expression of Bcrp1 mRNA **(D)** and BCRP1 protein **(E)** in vascular region in porcine dental pulp sections. Counterstain (blue) is Hoechst 33342. Scale bars = 50 μm. Abbreviations: Bcrp1, breast cancer resistant protein 1; prim, primary; SP, side population; Tert, telomerase reverse transcriptase.

real-time RT-PCR. Those levels of expression were much higher in SP cells compared with non-SP cells (Fig. 2B), suggesting that this population was highly enriched for stem cells. Stat3 and Bmi1 mRNA were still expressed in SP cells on day 116 and disappeared in non-SP cells and primary pulp cells (Fig. 2C), suggesting the self-renewal and replicative capacity of SP cells. The expression of Bcrp1 mRNA and BCRP1 protein was detected by in situ hybridization and immunohistochemistry in the perivascular region of porcine dental pulp tissue (Fig. 2D, 2E).

Cell Surface Antigen Markers for Stem Cells

To examine the "stemness" of the porcine pulp SP cells, mRNA expression of cell surface antigen markers for stem cells were examined by real-time RT-PCR, since antibodies cross-reacting against CD are not available for porcine cells. CD105 and CD150 mRNA were highly expressed in SP cells compared with non-SP cells like other tissue stem cells (Table 2). Much lower expression of CD45, a gene typical of hematopoietic cells, was detected in SP cells than in non-SP cells. Expression of the differentiation markers Colα1(I) and Colα1(III) was much lower in SP cells than in non-SP cells. Markers of odontoblast differentiation Dspp and enamelysin were not detected in SP cells. These results suggest that SP cells contain an undifferentiated population, as do other tissue stem cells. CD146 is known to be highly expressed in smooth muscle cells and endothelial cells, as are CD31 and Vegfr2 in endothelial cells and NG2, desmin, and α-smooth muscle cell actin in microvascular pericytes. The expression of α-smooth muscle cell actin in SP cells was much lower than in non-SP cells. Endothelial markers CD31 and Vegfr2 were expressed in SP cells (Table 2).

Differentiation of SP Cells into Odontoblast Lineage

Total cell numbers were rapidly decreased in BMP2-supplemented pellets and control non-supplemented pellets of pulp SP cells by day 1. They gradually decreased until day 10 and did not change thereafter. There was no difference in cell number between BMP2 pellets and control pellets (Fig. 3A). The alkaline phosphatase activity gradually increased after day 7 and was significantly higher on day 10 in BMP2 pellets than in control pellets (Fig. 3B). The collagenous extracellular matrix stained by Masson trichrome was stronger in the BMP2-supplemented pellets than those in control pellets starting on day 14 (Fig. 3C, 3D). Alizarin red staining showed mineralization in the BMP2 pellets on day 35 (Fig. 3E, 3F). We next investigated the functional effect of BMP2 application on the expression of α1(I) collagen and odontoblast markers *Dspp*, *enamelysin*, and *Dmp1* on days 10, 14, and 28. Quantitative analysis by real-time RT-PCR showed that the expression of α1(I) collagen, Dspp, and enamelysin was significantly increased in the BMP2-supplemented pellet cultures compared with the control on days 14 and 28 (Fig. 3G). These results indicated that BMP2 supplement enhanced differentiation of pulp SP cells into odontoblasts.

Multidifferentiation Capability of Pulp SP Cells

The chondrogenic potential of SP cells was examined. The porcine pulp SP cells obtained from the fourth passage culture were maintained in pellet cultures for 45 days. The amount of cartilage proteoglycan stained with Alcian Blue was much greater in the pellets induced from SP cells compared with those from non-SP cells, primary pulp cells (Fig. 4A–4C), and non-induction pellet (data not shown). The expression of aggrecan and type II collagen mRNA was much stronger in the SP cells than that in the primary pulp cells. Almost 30% of SP cells were converted into chondrocytes. No expression was seen in the non-SP cells and uninduced SP cells (Fig. 4D).

Next, the adipogenic potential of pulp SP cells was investigated in the third-passage cultures, which were cultured under the adipogenic condition for 28 days. The pulp SP cells showed intense staining with oil red O compared with non-SP and primary pulp cells (Fig. 4E–4G). aP2 and PPARγ mRNA was expressed in SP cells on day 28, but not in uninduced SP cells, non-SP cells, or primary pulp cells (Fig. 4H).

Finally, the neurogenic potential of SP cells was examined. The neurospheres, which were proliferating clusters of cells detached, were seen 15 days after neuronal induction of the SP cells from third-passage culture (Fig. 4I, 4J). Ninety percent of SP cells formed neurospheres. Sox2 mRNA was expressed in the neurospheres induced from SP cells but not in cells from non-SP cells or primary cells (Fig. 4M). Those neurosphere cells from SP cells were dissociated and seeded to the gelatin-coated dish to adhere and immunostained with antibody to neuromodu-

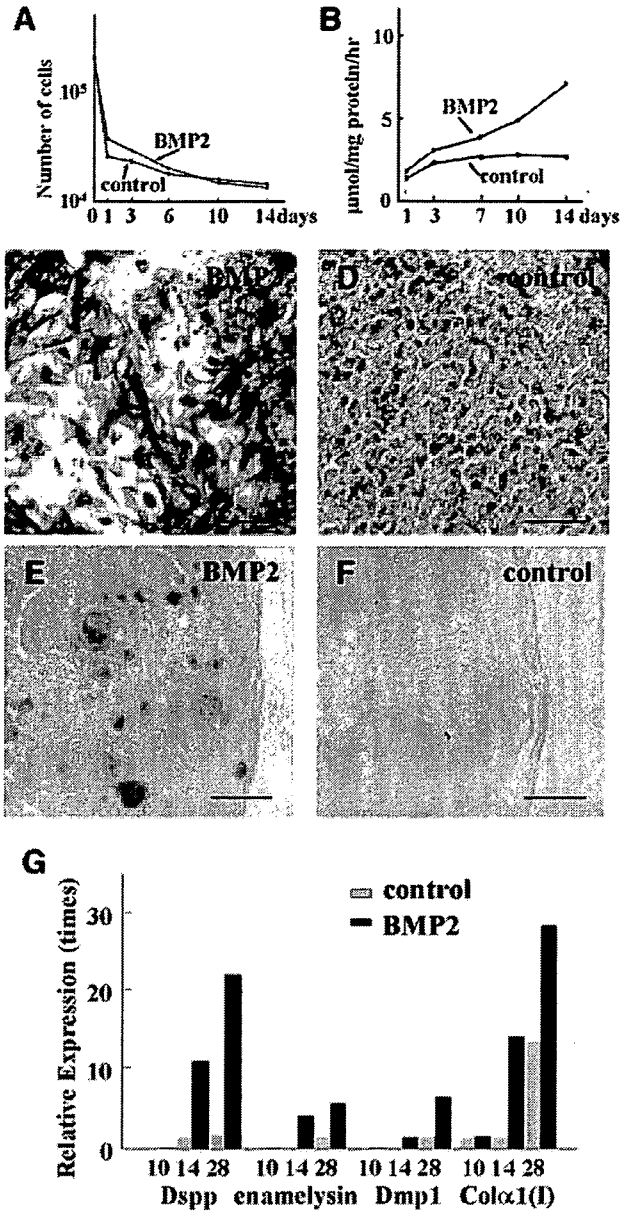

Figure 3. Differentiation of pulp SP cells into odontoblast lineage in the three-dimensional pellet culture. **(A):** The changes in cell numbers of porcine pulp SP cells in pellet culture treated with BMP2 compared with control. Each point is expressed as the mean ± SD of six determinations. **(B):** The changes in alkaline phosphatase activity in pellet culture treated with BMP2 and control. Each point is expressed as the mean ± SD of six determinations. In pellet cultures treated with BMP2, there was increased alkaline phosphatase activity compared with control. Morphological changes in the pellet were observed on day 14 (Masson trichrome stain). Shown are results of treatment with BMP2 **(C)** and untreated control **(D)**. Scale bars = 50 μm. Pellets treated BMP2 had increased collagen matrix compared with control. Alizarin red staining shows mineralization of BMP2 supplemented pellet on day 35. Shown are BMP2-treated pellet **(E)** and control pellet **(F)**. Scale bars = 50 μm. Note that mineralization was increased in the pellet supplemented with BMP2 compared with control. **(G):** Real-time reverse transcription-polymerase chain reaction, analysis of Dspp, enamelysin/matrix metalloproteinase 20, Dmp1, and α1(I)collagen expression in the pellet culture treated with BMP2 compared with control. The experiment was repeated four times, and one representative experiment is presented. Abbreviations: BMP, bone morphogenetic protein; Dmp1, dentin matrix protein 1; Dspp, dentin sialophosphoprotein; prim, primary; SP, side population.

lin after subconfluence (Fig. 4N). They were immunoreactive for neuromodulin in cytoplasm (Fig. 4O). The cells had rounded soma and a few of long, thin processes with neuronal morphology. These processes contacted each other at the end or soma and showed synapse-like morphology. The expression of neuromodulin and neurofilament mRNA was also detected in SP cells but not in uninduced SP cells (Fig. 4P).

