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Enhanced Generation of Endothelial Cells From CD34+ Cells of the Bone Marrow in Rheumatoid Arthritis

Possible Role in Synovial Neovascularization

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Objective. **To examine the capacity of bone marrow CD34+ cells to generate endothelial cells, in order to assess the role of bone marrow in neovascularization in the synovium of rheumatoid arthritis (RA).**

Methods. **CD34+ cells purified from the bone marrow of 13 patients with active RA and 9 control subjects (7 osteoarthritis [OA] patients and 2 healthy individuals) were cultured in the presence of stem cell factor (10 ng/ml) and granulocyte–macrophage colony-stimulating factor (1 ng/ml). After 18 days of incubation, the generation of endothelial cells was assessed by flow cytometry. The generation of endothelial cells was compared with the degree of vascularization in the synovial tissues and with the microvessel densities in the synovium, as determined by microscopy. The expression of vascular endothelial growth factor receptor 2/kinase insert domain receptor (KDR) messenger RNA (mRNA) in CD34+ cells was examined by quantitative reverse transcription–polymerase chain reaction.**

Results. **The generation of CD14+ cells from bone marrow–derived CD34+ cells from RA patients was comparable to that from control subjects. However, the generation of von Willebrand factor (vWF)–positive cells and CD31+/vWF+ cells from RA bone marrow–derived CD34+ cells was significantly higher than that from control subjects ($P = 0.004$ and $P = 0.030$, respectively). The generation of vWF+ cells from bone marrow CD34+ cells correlated significantly with the microvessel densities in the synovial tissues ($r = 0.569$, $P = 0.021$). Finally, RA bone marrow CD34+ cells expressed KDR mRNA at higher levels than OA bone marrow CD34+ cells.**

Conclusion. **These results indicate that RA bone marrow CD34+ cells have enhanced capacities to differentiate into endothelial cells in relation to synovial vascularization. The data therefore suggest that bone marrow CD34+ cells might contribute to synovial neovascularization by supplying endothelial precursor cells and, thus, play an important role in the pathogenesis of RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of synovial lining cells (1). Synovial lining cells consist of type A (macrophage-like) synoviocytes and type B (fibroblast-like) synoviocytes. Recent studies have suggested that type A synoviocytes are derived from monocyte precursors in the bone marrow (2). Accordingly, it has been shown that the spontaneous generation of CD14+ cells from bone marrow–derived CD14– progenitor cells is accelerated in RA, resulting in the facilitated entry of such CD14+ cells into the synovium (3). On the other hand, type B synoviocytes have a morphologic appearance of fibroblasts as well as the capacity to produce and secrete a variety of factors, including proteoglycans, cytokines, arachidonic acid metabolites, and matrix met-

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alloproteinases (MMPs), that lead to the destruction of joints (4). Whereas type B synoviocytes are thought to arise from the sublining tissue or other support structures of the joint (4), recent studies have suggested that they are also derived from bone marrow progenitor cells (5). Increasing attention has therefore been given to the role of the bone marrow in the pathogenesis of RA (6).

In the RA joint, the massive proliferating synovium forms an invading tissue called pannus, which results in the destruction of cartilage and bone. A number of studies have shown that persistent neovascularization is a crucial support to the continuous proliferation of the synovium, through delivery of nutrients and recruitment of inflammatory cells into the synovium (7,8). It was a long-held belief that vessels in the embryo developed from endothelial progenitors (vasculogenesis), whereas spouting of vessels in the adult resulted only from division of differentiated endothelial cells (angiogenesis) (9). Thus, the neovascularization in RA synovium has been attributed to angiogenesis, a process characterized by spouting of new capillaries from preexisting blood vessels (10).

Asahara et al (11), however, isolated endothelial progenitor cells from adult human peripheral blood using magnetic bead selection of CD34+ hematopoietic cells, and thus demonstrated that human peripheral blood CD34+ cells differentiated in vitro into endothelial cells, which expressed endothelial markers, including CD31. In addition, those investigators found that human CD34+ cells were incorporated into neovascularized hind limb ischemic sites in animal models (11). Since the time these observations were reported, it has also been found that endothelial progenitor cells capable of contributing to capillary formation can be derived from the bone marrow, possibly playing a role in the de novo formation of capillaries without preexisting blood vessels (12–14). Thus, the accumulating evidence has suggested that bone marrow–derived endothelial cells might be involved in several disorders characterized by excessive angiogenesis, such as myocardial infarction (15). However, the role of bone marrow in RA synovial neovascularization has not been explored.

It has been demonstrated that early endothelial progenitor cells in bone marrow express CD34, CD133, and vascular endothelial growth factor receptor 2 (VEGFR-2)/kinase insert domain receptor (KDR) (15). In general, early endothelial progenitor cells in the bone marrow are positive for CD34/CD133/VEGFR-2, whereas circulating endothelial progenitor cells are positive for CD34/VEGFR-2/CD31, negative for CD133, and are beginning to express von Willebrand factor (vWF) (15). Thus, it appears that vWF is expressed on fully matured endothelial cells.

The current studies were undertaken to explore whether CD34+ cells derived from the bone marrow of RA patients might have an enhanced capacity to generate endothelial cells so that we could assess the role of the bone marrow in the neovascularization of RA synovium. The results clearly indicate that bone marrow–derived CD34+ cells from RA patients differentiate into vWF+ endothelial cells upon stimulation with stem cell factor (SCF) and granulocyte–macrophage colony-stimulating factor (GM-CSF) much more effectively than do those from control subjects. The data therefore suggest that bone marrow CD34+ cells might play a role in the synovial hyperplasia in RA through mobilization of endothelial progenitor cells into the synovium, where angiogenesis is activated.

MATERIALS AND METHODS

Patients and samples. Bone marrow samples from 13 RA patients (1 man and 12 women; mean age 58.2 years [age range 45–72 years]) were obtained during joint operation through aspiration from the iliac crest. All RA patients met the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria (16). As controls, bone marrow samples were similarly obtained from 7 patients with osteoarthritis (OA) (7 women; mean age 70.6 years [age range 67–74]). All study patients gave their informed consent for study. In addition, bone marrow–derived CD34+ cells from 2 healthy individuals (2 men; ages 27 years and 24 years) were purchased from BioWhittaker (Walkersville, MD). Synovial tissues were also obtained from 10 of the RA patients and 6 of the OA patients during the same joint operation.

A second group of bone marrow samples was obtained from an additional 10 RA patients (3 men and 7 women; mean age 62.6 years) and an additional 4 OA patients (2 men and 2 women; mean age 72.6 years). These bone marrow samples were used in analyses of the expression of KDR messenger RNA (mRNA). These patients also gave their informed consent for study. In addition, samples from 7 of the RA patients and 6 of the OA patients from the first group described above were included in the study of KDR mRNA expression.

Culture medium and reagents. RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), and 10% fetal bovine serum (Life Technologies) was used for all cultures. Recombinant human GM-CSF and SCF were purchased from PeproTech (London, UK).

Preparation and culture of bone marrow–derived CD34+ cells. Mononuclear cells were isolated by centrifugation of heparinized bone marrow aspirates over sodium

Figure 1. Histologic features (A) and immunohistochemistry of CD31 cells (B) in the synovium of patients with osteoarthritis (OA) and rheumatoid arthritis (RA). Four representative samples of synovium from OA (a and b) and RA (c and d) patients are presented, showing trace (a), mild (b), moderate (c), and strong (d) neovascularization. H&E = hematoxylin and eosin. (Original magnification × 25.)

diatrizoate-Ficoll gradients (Histopaque; Sigma, St. Louis, MO). CD34+ cells were purified from the mononuclear cells through positive selection using magnetic beads (Dynal CD34 progenitor cell selection system; Dynal, Oslo, Norway). CD34+ cells thus prepared were ~95% CD34+ cells and <0.5% CD19+ B cells, as previously described (5).

CD34+ cells were incubated in a 24-well microtiter plate with flat-bottomed wells (no. 3524; Costar, Cambridge, MA) at a density of 1.0×10^5/well in the presence of SCF (10 ng/ml) and GM-CSF (1 ng/ml). After 18 days of incubation, the cells were stained with various antibodies and analyzed by flow cytometry.