Dentin Regeneration by Transplantation of Bmp2-Transduced Cells In Vivo

The autologous transplantation of the pellet (cell aggregate) of SP cells was performed on the amputated pulp of canine teeth 14 days after three-dimensional culture supplemented with BMP2. The pulp SP cells differentiated into osteodentinoblasts and secreted osteodentin matrix around them 1 month after surgery (Fig. 5A, 5B). A higher amount of osteodentin was observed in the BMP2 pellet than in the control pellet (Fig. 5A–5D). The in situ hybridization in serial sections showed mRNA expression of Dspp and enamelysin (Fig. 5E, 5F) in odontoblasts/osteodentinoblasts confined in osteodentin matrix stained with eosin (Fig. 5G) and Masson trichrome (Fig. 5H). There was no expression of Dspp or enamelysin in the control pellet (data not shown). These results suggest that the pulp SP cells responded to BMP2 to differentiate into odontoblasts/osteodentinoblasts.

Discussion

The present work describes the isolation and characterization of SP cells from dental pulp and their multilineage differentiation. SP cells were detected in adult pulp tissue from several species, including human, bovine, canine, and porcine species. They were purified by flow cytometry on the basis of efficient efflux of vital fluorescent dye Hoechst 33342 [35]. The dye efflux property of SP cells is mediated by the multidrug resistance transporter of ATP binding cassette G2 (ABCG2)/breast cancer resistance protein 1 (Bcrp1). In the porcine dental pulp, the SP cell fraction was approximately 0.2% of the gated population of primary pulp cells. The SP cells were sensitive to verapamil and expressed a higher level of Bcrp1 compared with non-SP and primary pulp cells. The three key characteristics of the SP cells are self-renewal capacity, expression of stem cell surface markers, and multilineage differentiation. The cumulative cell number was higher and proliferative life span was longer in pulp SP cells compared with non-SP cells. The polycomb gene was highly expressed in the SP cells. The *Bmi1* gene has been implicated in self-renewal [36]. Similarly, *Oct4* and *Stat3*, implicated in maintenance of self-renewal and pluripotency [37], were highly expressed in SP cells compared with non-SP cells. Finally, telomerase reverse transcriptase (Tert) mRNA was also highly expressed in pulp SP cells. TERT has a crucial function of maintenance of telomere length, preserving genomic stability and the long-term viability of highly proliferative organs [38, 39]. These results suggest that the pulp SP cells have self-renewal capacity.

The human pulp mesenchymal stem cells are currently demonstrating the similarity in positive expression of mesenchymal progenitor-related antigens, such as SH2, SH3, SH4, CD29, and CD166, to bone marrow mesenchymal stem cells [14]. The presence of cell surface markers in porcine pulp SP cells was examined by RT-PCR, as there is a paucity of specific CD antibodies for porcine cells. It is well known that SP cells

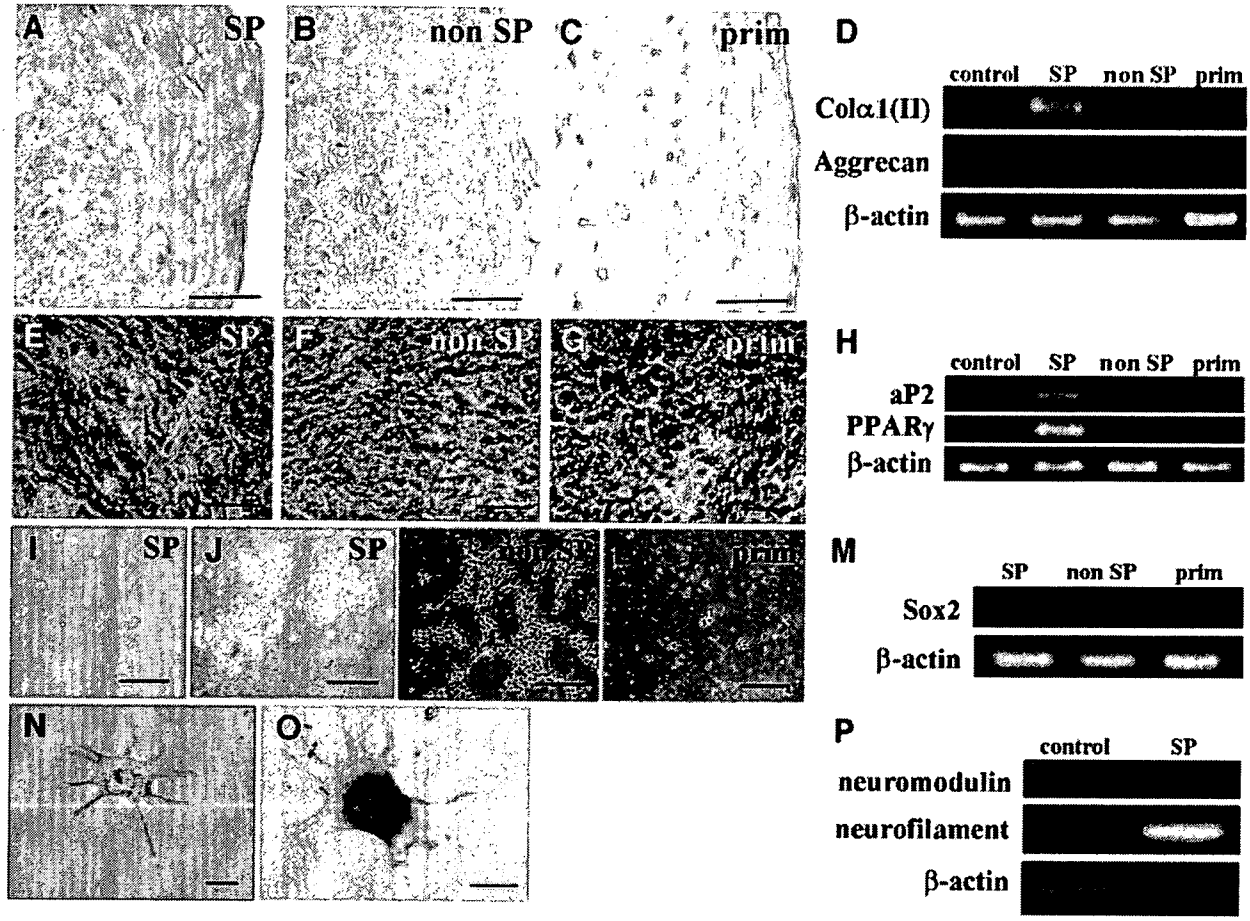

Figure 4. Induced differentiation of chondrogenic, adipogenic, and neurogenic lineages of dental pulp SP cells. Histological appearance of the pellet cultures of SP cells on day 28 **(A)**, non-SP cells **(B)** and primary cells **(C)** stained by Alcian blue. Scale bars = 50 μm. **(D):** The expression of Colα1 (II) and Aggrecan mRNA, markers of chondrogenic differentiation is prominent in SP cells compared with non-SP cells. **(E):** Oil red O staining of adipogenic differentiation on day 28. Note the presence of adipocytes in the SP cell fraction **(E)** but not in non-SP cells **(F)** and a few in primary cells **(G)**. Scale bars = 100 μm. Markers of adipogenic differentiation aP2 and PPARγ mRNA were observed only in SP cells **(H)**. **(I):** Appearance of SP cells on day 3. Neurosphere formation on day 15 was seen only in SP cells **(J)** and not in non-SP cells **(K)** or primary cells **(L)**. Scale bars = 500 μm. The expression of Sox2 mRNA confirms the neurosphere formation from SP cells only **(M)**. A neuron-like cell induced after dissociation of the neurosphere from SP cells and plating on the gelatin-coated dish for 13 days **(N)**. Immunostaining of neuromodulin **(O)** and the expression of neuromodulin and neurofilament mRNA were observed in SP cells **(P)**. Scale bars = 5 μm. Abbreviations: aP2, adaptor protein 2; Colα1, collagen α1; PPARγ, peroxisome proliferator-activated receptor γ; prim, primary; SP, side population.