Immunofluorescence staining and analysis. Cultured CD34+ cells were stained with saturating concentrations of antibodies, including fluorescein isothiocyanate (FITC)–conjugated anti–HLA-DR monoclonal antibody (mAb) (mouse IgG2b; Immunotech, Marseilles, France), FITC-conjugated sheep anti-vWF IgG (Cosmo Bio, Tokyo, Japan), phycoerythrin (PE)–conjugated anti-CD14 mAb (mouse IgG2a; Immunotech), PE-conjugated anti-CD31 mAb (mouse IgG1; Immunotech), PE-conjugated murine IgG1 and IgG2a control mAb or FITC-conjugated murine IgG2b control mAb, which were raised against *Aspergillus niger* glucose oxide, an enzyme that is neither present nor inducible in mammalian tissues (Dako, Glostrup, Denmark), or FITC-conjugated control sheep IgG purified from normal sheep serum (Rockland, Gilbertsville, PA).

Briefly, the cells were washed with 2% normal human serum in phosphate buffered saline (PBS), pH 7.2, and 0.1% sodium azide (staining buffer), and the cells were stained with saturating concentrations of a variety of antibodies at 4°C for 30 minutes. The cells were then washed 3 times with staining buffer and fixed with 1% paraformaldehyde in PBS for at least 5 minutes at room temperature. Cells were analyzed using an Epics XL flow cytometer (Coulter, Hialeah, FL) equipped with an argon-ion laser at 488 nm. A combination of low-angle and 90° light scatter measurements (forward scatter versus side scatter) was used to identify bone marrow cells. The percentages of cells that stained positive for each mAb were determined by integration of cells above a specified fluorescence channel, which was calculated in relation to an isotype-matched control mAb.

Synovial histopathology and determination of microvessel densities. Synovial tissues were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. To visualize endothelial cells in the synovium, the paraffin-embedded sections were also stained with murine anti-CD31 mAb (clone JC/70A; Dako) and then developed using a Dako Envision kit, which includes horseradish peroxidase and diaminobenzidine. The degree of neovascularization was analyzed under light microscopy and scored as 0 (trace), 1 (mild), 2 (moderate), or 3 (strong) (Figures 1A and B). Grades were assigned by 2 independent observers (SH and TY) who had no knowledge of the diagnosis of the patients from whom the tissues had been obtained. When grades differed (2 of 16 cases), the synovium was reexamined, and a consensus was reached.

Sections were photographed with an Olympus DP11 digital camera (Olympus, Tokyo, Japan), and the CD31+ microvessel densities were determined by counting the vascular structures with a clearly defined lumen or linear shape as seen on the photographs. The final microvessel density was calculated as the mean score of the 3 1-mm^2 fields with the highest individual scores (17).

Measurement of cytokines in the culture supernatants. Concentrations of tumor necrosis factor α (TNFα) and vascular endothelial growth factor (VEGF) in the culture supernatants were measured by enzyme-linked immunosorbent assay

(ELISA) using a human TNFα ELISA kit (PeproTech) and a human VEGF immunoassay kit (BioSource International, Camarillo, CA).

RNA isolation and real-time quantitative polymerase chain reaction. Total RNA was isolated from purified bone marrow CD34+ cells using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Complementary DNA (cDNA) samples were prepared from 1 μg of total RNA using the SuperScript reverse transcriptase preamplification system (Life Technologies) with oligo(dT) primers and were subjected to PCR. Real-time quantitative PCR was performed using the LightCycler rapid thermal cycler system (Roche Diagnostics, Lewes, UK) with primer sets for VEGFR-2/KDR or β-actin (Nihon Gene Research Laboratories, Sendai, Japan) and LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics).

The primer sequences were as follows: for KDR, 5′-CAGACGGACAGTGGTATGGT-3′ (forward) and 5′-GCCTTCAGATGCCACAGACT-3′ (reverse); and for β-actin, 5′-GCAAAGACCTGTACGCCAAC-3′ (forward) and 5′-CTAGAAGCATTTGCGGTGGA-3′ (reverse). The PCR reaction conditions were as follows: denaturing at 95°C for 10 minutes for 1 cycle, followed by 40 cycles of denaturing at 95°C for 10 seconds, annealing at 62°C for 10 seconds, and extension at 72°C for 5 seconds (KDR) or 10 seconds (β-actin). Quantitative analysis was performed using LightCycler software version 3.5. All results for KDR were calibrated to the copy number of β-actin from each cDNA sample.

RESULTS

Synovial histopathologic features in RA. A number of studies have confirmed that the microscopic appearance of RA synovial tissue is variable (18). Consistent with those studies, Figures 1A and B show representative patterns of neovascularization seen in RA synovium in this study. It appears that the overall degree of neovascularization is correlated with the degree of exudation, cellular infiltration, and granulation tissue development, which are characteristic features of RA synovium (18). Thus, synovium with marked granulation and cellular infiltration showed the maximal degree of neovascularization (Figures 1A and B part

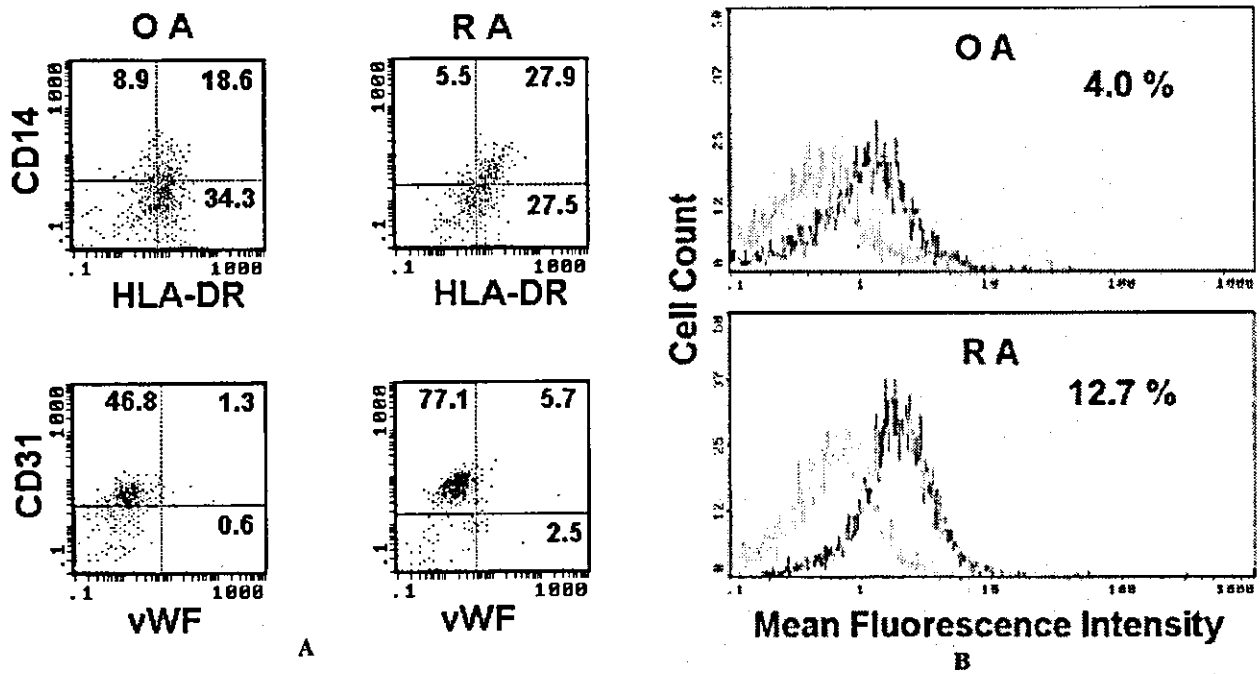

Figure 2. Representative 2-color and single-color flow cytometric analyses of the phenotypes of CD34+ cells stimulated with stem cell factor (SCF) and granulocyte–macrophage colony-stimulating factor (GM-CSF). CD34+ cells from the bone marrow of a rheumatoid arthritis (RA) and an osteoarthritis (OA) patient were stimulated for 18 days with SCF and GM-CSF, stained with phycoerythrin (PE)–conjugated anti-CD14 and fluorescein isothiocyanate (FITC)–conjugated anti–HLA-DR or with PE-conjugated anti-CD31 and FITC-conjugated von Willebrand factor (vWF), and analyzed by flow cytometry. **A**, The quadrant boundaries were determined by analysis of isotype-matched control cells. Values shown are percentages of cells. **B**, Single-color analysis of the expression of vWF (left histogram). Right histogram shows control staining. Percentages shown are for vWF+ cells. Figure 2B can be viewed in color in the online issue, which is available at http://www.arthritisrheum.org.