isolated from bone marrow express stem cell markers such as Sca-1 (stem cell antigen), Thy1 (CD90), and the panhematopoietic markers CD45 and c-kit (CD117) [16, 40, 41]. However, they do not express differentiated markers characteristic of hematopoietic lineage, as in other tissue stem cells, such as skeletal muscle, pancreas, cardiac muscle, and intestine [42]. Pulp SP cells expressed mRNA for stem cell marker mRNA, *CD105* (*endoglin*) [43] and *CD150* [44]. CD150 has recently been demonstrated to be a useful marker for distinguishing stem cells from other progenitors. The primitiveness of pulp SP cells could thus be predicted based on the expression of CD150. On the other hand CD45 expression was very low compared with non-SP cells. The pulp SP cells expressed markers of endothelial cells, CD31 and Vegfr2. They lacked differentiated odontoblast markers Dspp and enamelysin and expressed low levels of Colα1(I) and Colα1(III). The origin and lineage of pulp SP cells are not clear. The presence of endothelial markers in isolated dental pulp SP cells and the localization of BCRP1 in perivascular region are suggestive of potential perivascular origin of some pulp SP cells. This is noteworthy in view of the implication of perivascular niche in mesenchymal stem cells of human dental pulp [45].

Accumulating evidence indicates that tissue stem cells have the potential to differentiate into other unrelated organs. For example, the multipotency of bone marrow stem cells to engraft into cardiac muscle, vascular endothelium, liver, and skeletal muscle has been described [46–49]. Mesenchymal stem cells of the bone marrow have the potential to differentiate into adipocytes, muscle, and bone [50, 51]. SP cells isolated from porcine dental pulp were multipotent, giving rise to adipogenic, chondrogenic, and neurogenic tissues in addition to odontoblasts. The porcine SP cells were chondrogenic, whereas unfractionated human dental pulp "stem" cells [13, 14] were not. It is possible that the SP cells were more in the hierarchy of stem cell lineage. The expression of odontoblast differentiation markers *Dspp* and *enamelysin* was increased in the BMP2-treated pellet

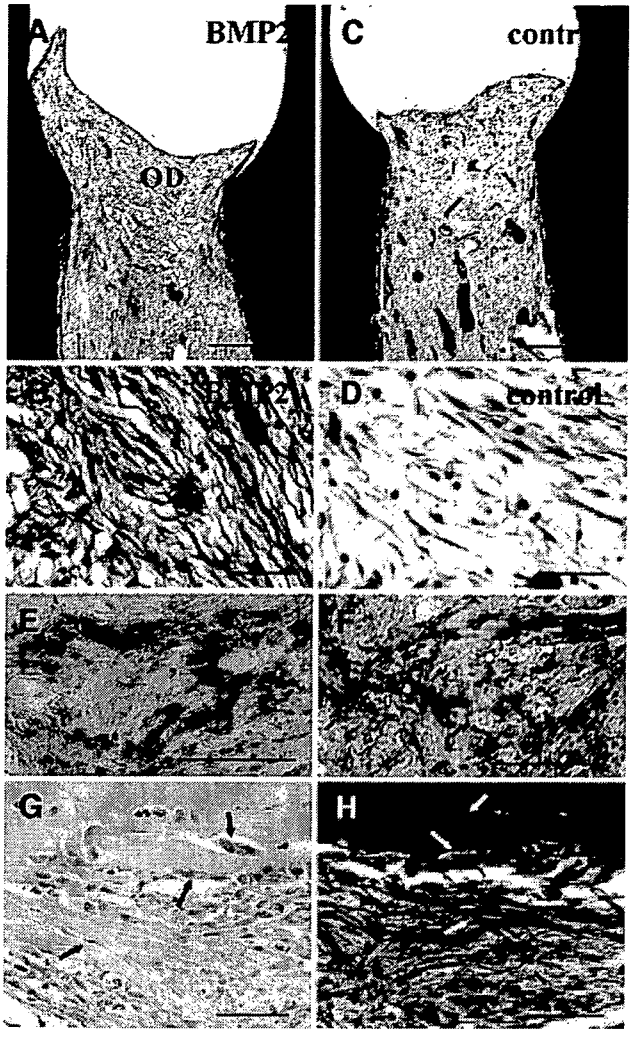

Figure 5. The autogenous transplantation in vivo of the pellet culture of canine side population (SP) cells on the canine amputated pulp (Masson trichrome stain). **(A, B):** Formation of OD in amputated pulp 1 month after autogenous transplantation of SP cell pellet treated with BMP2. **(C, D):** Small amount of osteodentin formation (OD) in the control. Scale bars = 500 μm **(A, C)**, 50 μm **(B, D)**. Shown is in situ hybridization analysis of canine tooth transplanted with SP cell pellet treated with BMP2 dentin sialophosphoprotein **(E)** and enamelysin **(F)**. Adjacent sections were stained with H&E **(G)** and Masson trichrome **(H)**. Note the odontoblasts/osteodentinoblasts (arrows) confined in osteodentin matrix. Scale bars = 50 μm. Abbreviations: BMP, bone morphogenetic protein; OD, osteodentin.

cultures (cellular aggregates) compared with the untreated control, indicating that BMP2 enhanced differentiation of pulp SP cells into odontoblasts. Osteodentin matrix formation was also increased in the BMP2-treated pellet cultures compared with the control.

Finally, odontoblastic differentiation in vitro from pulp SP cells has to be extended to in vivo experiments to demonstrate osteodentin and tubular dentin formation. To experimentally investigate the dentinogenesis in vivo, a switch was made to the dog, as previously our laboratory has demonstrated the BMP-induced dentinogenesis in the canine teeth of dogs [27, 52]. An experimental model of surgical amputation to expose the dental pulp of canine teeth in dogs was used. Since the dogs were outbred, autogenous SP cells isolated from the individual dogs were surgically implanted in the same dog. Pellet cultures of BMP2-treated dog SP cells induced osteodentin formation in surgically-created defects on amputated dental pulp. The terminal differentiation of odontoblasts was demonstrated by in situ hybridization of *Dspp* and *enamelysin*. Induction of osteodentin by BMP2-treated SP cells extends the previous work by our team and others of BMP-dependent osteodentin and tubular dentin in dogs [27, 52–54]. Thus, the response of the SP cells to BMP2 was similar to that of the mixed population of cells in the canine dental pulp.

The utility of pulp SP cells to induce in vivo dentin formation in dogs deserves additional comments with special reference to regeneration of tissues. SP cells derived from the liver and bone marrow were successfully used to regenerate liver [49]. Bone marrow-derived SP cells contributed to repair of lungs and tracheal epithelium [55, 56]. Transplantation of SP cells from adult mouse skin into *Mdx* mice with a mutation in the dystrophin gene resulted in expression of normal dystrophin by homing to and fusion with mutated myofibers [57]. The results in the present investigation of dentin formation by SP cells in surgically amputated teeth, along with earlier results in liver, lung, and skeletal muscle, demonstrate the regenerative potential of SP cells. In conclusion, pulp-derived SP cells induced to differentiate with morphogens such as BMP2 may be of potential clinical utility in the cell therapy for endodontics and dentistry.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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Side Population Cells Isolated from Porcine Dental Pulp Tissue with Self-Renewal and Multipotency for Dentinogenesis, Chondrogenesis, Adipogenesis, and Neurogenesis

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ORIGINAL ARTICLE

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Production of IL-7 is increased in ovariectomized mice, but not RANKL mRNA expression by osteoblasts/stromal cells in bone, and IL-7 enhances generation of osteoclast precursors in vitro

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Abstract Osteoclastogenic cytokines produced by T and B lineage cells and interleukin (IL)-7-induced expansion of the pool size of osteoclast precursors have been suggested to play an important role in acceleration of osteoclastogenesis induced by estrogen deficiency. However, the contribution of increased RANKL produced by osteoblasts/stromal cells to increase osteoclastogenesis in a mouse model of estrogen-deficient osteoporosis and in vitro effects of IL-7 on osteoclast precursor generation remain controversial. Thus, we investigated the effect of ovariectomy (OVX) of mice on production of RANKL, osteoprotegerin (OPG), and IL-7 in bone and the effect of IL-7 on osteoclast precursor generation in vitro. OVX did not significantly stimulate mRNA expressions of RANKL and OPG in whole femurs. Because the epiphysis, but not the femoral shaft (diaphysis) or bone marrow, is the main site of osteoclastogenesis, it is important to specifically analyze mRNA expression by osteoblasts/stromal cells at these parts of the femur. Therefore, we isolated RNA from bone marrow cell-free epiphysis, diaphysis, and flushed-out bone marrow and examined mRNA expression. The results showed no significant changes of RANKL and OPG mRNA expression in any part of the femur. In addition, OVX did not significantly affect RANKL and OPG mRNA expression by the adherent stromal cells isolated from flushed-out bone marrow cells but did stimulate RANKL mRNA expression by $B220^+$ cells in the nonadherent cell fraction. On the other hand, OVX increased IL-7 mRNA expression in the femur as well as IL-7 concentrations in bone fluid. In cultures of unfractionated bone cells isolated by vigorous agitation of minced whole long bones to release the cells tightly attached to the bone surfaces, but not in cocultures of clonal osteoblasts/stromal cells and flushed-out bone marrow cells, IL-7 stimulated generations of osteoclasts as well as osteoclast precursors. These data suggest that increased RANKL production by osteoblasts/stromal cells is unlikely to play a central role in acceleration of osteoclastogenesis in estrogen deficiency of mice and that IL-7 stimulates osteoclast precursor generation, presumably through an action of IL-7 on the cells attached to bone rather than on cells contained in the bone marrow cell population.