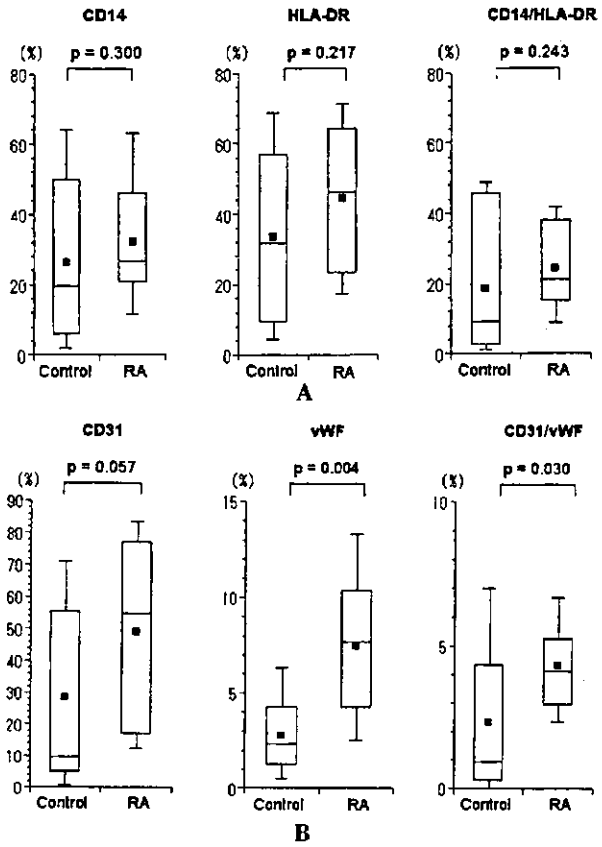

Figure 3. Phenotypic features of bone marrow–derived CD34+ cells stimulated with SCF and GM-CSF. Bone marrow CD34+ cells from 13 RA patients and 9 control subjects were stimulated for 18 days with SCF and GM-CSF, stained with **A**, PE-conjugated anti-CD14 and FITC-conjugated anti–HLA–DR or **B**, PE-conjugated anti-CD31 and FITC-conjugated anti-vWF, and analyzed by flow cytometry. Data are shown as box plots. Horizontal lines constituting the top, middle, and bottom of the boxes show the 75th, 50th, and 25th percentiles, respectively. Lines outside the boxes show the 90th and 10th percentiles. Solid squares inside the boxes show the mean. *P* values were determined by Mann-Whitney U test. See Figure 2 for definitions.

d), whereas the synovium with trace amounts of exudation and cellular infiltration lacked neovascularization. The results therefore suggest that neovascularization might play a role in development of inflamed synovium in RA.

Generation of vWF+ cells from bone marrow–derived CD34+ cells. After stimulation of bone marrow CD34+ cells (1.0×10^5) with SCF and GM-CSF for 18 days, the mean ± SD number of recovered cells was $3.85 \pm 2.97 \times 10^5$ for RA patients and $3.79 \pm 3.18 \times 10^5$ for control subjects ($P = 0.6401$ by Mann-Whitney U test). Figures 2A and B show, respectively, the representative dual-parameter 4-quadrant scattergrams and single-color histograms of the bone marrow CD34+ cells stimulated with SCF and GM-CSF for 18 days.

A similar percentage of cultured CD34+ cells from the RA patient and the OA control patient expressed CD14 and HLA–DR. Although in Figure 2A, bone marrow CD34+ cells from the RA patient generated higher percentages of CD14+/HLA–DR+ cells, there was no significant difference in the percentages of CD14+ cells and CD14+/HLA–DR+ cells generated by bone marrow CD34+ cells from the 9 control subjects and the 13 RA patients (Figure 3A). In contrast, bone marrow CD34+ cells from the RA patient gave rise to higher numbers of vWF+ cells and CD31+/vWF+ cells than did those from the OA control patient (Figures 2A

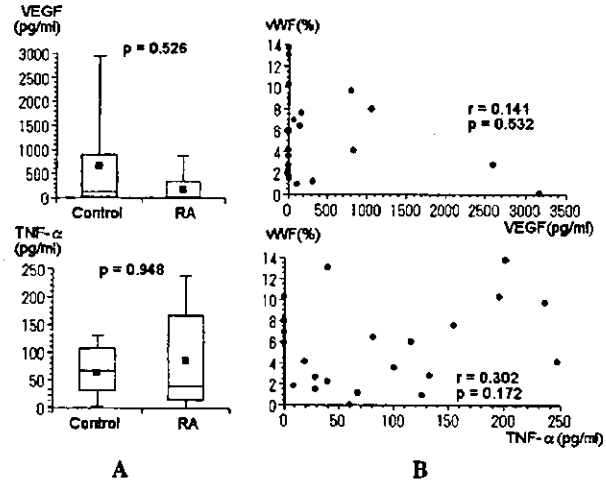

Figure 4. Production of vascular endothelial growth factor (VEGF) and tumor necrosis factor α (TNFα) by bone marrow–derived CD34+ cells and correlation with the generation of von Willebrand factor (vWF)–positive cells. Bone marrow CD34+ cells from 13 rheumatoid arthritis (RA) patients and 9 control subjects were stimulated for 18 days with stem cell factor and granulocyte–macrophage colony-stimulating factor. Cells were analyzed for vWF expression by flow cytometry; supernatants were assayed for VEGF and TNFα by enzyme-linked immunosorbent assay. **A**, Data are shown as box plots. Horizontal lines constituting the top, middle, and bottom of the boxes show the 75th, 50th, and 25th percentiles, respectively. Lines outside the boxes show the 90th and 10th percentiles. Solid squares inside the boxes show the mean. *P* values were determined by Mann-Whitney U test. **B**, Correlations between the generation of vWF+ cells and the production of each cytokine was evaluated by simple regression analysis.

and B). Accordingly, bone marrow CD34+ cells from the 13 RA patients generated significantly higher numbers of vWF+ cells as well as CD31+/vWF+ cells than did those from the 9 control subjects, although there was no significant difference in the number of CD31+ cells (Figure 3B). These results indicate that upon stimulation with SCF and GM-CSF, bone marrow CD34+ cells from RA patients have enhanced capacities to generate endothelial cells compared with control subjects, whereas the capacities of RA bone marrow CD34+ cells to give rise to CD14+ monocyte-lineage cells were comparable to those of the control subjects.

Relationship between the generation of vWF+ cells and the production of VEGF and TNFα. It was possible that the enhanced generation of vWF+ cells from RA bone marrow–derived CD34+ cells might be a result of the increased production of angiogenic cytokines. In fact, it has been reported that VEGF (19,20) and TNFα (21–24) play a major role in regulating neovascularization in RA. Our next experiments therefore examined the capacity of bone marrow CD34+ cells stimulated with SCF plus GM-CSF to produce VEGF and TNFα. As can be seen in Figure 4, there were no significant differences in the production of VEGF and TNFα by RA bone marrow CD34+ cells and control bone marrow CD34+ cells. In addition, the generation of vWF+ cells was not significantly correlated with the production of VEGF or TNFα. The results therefore suggest that the enhanced generation of vWF+ cells from RA bone marrow CD34+ cells might not be due to the increased production of angiogenic cytokines but, more likely, it may be due to the intrinsic abnormalities of the bone marrow CD34+ cells.

Relationship between the capacity of bone marrow–derived CD34+ cells to generate endothelial cells and the vascularization of the synovium. To further confirm the role of the bone marrow in synovial neovascularization, we next examined the relationship between the capacity of bone marrow CD34+ cells to generate vWF+ cells and the degree of vascularization in the synovium in synovium samples obtained from 10 of the RA patients and 6 of the OA patients on the same day as the bone marrow samples. The degree of vascularization in the synovium was analyzed under light microscopy and scored as described in Materials and Methods. The degree of synovial vascularization in the RA patients was significantly elevated compared with that in the OA patients. In addition, the capacity of bone marrow CD34+ cells to generate vWF+ cells was significantly correlated with the degree of vascularization in the synovium in these 16 patients (data not shown).