Key words estrogen · RANKL · osteoprotegerin · interleukin-7 · osteoclasts

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Introduction

Estrogen deficiency causes bone loss mainly as a consequence of excessive bone resorption by osteoclasts. Because estrogen deficiency is accompanied by accelerated osteoclastogenesis, regulation of osteoclastogenesis is central to the understanding of the pathogenesis and treatment of estrogen deficiency-induced osteoporosis [1–3]. In addition, estrogen directly regulates the bone-resorbing activity of osteoclasts [4].

Accumulated evidence has suggested that osteoclast differentiation is principally stimulated by an increase in the ratio of RANKL to osteoprotegerin (OPG), a soluble decoy receptor of RANKL, in bone [5–7]. Recent studies have revealed that RANKL is produced by at least three types of cells: osteoblasts/stromal cells, T cells [8,9], and pre-B cells [10]. In addition, T cells produce another osteoclastogenic cytokine, tumor necrosis factor (TNF)-α [11–13]. Recent reports have shown that ovariectomy (OVX) increases the production of interleukin (IL)-7 [14], which stimulates pro-

duction of RANKL and/or TNF-α by T cells [13,15], and that neutralization of IL-7 by administration of its specific antibody prevents bone resorption induced by OVX [14]. OVX-induced bone loss was absent in T-cell-deficient nude mice [11,12], and was restored by adoptive transfer of T cells from normal mice, but not by T cells from TNF-α knockout mice [12]. Production of RANKL by $B220^+$ pre-B cells was also upregulated in ovariectomized mice and by addition of isolated $B220^+$ cells from ovariectomized mice, but not cells from sham mice, to $B220^-$ cell cultures enhanced in vitro osteoclastogenesis in the presence of prostaglandin (PG)E_2 [10]. These data suggest that TNF-α and/or RANKL produced by T and B lineage cells plays an important role in acceleration of osteoclastogenesis caused by estrogen deficiency.

On the other hand, the involvement of increased RANKL produced by osteoblasts/stromal cells in accelerated osteoclastogenesis caused by estrogen deficiency is controversial. It has been reported that the expression of RANKL mRNA by osteoblasts/stromal cells is stimulated after estrogen withdrawal [16]. Upregulation of RANKL expression on osteoblasts/stromal cells as well as on immune cells in human bone marrow isolated from postmenopausal women has been demonstrated [17]. However, there also exists compelling evidence demonstrating that estrogen deficiency does not alter substantially RANKL [18,19] and OPG [19] mRNA expression in bone. In addition, estrogen has failed to consistently regulate osteoclastogenesis induced by osteoblast/stromal cell-derived RANKL in vitro. Treatment of ovariectomized mice with the pro-drug of 1α, 25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], which upregulates RANKL expression by osteoblasts/stromal cells in vitro, suppressed osteoclastogenesis without significantly altering RANKL and OPG mRNA expression in bone [19]. Because osteoblasts/stromal cells have been considered to play a pivotal role in regulation of osteoclastogenesis under physiological conditions, it is essential to establish whether osteoblasts/stromal cells would be involved in the increase of RANKL production by estrogen deficiency.

Alternatively or in addition, recent studies have implicated that estrogen deficiency stimulates osteoclastogenesis by increasing the pool size of osteoclast precursors [19–21]. Although it is a widespread belief that osteoclast precursors are of the monocyte/macrophage lineage, it has been consistently reported that osteoclasts can be generated from a subfraction of $B220^+$ pre-B cells [20–23]. Because proliferation of $B220^+$ pre-B cells is stimulated by IL-7, it raises the intriguing possibility that IL-7 induced by OVX stimulates osteoclastogenesis by expanding the pool size of $B220^+$ osteoclast precursors and that estrogen deficiency selectively augments the number of osteoclast precursors in the $B220^+$ cell fraction but not in the $B220^-$ fraction [21]. However, the effect of IL-7 on generation of osteoclast precursors in vitro has remained controversial [24].

The objective of our study was to determine precisely at mRNA levels whether estrogen deficiency would affect production of RANKL and OPG by osteoblasts/stromal cells in vivo and to investigate whether IL-7 would stimulate the generation of osteoclast precursors in vitro.

Materials and methods

Ovariectomy

Seven-week-old ddy mice were purchased from Nippon SLC (Shizuoka, Japan) and sham-operated or ovariectomized. The mice were allowed free access to water and commercial standard rodent chow (CE-2) under standard laboratory conditions at 24° ± 2°C and 50%–60% humidity. After surgery, blood samples were collected for enzyme-linked immunoabsorbent assay (ELISA). Femurs were isolated and immediately stocked in RNlator (Qiagen, Tokyo, Japan) at –80°C. In some experiments, femurs were used for bone marrow cell preparations. These animal studies were carried out in accordance with the ethical guidelines for animal care and approved by the Animal Care Committee of Meikai University.

Separation of adherent cells and nonadherent cells in bone marrow cells

The epiphysis of each femur was separated from the femoral shaft (diaphysis) by cutting with scissors, and bone marrow cells were isolated from the diaphysis by flushing with α-minimal essential medium (α-MEM) by means of a 26-G needle. The bone marrow cells were seeded at $1 \times 10^6/cm^2$ into culture dishes and cultured in α-MEM with 5% fetal bovine serum (FBS) for 24 h. Nonadherent cells were separated by harvesting culture medium and were further harvested by gentle washing with PBS. The nonadherent cells were pelleted by centrifugation, and adherent cells that remained on the dishes were used for further analyses [10].

Isolation of $B220^+$ cells

$B220^+$ cells were isolated from nonadherent cells by using a magnetic cell-sorting system (MACS; Miltenyi Biotec, Gladbach, Germany), as previously described [10,20].

Preparation of total RNA

Total RNA of separated cells was isolated by using an RNeasy Mini Kit (Qiagen), in accordance with the instruction manual. Total RNA of femurs from each mouse was isolated by using a Concert Micro-to-Midi Total RNA purification System (Invitrogen, Tokyo, Japan). Briefly, the epiphysis, diaphysis, flushed-out bone marrow cells, and whole femur were homogenized in lysis buffer and then used for total RNA purification as described in the instruction manual. Total RNA was treated with RNase-free DNase-I (Takara Bio, Tokyo, Japan) at 37°C for 90 min, purified with an RNeasy Mini kit, and used for cDNA synthesis.

Real-time RT-PCR

cDNA was synthesized from total RNA (5 μg) of each sample by random priming with Super Script-II reverse

transcriptase (RT; Lifetech Oriental, Tokyo, Japan). Reaction mixtures without the RT were also prepared and used as negative controls. An aliquot of each cDNA synthesis reaction mixture was diluted and used for real-time Polymerase chain reaction (PCR) quantitation. An equal volume of aliquot from each cDNA was mixed, serially diluted, and used as standard cDNAs. TaqMan probes/primers for RANKL, OPG, IL-7 and 18S rRNA, and PCR enzyme mix for real-time PCR were purchased from Applied Biosystems Japan (Tokyo, Japan). The real-time PCR quantitation was performed in triplicate by using a GeneAmp SDS5700 and GeneAmp SDS5700 software (Applied Biosystems Japan) according to the instruction manuals. The relative amount of target was calculated from standard curves generated in each PCR, and quantitation data with a coefficient of variance (CV) less than 10% were used for further analyses. Each calculated mRNA amount was standardized by reference to 18S rRNA. Data were expressed as the mean ratio to the control value ± SD, as described in the figure legends.

ELISA

The femurs and tibias were punctured at the epiphysis with a 22-G needle, and the fluids as well as bone marrow cells in the bones were flushed out by centrifugation (3000*g*) for 30s. Total volume of the flushed-out bone marrow cell suspension from one mouse (two femurs and two tibias) was adjusted to 150μl with α-MEM and further centrifuged at 1000*g* for 3min. The supernatant was harvested as bone fluid. The amount of soluble RANKL or IL-7 in each bone fluid and blood sample was measured by using a Quantikine M kit (R & D Systems, Minneapolis, MN, USA) according to the instruction manual.

Bone marrow cell cultures

The bone marrow cells (1×10^5) were seeded into each well of 96-well plates and cultured in α-MEM containing 10% FBS, macrophage-colony stimulating-factor (M-CSF; 5ng/ml, R & D systems), soluble RANKL (5ng/ml; R & D Systems), and various concentrations of IL-7 (0–30ng/ml; R & D Systems) for 4–5 days. In other experiments, the cells were cultured in α-MEM containing 10% FBS, M-CSF (5ng/ml), and various concentrations of IL-7 (0–30ng/ml) for 3–4 days, followed by further cultivation with M-CSF (5ng/ml) and soluble RANKL (5ng/ml) for 3 days. After cultivation, the cells were stained for tartrate-resistant acid phosphatase (TRAP) activity using a leukocyte acid phosphatase kit (Sigma-Aldrich Japan, Tokyo, Japan). The $TRAP^+$ multinucleated cells (MuNCs) containing three or more nuclei were counted under an inverted microscope.

Cocultures of bone marrow cells and stromal cells

The stromal layer was prepared by culturing MC3T3-G2/PA6 or ST2 cells as previously described [20]. The bone marrow cells (1×10^4) were seeded onto the stromal layer and cocultured in α-MEM containing 10% FBS and various concentrations of IL-7 (0–30ng/ml) for 3 days, followed by further cultivation with PGE_2 (0.5–2μg/ml) or $1,25(OH)_2D_3$ ($0.5–1 \times 10^{-8}$M) for 7 days. In other experiments, the cells were cocultured in α-MEM containing 10% FBS, PGE_2 (0.5–2μg/ml), or $1,25(OH)_2D_3$ ($0.5–1 \times 10^{-8}$M) and various concentrations of IL-7 (0–30ng/ml) for 7 days. After cultivation, $TRAP^+$ MuNC formation was evaluated as already described.

Unfractionated bone cell cultures

Unfractionated bone cells were prepared as previously described [20,25]. Briefly, the long bones of 12-day-old ddy mice were minced into small fragments, and cells were released from the fragments in α-MEM containing 5% FBS by vigorous agitation using a vortex mixer. After sedimentation of the bone fragments, the supernatant was used as the unfractionated bone cell suspension. Although bone marrow cells flushed from long bones contain virtually no $TRAP^+$ osteoclasts and few stromal cells, the unfractionated bone cell population contains osteoclasts approximately 0.01%–0.05% of the total cell number and a large number of osteoblasts/stromal cells [25], as evidenced by cytospin examination and by the fact that unfractionated bone cells seeded and cultured at a high density (5×10^5 per each well of 96-well plate) on dentin slices start to form resorption pits in the absence of osteoclastogenic factors within 2 days [25]. The unfractionated bone cells (5×10^4 cells) were seeded into each well of 96-well plates and cultured in α-MEM containing 5% FBS with the indicated factors for 4 days. Then the medium was exchanged for fresh medium containing the factors, and the cells were further cultured for another 4 days. $TRAP^+$ MuNC formation was evaluated as already described. After culturing the cells for the initial 4 days, only a few cells were $TRAP^+$ and virtually no $TRAP^+$ cells were found at the end of the culture when the cells were cultured in the absence of osteoclastogenic factors. Therefore, $TRAP^+$ cells at the end of the culture period were considered newly formed $TRAP^+$ cells. These newly formed $TRAP^+$ MuNCs have various characteristics of bona fide osteoclasts such as calcitonin receptors and bone-resorbing activity [25].

Frequency analysis of clonogenic osteoclast precursors

Frequency analysis of clonogenic osteoclast precursors in unfractionated bone cells pretreated with test factors was performed based on a limiting dilution assay [20,26] with some modifications. The unfractionated bone cells were seeded into each well of 96-well plates at 1, 15, or 50 cells per well and precultured for 4 days in α-MEM containing 5% FBS and M-CSF (5ng/ml) with or without IL-7 (10ng/ml). The cells were then washed with α-MEM, further cultured for 3 days in α-MEM containing 5% FBS, M-CSF (5ng/ml), and soluble RANKL (5ng/ml), and then stained for TRAP. The wells containing $TRAP^+$ MuNCs were

counted as osteoclast positive. Plates with appropriate numbers of osteoclast-positive wells (6–20 wells in 96 wells) from each experimental group were selected, and 1/frequency of osteoclast precursors was calculated according to the formula 1/frequency = $N/\{\ln[T/(T-P)]\}$, where N is the number of cells seeded in a well (1, 15, or 50 cells), T is the number of wells per group (96 wells), and P is the number of osteoclast-positive wells. In preliminary experiments, we observed that no $TRAP^+$ MuNCs are formed when cells are pre-cultured in the absence of M-CSF and found no effects of treatment with higher doses of soluble RANKL (10ng/ml) and M-CSF (10ng/ml) on frequency.

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) and Fisher's probable least squares difference test. For the analysis of IL-7 quantitation, the Mann–Whitney U test was used (see Fig. 4B). A value of $P < 0.05$ was considered statistically significant.

Results

Quantitation of RANKL and OPG mRNA expressions in femurs

Bone resorption as well as the number of osteoclasts in femurs is already increased at 14 days after OVX [27]. To evaluate the effects of estrogen withdrawal on the expression of RANKL and OPG mRNA, we quantitated the expression of each in femurs at 7–9 days after the surgery. As shown in Fig. 1A, OVX did not significantly stimulate RANKL mRNA expression in the femurs. Similarly, OPG mRNA expression was not significantly altered, resulting in no substantial change in the RANKL/OPG mRNA ratio (Fig. 1B). Similar results were obtained when we examined the animals 4–5 days after the surgery (data not shown). Because many of the osteoclasts reside in the epiphysis, but not so many in the diaphysis and virtually none in the flushed-out bone marrow, regulation of RANKL and/or OPG expression may be different at each site. Therefore, we divided femurs into the three parts and quantitated the expression individually. RANKL mRNA was highly expressed in the epiphysis compared with its expression in the diaphysis and flushed-out bone marrow (Fig. 2A). OPG mRNA was highly expressed in both the epiphysis and diaphysis (Fig. 2B). OVX did not alter either expression significantly (Fig. 2A,B) and resulted in no substantial change in the RANKL/OPG mRNA ratio in any part of the femur (Fig. 2C).

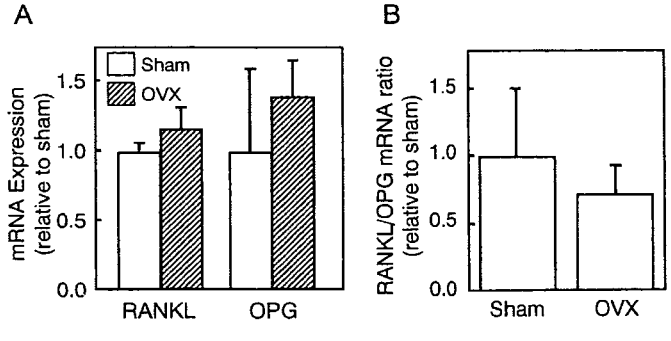

Fig. 1. A Quantitation of relative amount of RANKL mRNA (*RANKL*) and osteoprotegerin (OPG) mRNA (*OPG*) expressed by femurs 7–9 days after sham operation (*Sham*) or ovariectomy (*OVX*). **B** Ratio of RANKL mRNA/OPG mRNA. Data are expressed as relative to sham (mean ± SD of 10–12 mice)