To evaluate the synovial vascularization in a more objective manner, we calculated the CD31+ microvessel densities in the synovium. As shown in Figure 5, the synovial microvessel densities were significantly higher in RA patients than in OA patients. Moreover, the microvessel densities were also significantly correlated with the generation of vWF+ cells from bone marrow–derived CD34+ cells in the 16 patients with OA or RA. The results therefore suggest that vasculogenesis occurring through mobilization of endothelial progenitor cells from the bone marrow might be involved, at least in part, in the synovial neovascularization, and may thus play a significant role in the pathogenesis of RA.

Expression of mRNA for VEGFR-2/KDR in bone marrow–derived CD34+ cells. Recent studies have revealed that the vascular activation by VEGF/KDR was significantly higher in RA than in OA, although activation of the hypoxia inducing factor α (HIFα) pathway was comparable in RA and OA (25). It was therefore possible that there might be differences in the activation

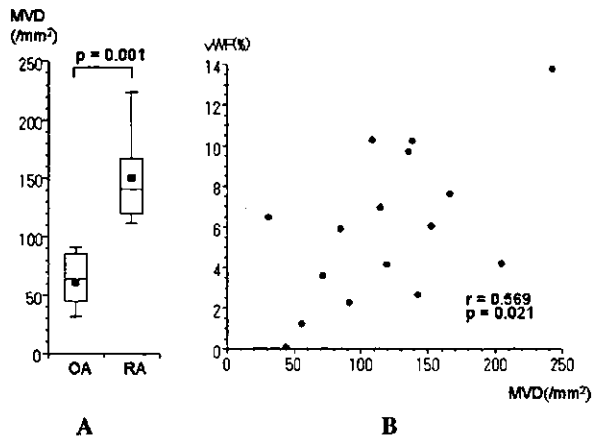

Figure 5. Correlation between synovial microvessel densities (MVD) and the generation of von Willebrand factor (vWF)–positive cells from bone marrow–derived CD34+ cells. Synovial microvessel densities were compared between 10 rheumatoid arthritis (RA) patients and 6 osteoarthritis (OA) patients. **A**, Data are shown as box plots. Horizontal lines constituting the top, middle, and bottom of the boxes show the 75th, 50th, and 25th percentiles, respectively. Lines outside the boxes show the 90th and 10th percentiles. Solid squares inside the boxes show the mean. *P* values were determined by Mann-Whitney U test. **B**, Correlations between the microvessel densities and the generation of vWF+ cells from bone marrow CD34+ cells in the 16 patients was determined by simple regression analysis.

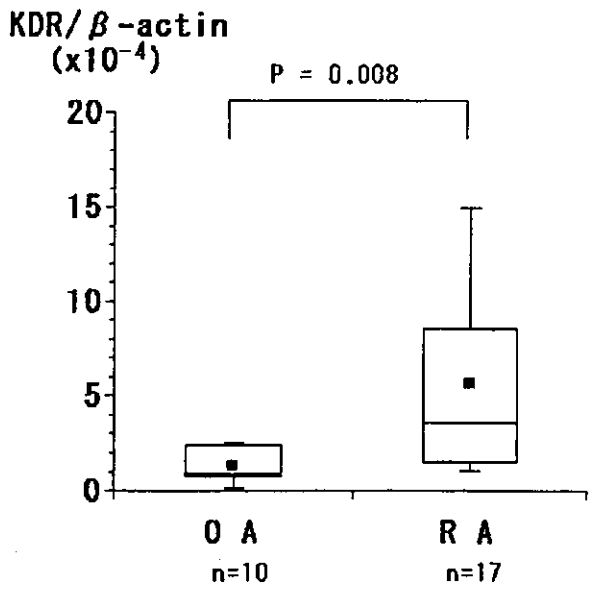

Figure 6. The expression of vascular endothelial growth factor receptor 2 (VEGFR-2)/kinase insert domain receptor (KDR) mRNA in bone marrow–derived CD34+ cells. RNA was extracted from bone marrow CD34+ cells obtained from rheumatoid arthritis (RA) and osteoarthritis (OA) patients and subjected to quantitative reverse transcription–polymerase chain reaction for VEGFR-2/KDR. The copy numbers for VEGFR-2/KDR mRNA were calibrated to those for β-actin. Data are shown as box plots. Horizontal lines constituting the top, middle, and bottom of the boxes show the 75th, 50th, and 25th percentiles, respectively. Lines outside the boxes show the 90th and 10th percentiles. Solid squares inside the boxes show the mean. *P* values were determined by Mann-Whitney U test.

of the VEGF/KDR pathway in bone marrow CD34+ cells. To explore this possibility, our final experiments examined the expression of KDR mRNA in bone marrow CD34+ cells as measured by quantitative reverse transcription–PCR. As can be seen in Figure 6, the expression of VEGFR-2/KDR mRNA in bone marrow CD34+ cells from 17 patients with RA was significantly higher than that in bone marrow CD34+ cells from 10 patients with OA. The results therefore suggest that up-regulation of KDR mRNA in bone marrow CD34+ cells in RA might result in their enhanced capacity to generate endothelial cells.

DISCUSSION

A number of studies have indicated that neovascularization is crucial to the synovial hyperplasia of RA (7,8). Postnatal neovascularization has been attributed to so-called angiogenesis, a process characterized by the sprouting of new capillaries from preexisting blood vessels (10). Thus, it has been shown that the expression of angiogenic factors, such as VEGF and basic fibroblast growth factor in synovial lining cells and stromal cells, is increased in RA synovium and plays a pivotal role in the angiogenesis (19,20,23). It is noteworthy that recent studies have demonstrated that endothelial progenitor cells of bone marrow origin play a significant role in the de novo formation of capillaries without preexisting blood vessels, so-called vasculogenesis (11–14). Moreover, bone marrow–derived endothelial precursor cells have been shown to home to neovascularized hind limb ischemic sites in animal models (11). Results of the current studies have shown that the generation of vWF+ endothelial cells from bone marrow CD34+ cells is up-regulated in RA. The data therefore suggest that mobilization of endothelial cells from bone marrow might also be enhanced and be involved in neovascularization of the RA synovium. It is thus likely that bone marrow–derived endothelial precursor cells might be homing to the synovium, where angiogenesis is enhanced (7,8).

It has been shown that bone marrow–derived endothelial progenitor cells make a significant contribution to angiogenic growth factor–induced neovascularization that may account for up to 26% of all endothelial cells (26,27). It is therefore likely that the enhanced capacity of bone marrow CD34+ cells to generate vWF+ cells might also play a critical role in the synovial neovascularization in RA. In fact, we found that the degree of synovial vascularization as well as the microvessel densities in RA synovium were much higher than those in OA synovium, findings that are consistent with those of a previous study (28). More important, the generation of vWF+ cells from bone marrow–derived CD34+ cells was significantly correlated with the degree of synovial vascularization as well as with the microvessel densities in arthritis patients. The data therefore raise the possibility that the mobilization of endothelial progenitor cells from bone marrow might also contribute to the enhanced synovial neovascularization in RA, although a direct role for these bone marrow–derived cells in synovial neovascularization remains to be elucidated. Further studies to explore in detail the capacity of endothelial progenitor cells generated from bone marrow CD34+ cells to undergo angiogenesis would be important.

Previous studies have shown that hematopoietic cytokines, such as SCF and GM-CSF, have potent

effects on endothelial cells and facilitate angiogenesis (29). In the current studies, we demonstrated that SCF and GM-CSF also induce the generation of endothelial cells from bone marrow CD34+ cells and, thus, participate in vasculogenesis. The mechanism of the enhanced generation of endothelial cells from RA bone marrow CD34+ cells stimulated with SCF and GM-CSF is still unclear. It is possible that the production of VEGF, presumably by CD14+ cells induced from CD34+ cells, might be enhanced in cultures of RA bone marrow–derived CD34+ cells. However, there were no significant differences in the production of VEGF between RA patients and control subjects. Moreover, the generation of vWF+ cells was not significantly correlated with the production of VEGF. It is therefore unlikely that the enhanced generation of vWF+ cells from RA bone marrow CD34+ cells might result from the up-regulation of VEGF production.