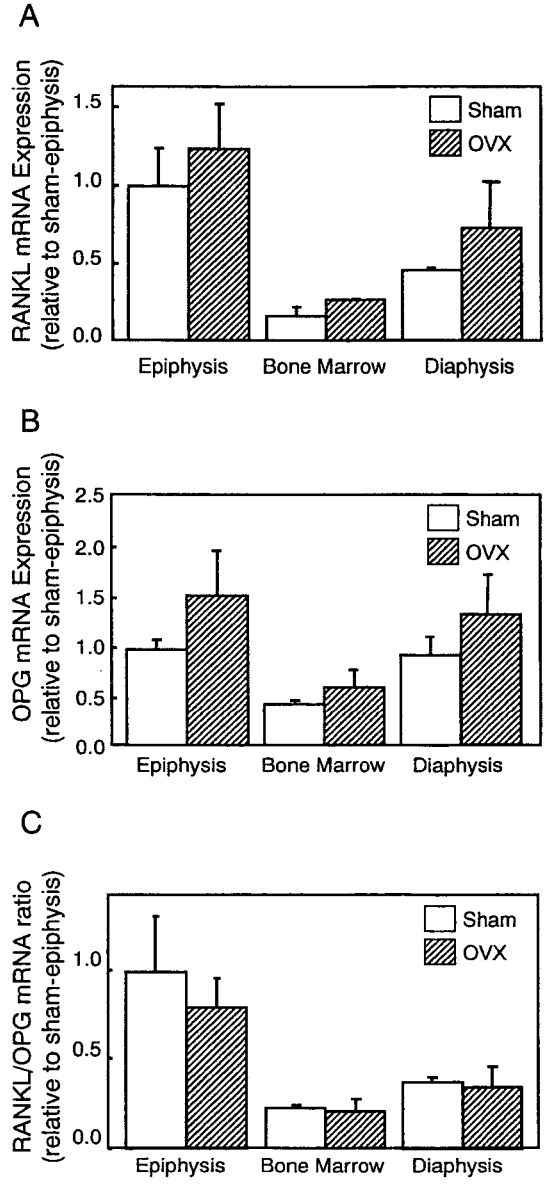

Fig. 2. Quantitation of relative amount of RANKL mRNA (**A**) and OPG mRNA (**B**) expressed in the epiphysis, flushed-out bone marrow, and diaphysis 7–9 days after sham-operation (*Sham*) or ovariectomy (*OVX*). **C** Ratio of RANKL mRNA/OPG mRNA. Data are expressed as relative to sham epiphysis (mean ± SD of 7–9 mice)

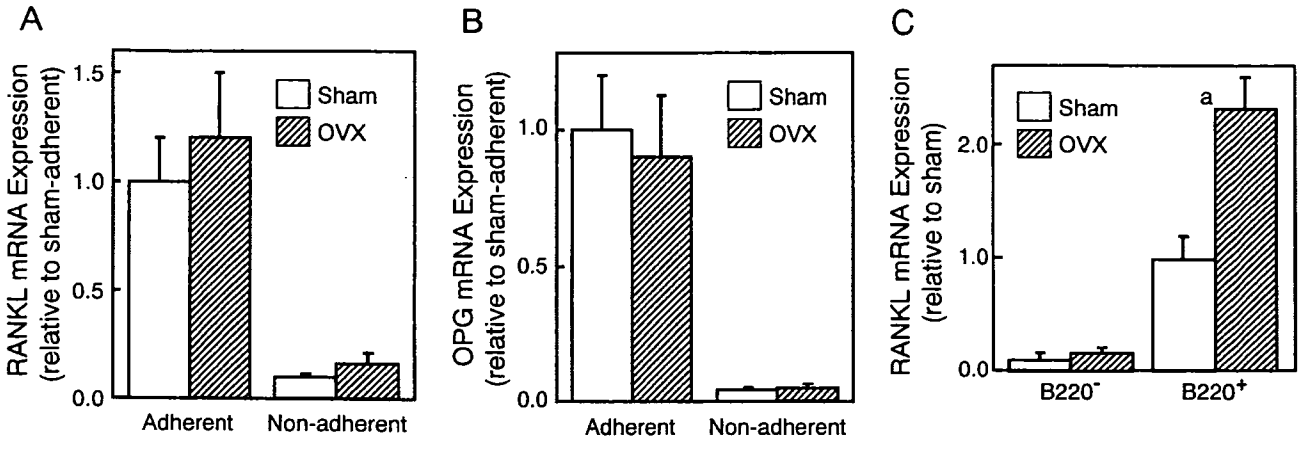

Fig. 3. Quantitation of relative amount of RANKL mRNA (**A**) and OPG mRNA (**B**) expressed by adherent or nonadherent cells separated by 24-h cultivation of bone marrow cells from sham-operated (*Sham*) or ovariectomized (*OVX*) mice 7 days after surgery. **C** Quantitation of relative amount of RANKL mRNA expressed by B220⁻ and B220⁺ cells purified from nonadherent cells. Data are expressed as relative to sham (mean ± SD of 6 mice). $^{a}P < 0.05$ (vs. sham mice)

Quantitation of RANKL and OPG mRNAs expressed by adherent bone marrow osteoblasts/stromal cells and nonadherent lymphohematopoietic cells

To further analyze the effect of OVX on RANKL and OPG mRNA expressions by sub-populations of cells in bone marrow, adherent stromal cells and nonadherent lymphohematopoietic cells were separated by culturing bone marrow cells for 24 h and subjected to mRNA quantitation. As shown in Fig. 3A,B, bone marrow stromal cells expressed RANKL and OPG mRNAs at levels more than five times higher compared with nonadherent lymphohematopoietic cells, and OVX did not upregulate either mRNA expression. The nonadherent cells slightly, but not significantly, increased RANKL mRNA expression by OVX. As we previously demonstrated the enhanced expression of RANKL by B220⁺ cells freshly isolated from ovariectomized mice [10], we checked RANKL mRNA expression by B220⁺ cells isolated from nonadherent cells after 24-h cultivation. The results showed that B220⁺ cells from ovariectomized mice expressed an amount of RANKL mRNA approximately twofold larger compared with that of sham-operated mice (Fig. 3C). Because the amount of RANKL mRNA expression by nonadherent cells, which include B220⁺ cells, is less than one-tenth of that of total bone marrow cells, it seems that the increased RANKL mRNA expression by B220⁺ cells does not significantly affect the amount of RANKL mRNA expression by total bone marrow cells. Taken together, it is suggested that RANKL and OPG mRNA expressed by the adherent cells including stromal cells is not significantly affected by estrogen deficiency.

Quantitation of IL-7

Because IL-7 was reported to be an important mediator of estrogen-deficiency induced bone resorption [15], we determined whether IL-7 would be increased by OVX. Consistent with a previous report [15], we found that the amounts of IL-7 mRNA in bone as well as IL-7 concentration in bone fluid were significantly increased at 7 days after OVX (Fig. 4A,B).

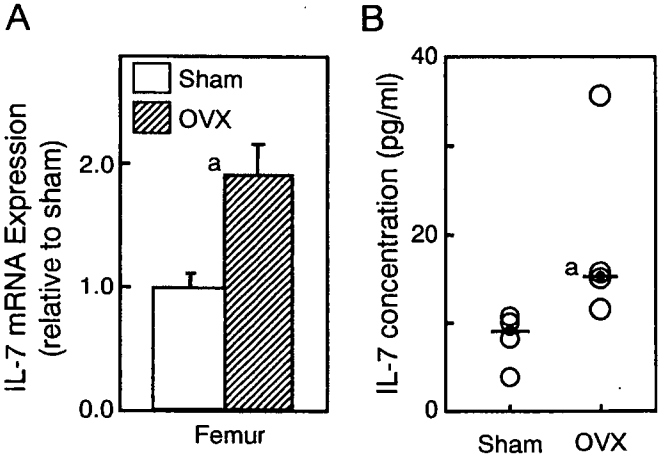

Fig. 4. **A** Quantitation of relative amount of interleukin 7 (IL-7) mRNA expressed by femurs 7 days after sham operation (*Sham*) or ovariectomy (*OVX*). Data are expressed as relative to sham (mean ± SD of 8 mice). $^{a}P < 0.05$ (vs. Sham). **B** Quantitation of IL-7 in bone fluids 7 days after sham operation (*Sham*) or ovariectomy (*OVX*). *Bar* indicates median value of each group (4 mice per group). $^{a}P < 0.05$ (vs. Sham)

Effect of IL-7 on osteoclastogenesis in unfractionated bone cell cultures

The foregoing results suggesting that IL-7, but not stromal cell-derived RANKL, increased by estrogen deficiency prompted us to examine the effects of IL-7 on osteoclastogenesis. As it has been shown that IL-7 does not stimulate osteoclastogenesis in cultures of flushed-out bone marrow cells [24], we tested several culture systems including cocultures of bone marrow cells and stromal cells (ST2