TNFα plays a crucial role in regulating not only inflammation, but also neovascularization in RA synovium (21–24). Anti-TNFα treatment in RA patients has been found to inhibit vascularity in the synovium (30,31). The results of the current studies revealed that significant amounts of TNFα were produced in cultures of bone marrow CD34+ cells. However, there were no significant differences in the production of TNFα by bone marrow CD34+ cells from RA patients and control subjects, nor was the generation of vWF+ cells significantly correlated with the production of TNFα. Therefore, the enhanced generation of vWF+ cells might not be accounted for by the increased production of TNFα, although it is still possible that up-regulation of the production of angiogenic factors other than VEGF and TNFα might be involved in the enhanced generation of vWF+ cells from RA bone marrow CD34+ cells. Alternatively, it is also possible that the reactivity of RA bone marrow CD34+ cells to various cytokines might be different from that of control bone marrow CD34+ cells. In this regard, previous studies have shown that RA bone marrow CD34+ cells have abnormal capacities to respond to TNFα and differentiate into fibroblast-like cells producing MMP-1 (5).

Neovascularization of the synovium is not unique to RA. It has also been observed in OA synovium and has been shown to play an important role in the development of new cartilage and mineralization (25,32,33). Of note, recent studies have revealed that levels of expression of the angiogenic factors VEGF and platelet-derived endothelial cell growth factor are increased in RA as well as in OA, relative to normal subjects, whereas the presence of an activated synovial vasculature was high only in RA (25). Moreover, the vascular activation by VEGF/KDR was significantly lower in OA than in RA patients, although the activation of the HIFα pathway was comparable in OA and RA patients (25). These observations suggest the presence of intrinsic abnormalities in synovial endothelial cells in RA patients. Of note, in the present study, RA bone marrow CD34+ cells displayed a higher capacity to generate vWF+ endothelial cells than did OA bone marrow CD34+ cells. Moreover, the expression of VEGFR-2/KDR mRNA in RA bone marrow CD34+ cells was significantly higher than that in OA bone marrow CD34+ cells. It is therefore likely that the differences in VEGF/KDR vascular activation at the level of bone marrow CD34+ cells between RA and OA patients might result in differences in their capacity to generate vWF+ cells, since signaling through the KDR plays a crucial role in the generation of endothelial cells (17,19). Further studies to delineate the precise sequelae of the up-regulation of KDR mRNA expression would be helpful for a complete understanding not only of the differences in synovial neovascularization in RA and OA, but also of the pathogenesis of RA.

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新規全気孔連通型HA多孔体 NEOBONE®を用いた骨欠損に対する治療

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Key words：hydroxyapatite ceramics, interconnected pore, bone graft

はじめに

整形外科分野において骨腫瘍，外傷など種々の病変による骨欠損に対し，古くから骨盤や腓骨などからの自家骨移植が優れた方法として一般的に用いられてきたが，採骨に伴う手術侵襲，採骨部の創や疼痛，採骨部の術後骨折などの合併症があるうえに，絶対的な供給量に問題がある。近年自家骨に代わり，アルミナ，ジルコニア，バイオグラス，ハイドロキシアパタイト(HA)などさまざまな素材が人工骨として使用されてきている。そのなかでも，HAは哺乳類の骨，歯の無機質成分に類似しており，その生体親和性，骨伝導能をみても人工骨として非常に適していると考えられる。それらのことより，1980年代より整形外科，歯科口腔外科領域において骨補填剤としてHA多孔体が使用されている。HA多孔体は当初その気孔内に新生骨が侵入し，母床骨と完全に同化することが期待されたが，HA多孔体が新生骨に完全に置換されるという報告はなく，むしろさまざまな臨床症例の解析から気孔内への新生骨侵入は数mm程度であることが明らかになってきた[1]。このような新生骨が侵入しない気孔は強度面で非常に不利であり，HA多孔体移植後骨折を合併した症例の報告も見受けられる[2]。これらは気孔と気孔を結ぶ連通構造に問題があり，深部への組織侵入，骨新生を阻害していたためと考えられる。

このような問題点を解決するためわれわれは物質・材料研究機構，東芝セラミックス株式会社，株式会社MMTとの共同開発で新しい製造法“起泡ゲル化技術”を取り入れ，新規HA多孔体を開発した。このHA多孔体は均一な気孔構造と適度な気孔間連通構造を特徴としている[3),4)]。本

Surgical treatment of bone defects with novel interconnected porous hydroxyapatite ceramics

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稿では，この新規HA多孔体(NEOBONE®)の特徴を述べ，臨床使用での良好な成績を紹介する。

新規HA多孔体(NEOBONE®)の開発

今回われわれが新規開発したNEOBONE®（図1ⓐ)は，気孔率75%，平均気孔径150ミクロン，初期強度12MPaであり，製法上の特徴は起泡ゲル化技術を取り入れたことである[3]。起泡ゲル化技術とは，①HAスラリーに起泡剤を加えて一定の条件で撹拌することにより泡沫状のスラリーを作製し，②ポリエチレンイミンと水溶性の多官能基エポキシ化合物を組み合わせた架橋重合反応により，短時間で泡沫状のスラリーを全域にわたって同時に固める手法である。

図1 ⓑにNEOBONE®の電子顕微鏡写真を示す。NEOBONE®の気孔は，ほぼ球形で比較的均一なサイズの気孔が規則正しく配列し，ほぼ全気孔が気孔間連通孔で連絡した構造を有していた。気孔隔壁は10～20ミクロンを有し，その表面はHA粒子が密に秩序よく配列されていた。水銀圧入ポロシメトリーによる気孔の連通状態の検討では，連通孔径分布は10～80ミクロン(平均40ミクロン)であり，NEOBONE®の気孔の約90%が細胞や組織が十分通過できる大きさの連通孔でつながっていた。力学的強度は初期圧縮強度で10MPa以上で，臨床応用されている同等の気孔率を有するHA多孔体と比較して優れた数値を示した。この強度は電子顕微鏡所見か

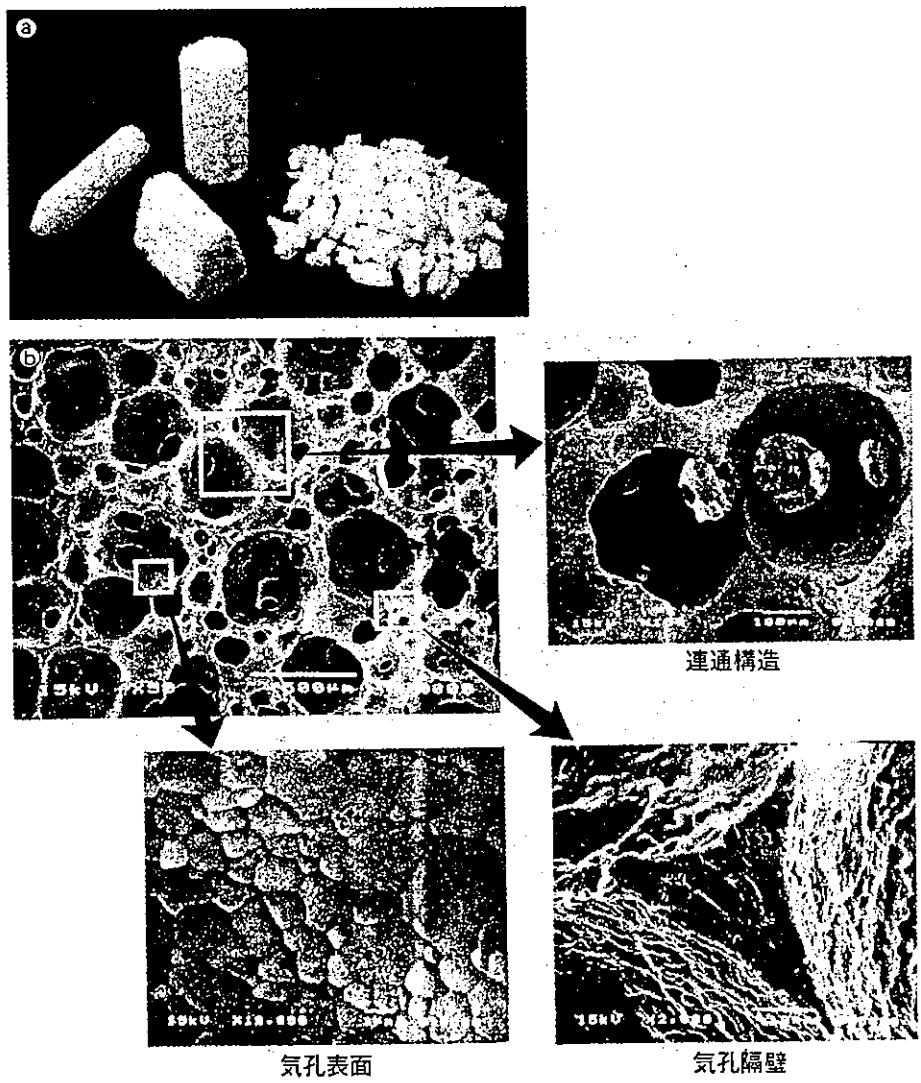


図1 NEOBONE®とその構造
ⓐ：NEOBONE®のマクロ像。
ⓑ：NEOBONE®の電子顕微鏡写真。

らもわかるように気孔が均一でほぼ球形であり，骨格部分のHA粒子間の結合が強固であることによって得られたと考えられる。

直径6mmのNEOBONE®円柱ブロックを白色家兎大腿骨に移植し骨伝導能を検討した(図2ⓐ)。大腿骨に移植した多孔体内への組織の侵入を，三次元的に捉えるため，表層からの距離によりゾーン分けを行い，母床骨に接している円柱ブロックの最外側部1mmをゾーンS，母床骨より最も離れている最深部1mmをゾーンDとした(図2 ⓑ)。移植後2週では，母床骨に接しているゾーンSで三日月状の無数の新生骨が観察できた。ゾーンDでは，細胞浸潤がほとんどなく，血塊が大部分を占めていた。移植後3週では，ゾーンSでは新生骨髄も確認することができたが，ゾーンDでは一部に新生骨を認めるものの，血管新生を伴う肉芽組織が主であった。

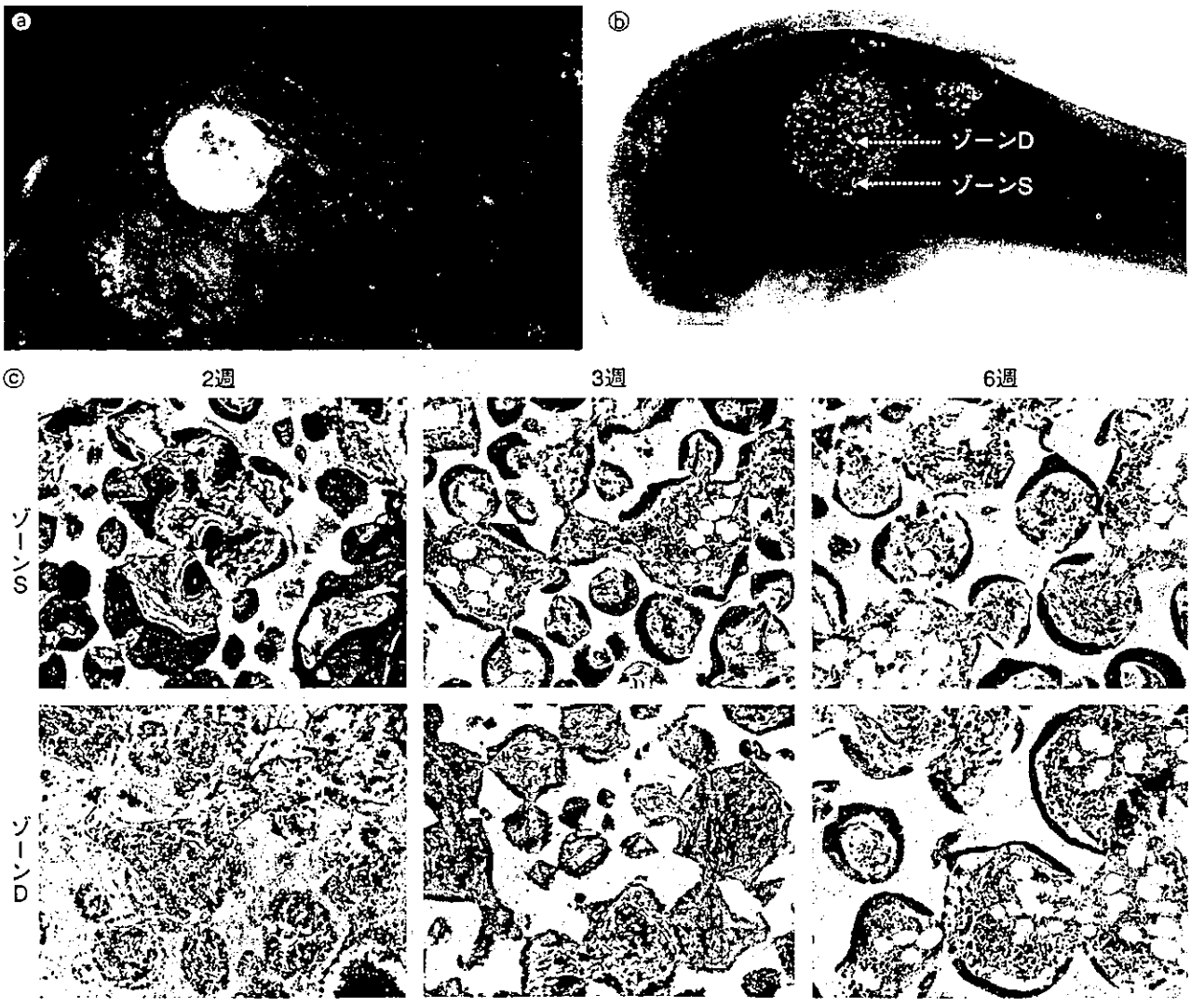
移植後6週では，ゾーンSからゾーンDまでのすべての気孔に豊富な新生骨髄を伴う新生骨を確認することができ，非常に優れた骨伝導能を示した(図2 ⓒ)。この骨新生に伴い圧縮強度は移植後9週で初期強度の3倍に達していた。

図2　NEOBONE®の骨伝導能

ⓐ：白色家兎大腿骨埋入実験(大腿骨顆部に直径6mmのドリルホールを開け，高さ15mmのNEOBONE®円柱を移植)。

ⓑ：ゾーン分け。

ⓒ：ゾーン別気孔内の骨形成(ヘマトキシリン・エオジン染色，×100)。



NEOBONE®の臨床使用

大阪大学医学部附属病院およびその関連施設において，新GCPに則った手続きを踏み，65症例66部位に対してNEOBONE®の臨床治験を行っている。

対象は，骨折および骨腫瘍の骨欠損，骨欠損を伴った関節リウマチ，変形性関節症である。術後経過をX線所見で観察すると，移植後2カ月で半数以上の症例にNEOBONE®内部，あるいはNEOBONE®/母床骨間に骨硬化像が確認され，移植後6カ月以内に6割以上の症例で明らかなX線透過性の減少がみられた[5]。

これらの結果は，市販HA多孔体の過去の使用報告[6]と比べて明らかに早く，臨床使用においてもNEOBONE®は優れた骨伝導能を有することが示された。

症例提示

【症例1】(図3)

28歳，男性。大腿骨近位部非骨化性線維腫。

大腿骨頭から頚部に広がる病巣を掻爬後，骨欠損部にNEOBONE®顆粒および直方体を用いて補填した。移植後2カ月で軽度の骨硬化像が，6カ月で強い骨硬化像がみられる。移植後24カ月では強い骨硬化像が保たれ，頚部内側骨皮質の連続性も確認できた。

【症例2】(図4)

36歳，男性。脛骨骨幹部開放骨折後遷延治癒。

脛骨遠位骨幹部の開放性骨折後約3カ月にわたり創外固定にて固定するが，まったく治癒傾向が認められなかったため，骨折部にNEOBONE®顆粒と自家骨海綿骨チップを移植した。術後2年のX線像では完全な骨癒合が得られている。