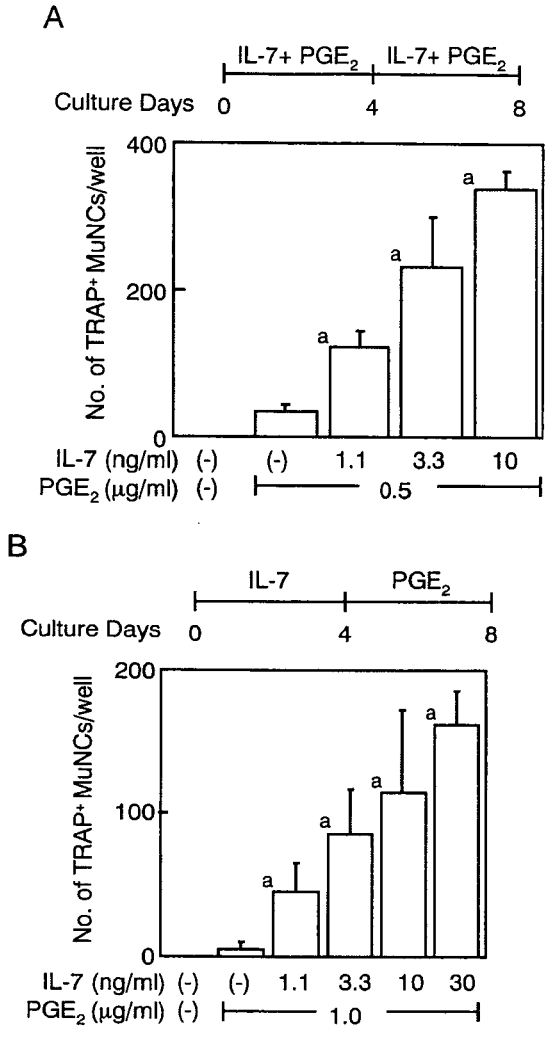

Fig. 5. Effects of IL-7 on osteoclastogenesis in cultures of unfractionated bone cells. **A** Unfractionated bone cells were treated with the indicated doses of IL-7 plus prostaglandin E_2 (PGE_2) for 8 days. **B** The bone cells were treated with indicated doses of IL-7 for 4 days, followed by treatment with the indicated dose of PGE_2 for 4 days. Data are expressed as the mean ± SD of 4 wells. $^aP < 0.05$ [vs. IL-7 (–)+ PGE_2 (0.5 μg/ml or 1.0 μg/ml)]

cells or MC3T3-G2/PA6 cells) and cultures of bone marrow cells. In all culture systems we tested, neither IL-7 in combination with PGE_2 or $1,25(OH)_2D_3$ (for cocultures) nor IL-7 in combination with M-CSF and soluble RANKL (for bone marrow cell cultures) could stimulate osteoclastogenesis (data not shown). Therefore, we utilized unfractionated bone cell cultures, which contain the cells attached tightly to the bone surfaces (see Materials and methods). We found that IL-7 alone does not affect osteoclastogenesis (data not shown), and that addition of PGE_2 (0.5 μg/ml) alone slightly stimulates formation in unfractionated bone cell cultures (Fig. 5A). When the bone cells were treated with IL-7 (0–10 ng/ml) in the presence of PGE_2 (0.5 μg/ml), IL-7 stimulated osteoclastogenesis dose-dependently. To clarify the effect of IL-7, we first pretreated the unfractionated bone cells with IL-7 for 4 days before PGE_2 (1.0 μg/ml) treatment to induce RANKL production. Pretreatment of

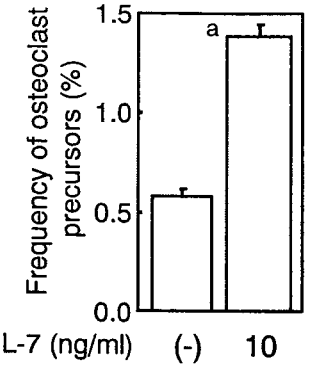

Fig. 6. Frequency analysis of clonogenic osteoclast precursors in unfractionated bone cells pretreated with M-CSF in the absence (–) or presence of 10 ng/ml IL-7. Data are expressed as the mean ± SD from three experiments. $^aP < 0.05$ [vs. IL-7 (–)]

unfractionated bone cells with IL-7 alone (0–10 ng/ml) augmented osteoclastogenesis induced by PGE_2 even in the absence of IL-7 (Fig. 5B). It should be noted that in this sequential treatment RANKL mRNA expression stimulated by PGE_2 was not significantly affected by the pretreatment of IL-7 (data not shown). These data suggest that IL-7 could stimulate osteoclastogenesis without affecting PGE_2-induced RANKL mRNA expression, at least in the unfractionated bone cell cultures.

Effect of IL-7 on frequency of clonogenic osteoclast precursors in cultures of unfractionated bone cells

A possible mechanism to stimulate osteoclastogenesis by IL-7 is that IL-7 augments the number of osteoclast precursors. To explore this possibility, we examined the effect of IL-7 on M-CSF-induced generation of osteoclast precursors by using frequency analysis. Theoretically, single unfractionated bone cells are pretreated with M-CSF alone or M-CSF plus IL-7 for 4 days and then the osteoclastogenic potential of the cells generated by the pretreatment is evaluated by stimulation with M-CSF plus soluble RANKL. The advantage of this assay is that even if the production profile of cytokines such as M-CSF, TNF-α, and RANKL varies between the presence or absence of IL-7 during the pretreatment, because very small numbers of cells are present in each well and osteoclastogenesis from the pretreated cells is induced by the addition of constant amounts of M-CSF and soluble RANKL, the effects of the variability can be neglected, which enables accurate evaluation of the number of osteoclast precursors generated by the pretreatment. The results of the frequency analysis revealed that the addition of IL-7 significantly increased the number of osteoclast precursors by approximately 2.4 fold (Fig. 6). Because an augmented number of osteoclast precursors could contribute to the increase in osteoclastogenesis independently of changes in osteoclastogenic factor production, these data suggest that IL-7-induced stimulation of osteoclastogenesis in the cultures of unfractionated bone cells is at least partly mediated by the stimulatory effect of IL-7 on the generation of osteoclast precursors.

Discussion

In this study, we showed that adherent cells in the epiphysis and diaphysis, as well as in bone marrow cells, which represent the majority of osteoblasts/stromal cells in bone, do not significantly alter RANKL and OPG mRNA expressions in the estrogen-deficient state. We found that production of IL-7 in bone is stimulated by OVX and that IL-7 augments osteoclastogenesis, at least via increasing the number of osteoclast precursors in cultures of unfractionated bone cells.

To evaluate the importance of RANKL produced by osteoblasts/stromal cells in epiphysis where many osteoclasts reside, we quantitated the target mRNA using RNA isolated from epiphysis or diaphysis freed from bone marrow, instead of using RNA from isolated osteoblasts/stromal cells, because of technical difficulties in separating the osteoblasts/stromal cells that are attached tightly to the bone matrix. Because we were unable to precisely quantitate RANKL on the cells at protein level, we could not rule out the possibility of upregulation of RANKL by a post-transcriptional mechanism. However, the fact that many cytokines implicated in acceleration of osteoclastogenesis caused by estrogen deficiency, such as IL-1, PGE_2, and TNF-α, increase RANKL production by osteoblasts/stromal cells via stimulation of RANKL mRNA expression [28,29], suggesting the possibility that the lack of enhancement of RANKL mRNA expression by cells at the epiphysis and diaphysis represents no substantial change in RANKL production by osteoblasts/stromal cells at the protein level. In addition, stromal cells separated from bone marrow cells also did not upregulate RANKL mRNA expression by OVX. These data are in contrast to a report showing an enhanced expression of cell surface RANKL on osteoblasts/stromal cells as well as on T and B lineage cells in bone marrow of postmenopausal women [17]. The discrepancy of RANKL expression by osteoblasts/stromal cells might be explained by species differences. In fact, substantial differences in responsiveness to estrogen between mouse and human bone have been reported [30,31].