図3 【症例1】

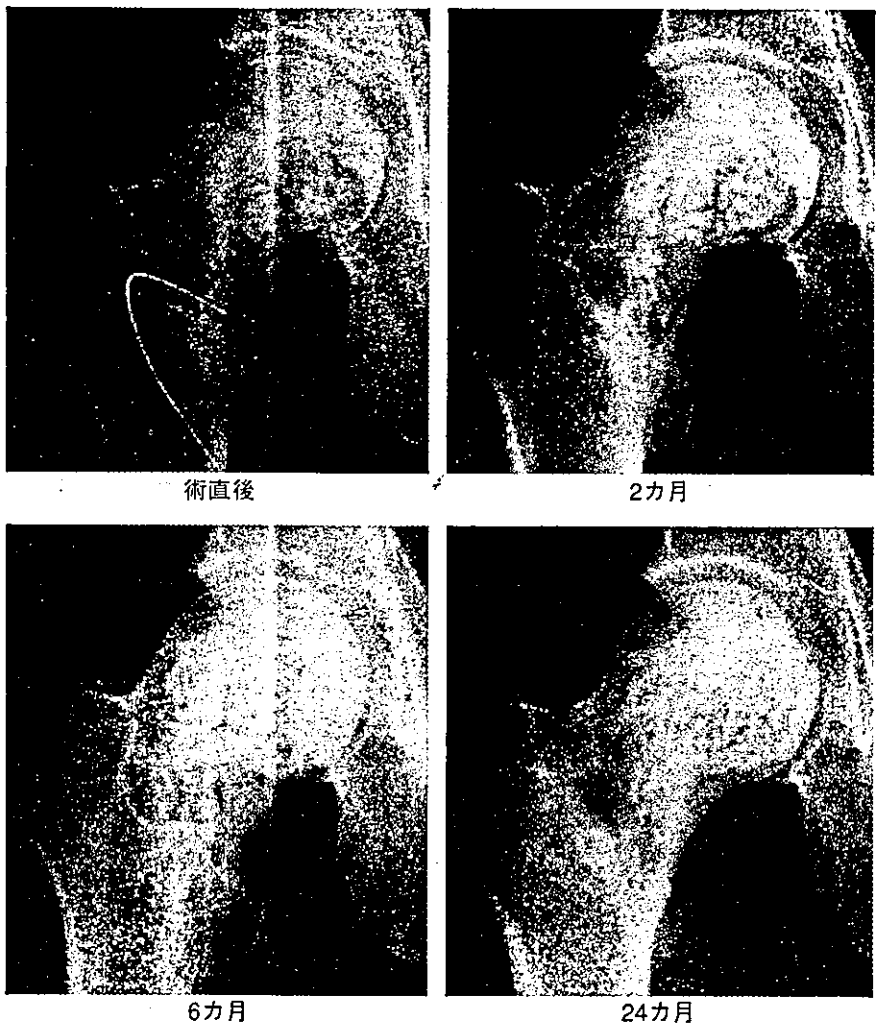
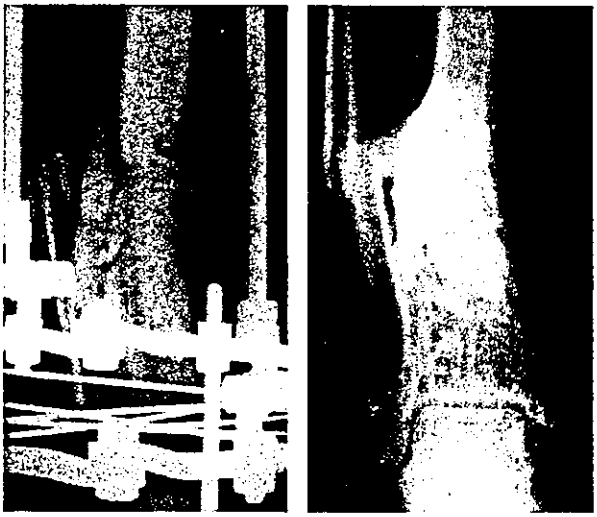


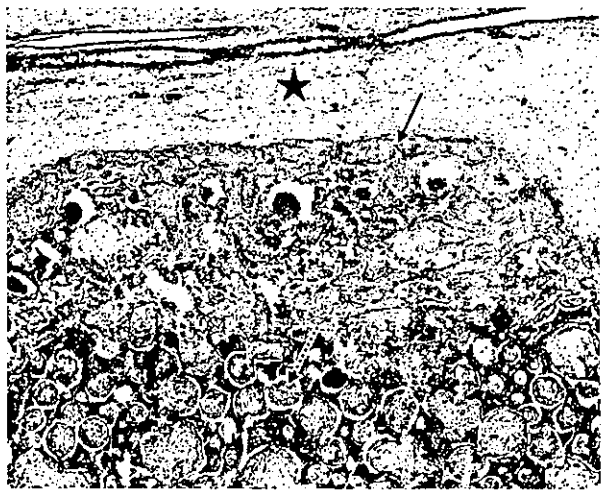
図4 【症例2】



術直後　　24カ月

図5 血管導入によるBMP含浸NEOBONE®気孔内の骨形成

NEOBONE®内に導入された血管(星印)に沿うように，旺盛な骨形成が起こり(実線矢印)ほぼすべての気孔にも新生骨が観察された(破線矢印)。



NEOBONE®の臨床応用に向けて

● 基礎的研究

骨形成にとって血管新生は非常に重要な因子であり[7]，血管内皮増殖因子(vascular endothelial growth factor；VEGF)などを使用した骨形成の増強などの研究が近年なされている[8]。

以前，本誌で触れたように，NEOBONE®に血管を導入することにより，NEOBONE®内の気孔に微細血管のネットワークを形成しえた[9]。この微細血管が骨形成に対してどのような効果を示すかを検討するために，NEOBONE®にBMP(bone morphogenetic protein)を含浸させ，気孔内の異所性骨形成で評価した。**図5**に示すように，血管を導入したBMP含浸NEOBONE®では，血管に隣接する部位を中心にほぼすべての気孔に骨形成が観察された。その新生骨形成量は血管導入をしなかった場合に比べ10倍以上であり，NEOBONE®への血管導入は骨形成に非常に有利に作用することが示された[10]。

● 臨床応用

巨大骨欠損部を補填する方法の1つとして，悪性骨腫瘍に対して術中体外放射線照射自家骨移植法にNEOBONE®を併用する試みを行っているので紹介する。

現在，悪性骨・軟部腫瘍の切除後の骨欠損に対しては，金属製人工関節を用いた再建術が主流となっている。しかしながら利用できる部位が大腿骨，脛骨近位，上腕骨に限られており，また膝蓋腱や腱板などの筋腱付着部の再建が困難であるという問題点がある。これらの問題を解決する方法として，血管柄付自家骨移植，同種骨移植，骨延長術などさまざまな再建法があるが[11), 12)]，関節，靱帯や腱付着部を温存しやすく機能温存に優れる術中体外放射線照射自家骨移植法をわれわれは積極的に試みている[13), 14)]。

術中体外放射線照射自家骨移植法(intraoperative extracorporeal autogenous irradiated bone grafting；IO RBG)とは，腫瘍広範切除中に腫瘍と一塊にして摘出した自家骨を体外にて放射線一括照射後，再度体内に戻す方法で，再生自家骨移植の一方法である。

組織学的検討では，摘出自家骨内の腫瘍組織や骨髄組織は長期間壊死骨の状態にあり，移植骨の再生の妨げになっていると考えられるため，IO RBGに血管導入したNEOBONE®を組み合わせることを最近試みている。