Another possible explanation is that only a small portion of osteoblasts/stromal cells in bone increases RANKL mRNA expression and that this increase is not detectable when RNA is isolated from whole osteoblasts/stromal cell population. Collectively, it is suggested that the majority of osteoblasts/stromal cells do not up-regulate RANKL production, at least at the mRNA level, by estrogen deficiency in mice. On the other hand, we found increased RANKL mRNA expression by $B220^+$ cells, as previously reported [10], and increased concentration of soluble RANKL in bone fluid and in serum of ovariectomized mice (data not shown). Therefore, we could not rule out an increase of soluble RANKL, which is presumably produced by the cells other than osteoblasts/stromal cells, and RANKL on nonadherent cells, such as $B220^+$ cells, in estrogen deficiency. We confirmed also the increase of IL-7 production in bone, as previously reported [15]. It has been shown that T cells produce soluble RANKL and TNF-α in response to IL-7 [13,14] and that TNF-α produced by T cells in ovariectomized mice is indispensable for acceleration of osteoclastogenesis [12]. It should be noted that TNF-α itself has relatively weak osteoclastogenic activity compared with RANKL, but that it additively enhances osteoclastogenesis induced by even a saturating amount of RANKL in vitro [11].

Taken together, these data support the hypothesis that TNF-α produced by T cells as well as soluble and membrane-bound RANKL produced by T and B lineage cells, rather than membrane-bound RANKL on osteoblasts/stromal cells, is increased by estrogen deficiency, leading to acceleration of osteoclastogenesis. In addition, these data also support the hypothesis that osteoclast precursors circulating in the bloodstream [32] are primed by an increased level of soluble RANKL and/or TNF-α, the production of which might possibly be stimulated in response to IL-7 in organs rich in T cells, such as spleen and thymus, and then migrate and differentiate in the bone microenvironment where osteoblasts/stromal cells covering bone matrix express a constant amount of RANKL on their cell surfaces as a basal condition to induce final osteoclastic differentiation.

A recent report has suggested that the potential of a purified $B220^+$ cell fraction to generate osteoclasts requires contaminated $B220^-$ cells [33]. However, we previously utilized a frequency analysis of osteoclast precursors, in which analysis the possibility that contaminated $B220^-$ cells in purified $B220^+$ cell fraction would influence the results could be excluded, and showed that the frequency of osteoclast precursors in purified $B220^+$ cells and that of $B220^-$ cells are at a comparable level. Therefore, it is theoretically possible to conclude that a major part of the osteoclasts generated in the purified $B220^+$ cell fraction is indeed derived from $B220^+$ cells. Our results showing that IL-7 stimulates osteoclastogenesis as well as generation of osteoclast precursors only in unfractionated bone cell cultures may have implications concerning the target cells of IL-7.

Recently, it has been reported that bone lining cells or endosteum covering calcified bone matrix is the hematopoietic stem cell niche and that hematopoietic stem cells are attached tightly to the endosteum [34–36]. Indeed, hematopoietic stem cells are released only after collagenase treatment of bone matrix but not by flushing-out the bone marrow [36]. In addition, localization studies have shown that myelomonocytic progenitors exist at the periendosteum and migrate to the central area of the bone marrow as they maturate [37]. It has been also reported that the proliferating μ-chain-negative immature $B220^+$ cells first appear at the subosteal region and then migrate into the central area of the bone marrow [38–40], suggesting that $B220^{-/+low}$ progenitor cells giving rise to $B220^+$ pro-B cells reside at the subosteal region. Importantly, a similar pattern of $B220^+$ cell localization has been reported in IL-7 transgenic mice with focal osteolysis [41]. From a methodological point of view, one of the differences between cultures of the unfractionated bone cells and cultures using bone marrow cells is that the unfractionated bone cells contain not only bone marrow cells, but also a population of cells that is released from the bone surface by vigorous

shaking of small pieces of bone fragments (see Materials and methods). Indeed, previous analyses have demonstrated that the unfractionated bone cell population is rich in cells that are attached tightly to the bone surface, such as osteoblasts/stromal cells, osteoclasts [25,26], and $TRAP^+$ $B220^+$ cells [20], when compared with the population of flushed-out bone marrow cells.

Because IL-7 promotes the proliferation and differentiation of the cells over a relatively wide range of differentiation stages from immature $B220^{-/+low}$ to $B220^{+high}$ and $B220^+$ osteoclast precursors are relatively immature among $B220^+$ cells [23], therefore our results may suggest that the immature $B220^{-/+low}$ existing at the subosteal region are possibly released in the unfractionated bone cells and that IL-7 acts on these cells to generate $B220^+$ osteoclast precursors. It was reported that RANKL stimulates not only osteoclastogenesis but also $B220^+$ cell proliferation when used together with IL-7 [42]. In estrogen-deficient mice, therefore, $B220^{-/+low}$ cells at the subosteal region would be well suited for their proliferation as well as osteoclastic differentiation because of the presence of increased amounts of IL-7 and TNF-α, in addition to M-CSF and RANKL expressed on the surfaces of osteoblasts/stromal cells covering the bone matrix, where osteoclasts exist and function. In this regard, the flushed-out bone marrow cells from ovariectomized mice may contain an increased number of $B220^+$ osteoclast precursors as a result of IL-7-stimulated proliferation of the cells at the subosteal region, but treatment of the flushed-out bone marrow cells with IL-7 in vitro may promote the differentiation of $B220^+$ cells along the B-cell lineage, thereby decreasing their potential of osteoclastic differentiation. This finding may explain the inhibitory effect of IL-7 on osteoclastogenesis in bone marrow cell cultures [24]. Further studies are required to reveal the precise nature of the target cells for IL-7.

It could be possible that treatment of the unfractionated bone cells with IL-7 results in increase of $B220^+$ cells that express RANKL; however, we could not detect a significant effect of IL-7 on RANKL mRNA expression, at least in our cultures (data not shown). Although our results do not rule out the involvement of $B220^+$ cell-derived RANKL for acceleration of osteoclastogenesis in vivo, a possible explanation might be that only a small portion of RANKL is produced by $B220^+$ cells, and that the increase of RANKL mRNA expression by $B220^+$ cells resulting from IL-7 treatment in our cultures could not significantly affect the total amount of RANKL mainly produced by stromal cells in response to PGE_2. Taken together, it is speculated that the stimulatory effect of IL-7 on osteoclastogenesis in the unfractionated bone cells might not be the result of the increase in osteoclastogenic factors but rather be caused the expansion of osteoclast precursors.

In conclusion, our study suggests that estrogen deficiency does not significantly alter RANKL and OPG expression by osteoblasts/stromal cells, but increases IL-7 production in bone, and that IL-7 augments a pool size of osteoclast precursors through the action of IL-7 on the cells attached to the bone surfaces rather than on those contained in the bone marrow cell population, thereby leading to stimulation of osteoclastogenesis. The increased osteoclastogenic factors produced by immune cells, rather than osteoblasts/stromal cells, and/or an augmented pool size of osteoclast precursors might be important for the pathogenesis and treatment of estrogen deficiency-induced osteoporosis.

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歯周組織再生医療とDDS技術に期待するもの

Periodontal regeneration and DDS

Keywords
歯周病
歯周組織再生
塩基性線維芽細胞増殖因子
歯根膜細胞

村上 伸也　島袋 善夫
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歯周病分子病態学・歯周病診断制御学

Summary

In order to enhance periodontal tissue regeneration, we have been investigating the biological activities of basic fibroblast growth factor (bFGF, FGF-2). Intraosseous bony defects were surgically created in beagle dogs and non-human primates and recombinant FGF-2 was topically applied to those defects. Six or eight weeks after application, in all sites where FGF-2 was applied, significant periodontal ligament (PDL) formation with new cementum and new bone formation was observed in amounts greater than in the control sites. No instances of epithelial down growth, ankylosis, or root resorption were observed in the FGF-2 sites. Based on the data of *in vitro* analysis, we speculate that FGF-2 plays important roles in wound healing by promoting angiogenesis, regulating production of extracellular matrices, and inducing the growth of immature PDL cells, and in turn accelerates periodontal regeneration. Introducing DDS which enables controlled release of plural cytokines may enhance the potential of "cytokine therapy" in the fields of not only periodontal regeneration but also craniofacial medicine.

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歯周病と歯周治療

歯周病はデンタルプラーク（細菌バイオフィルム）に起因する感染症であり，疾患の進行に伴い歯の支持組織である歯周組織が破壊される慢性炎症性疾患である（図１）。世界中において依然罹患率の高い疾患の一つであり，成人が歯を失う最大の原因に挙げられている。日本においても成人の約80％が罹患している「口」の生活習慣病として位置づけられている。歯周治療の原則は，原因であるデンタルプラークを歯根表面の壊死セメント質とともに機械的に除去することである。しかしながら，それだけでは創傷治癒の場にいち早く到達する歯肉上皮により創傷治癒が完了してしまい，歯周病の進行により失われたセメント質や歯槽骨の新生を伴った歯周組織再生は達成できない。中高年者，高齢者において「口」と「歯」が支えるQOLが歯