19歳，女性の右脛骨近位部の骨肉腫に対して(**図6** ⓐ)，通常の腫瘍用人工関節では膝伸展機

図6　NEOBONE®の臨床応用

19歳，女性。脛骨近位骨肉腫。

ⓐ：MRI T1強調像。

ⓑ：三次元切削器(Roland DG, MDX-20)にて切削したカスタムデザインNEOBONE®。

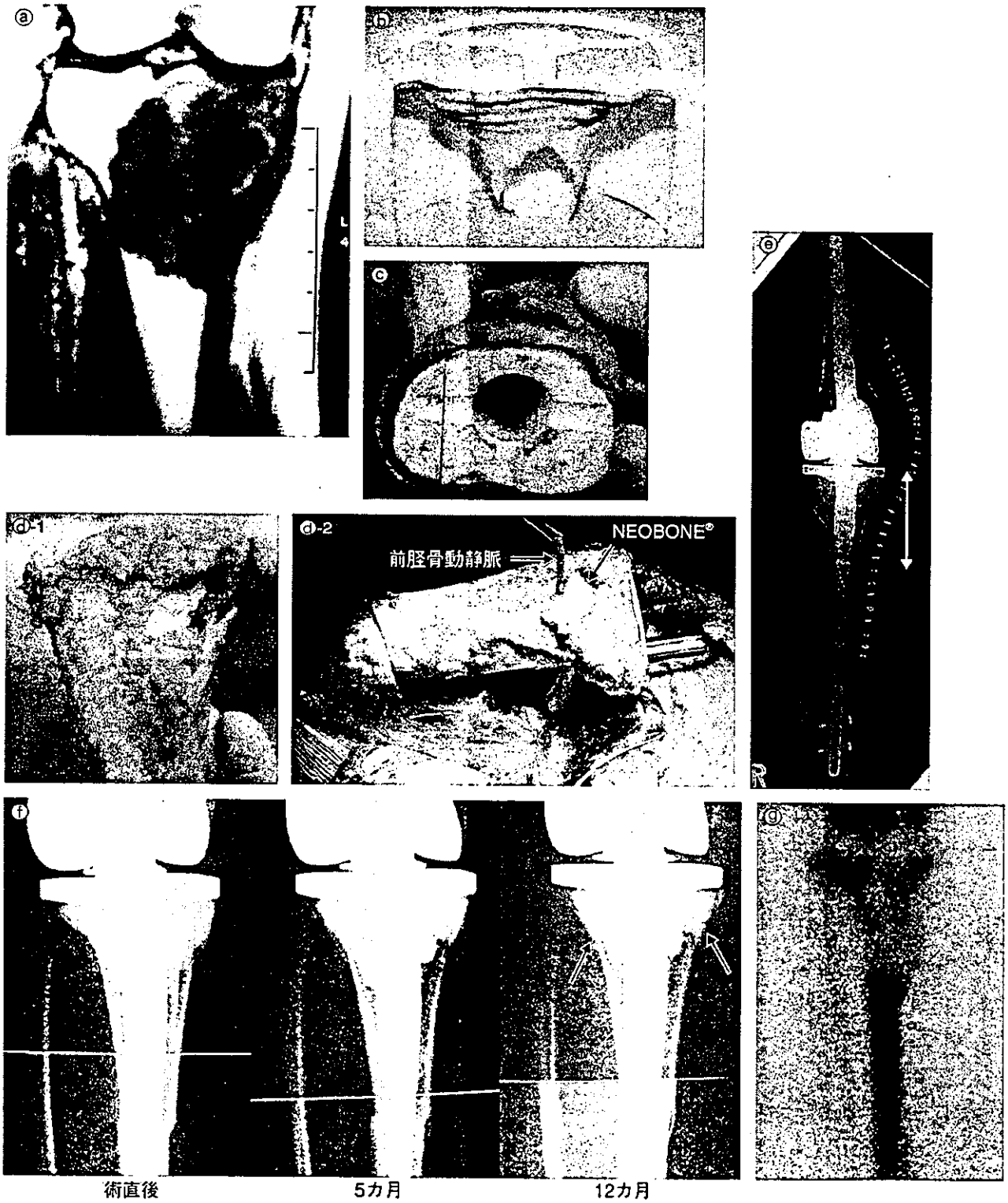
ⓒ：骨欠損部補填後。

ⓓ：NEOBONE®内に導入された前脛骨動静脈束。

ⓔ：術後X線像。矢印は放射線処理骨の範囲。

ⓕ：術後経過(術直後，術後5カ月，術後12カ月)に伴い血管を通しているNEOBONE®内の骨孔(実線矢印)に骨硬化像が出現し，放射線照射骨の内側，外側の骨皮質(破線矢印)のリモデリングが観察できた。

ⓖ：骨シンチグラム(術後12カ月)。



構の温存が困難であるため，LINK社製ローテーティングヒンジ型ロングステムTKAにIO RBGを併用して再建を行った。健側CT画像により髄腔の三次元CADモデルを作製しこのデータを元に，ブロック状のNEOBONE®を三次元切削器(Roland DG, MDX-20)にて切削することにより，患部の髄腔に合致するready-to-useのカスタムデザインNEOBONE®を作製した(図6 ⓑ)。これを放射線照射骨の骨欠損部(腫瘍，骨髄掻爬部)に補填し(図6 ⓒ)，後方から前方に貫くように前脛骨動静脈束をNEOBONE®内に挿入している(図6 ⓓ)。

術後，X線にて経過を追っていくと，放射線照射骨の内側，外側の骨皮質のリモデリングが起こり，血管を通したNEOBONE®内の骨孔に骨硬化像が出現し，術後1年の骨シンチグラフィでも，移植したNEOBONE®の部位に一致して強いup-takeを認めた(図6 ⓔ～ⓖ)。これまでの報告で放射線照射骨への，骨組織，血管の侵入は10カ月で正常骨組織との接合部から約1cm程度とされており[11]，これに比べると本症例は血管導入したNEOBONE®を併用することにより，これまでにない骨再生能力を獲得したと考えられる。

まとめ

今回，高度骨欠損に対する骨補填材として，“起泡ゲル化技術”という三次元構造を制御する新たな手法を用いた新世代HA多孔体NEOBONE®を紹介した。NEOBONE®はすでに市販され臨床応用されている従来のHA多孔体と比べ，①全気孔が細胞や組織が無理なく通過しうる大口径の気孔間連通孔で連結している，②高い気孔率であるが，気孔隔壁の構造は緻密体HAと同様の構造をもつため，臨床使用に十分耐える強度を有する，などという点で非常に優れた特性をもつ。

動物実験でも，早期より深部気孔内への骨伝導能が証明されたが，臨床使用においてもX線評価にて同様の優れた骨伝導能を示した。組織工学的アプローチとして，血管をNEOBONE®に導入することにより，骨再生を促進した基礎実験・臨床症例を示したが，今後その連通気孔構造をいかしてBMPやVEGFなどの分化・増殖因子や骨形成性細胞を気孔内に導入した生体活性型人工骨が臨床応用されれば，これまで困難であった難治性骨関節疾患の治療に貢献できることを期待する。

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II. 骨代謝調節系

骨芽細胞の機能・骨形成メカニズム　骨形成促進因子

骨形成因子(BMP)

Bone morphogenetic proteins

樋口周久　　吉川秀樹

Key words：骨形成因子，間葉系細胞，骨芽細胞

はじめに

1965年，Urist[1]は塩酸脱灰骨による異所性骨誘導現象を発見し，骨基質中には軟骨・骨形成の誘導能を有する因子(骨形成因子(bone morphogenetic protein: BMP))が存在することを証明した．1988年，Wozneyら[2]によって2種類のBMPのcDNAがクローニングされ，これを契機に約20種類のBMPファミリーの遺伝子が同定されている．当初，*in vivo*における骨形成誘導能を有する蛋白として見いだされたBMPは，一連の研究により骨形成以外に，初期発生過程や各種器官形成過程，あるいは細胞死など多彩な機能を有することが示されている[3]．現在，骨折治癒や骨癒合促進，骨欠損部の骨再生などに対してヒト組換え型BMP-2やBMP-7による基礎研究や臨床研究が盛んに行われており，またBMPの遺伝子導入による骨・軟骨再生研究が進められている．一方，心筋再生など広い領域での組織，器官再生への臨床応用も期待されている．

本稿では，多機能を有するBMPの作用機序および機能を，特に間葉系細胞，軟骨細胞，骨芽細胞分化に焦点を絞り概説する．

1. BMPの構造とシグナル制御

BMPは分子量約3万，約100-120アミノ酸をもつサブユニットがジスルフィド結合によって2量体を形成している分泌蛋白質である．TGF-βとアミノ酸配列で30-40%の相同性をもつTGF-βスーパーファミリーに属し，BMPサブファミリーを形成している(図1)．ほとんどのBMPは異所性に骨・軟骨を形成する作用があるが，骨・軟骨以外の細胞増殖や分化，発生初期における形態形成など多種の作用をもつことが明らかになっている[3]．BMPは細胞内で約300-400アミノ酸数をもつ前駆体として合成され，その後2量体形成，N末端の一部が切断除去され，成熟蛋白として分泌され作用する．成熟蛋白は標的となる細胞表面に存在する2種類に分類されるセリンスレオニンキナーゼ型レセプターに結合し，細胞内へシグナルを伝達する．BMPのレセプターへの結合はI型レセプターのリン酸化を引き起こし，細胞内のシグナル伝達分子である特異型SmadのSmad1/5/8を活性化する．活性化された特異型Smadは共有型SmadであるSmad4と複合体を形成して核内に移行し，軟骨形成や骨形成に必須であるRunx2/Cbfa1など他の転写因子と協調してBMP応答遺伝子の転写を調節している．

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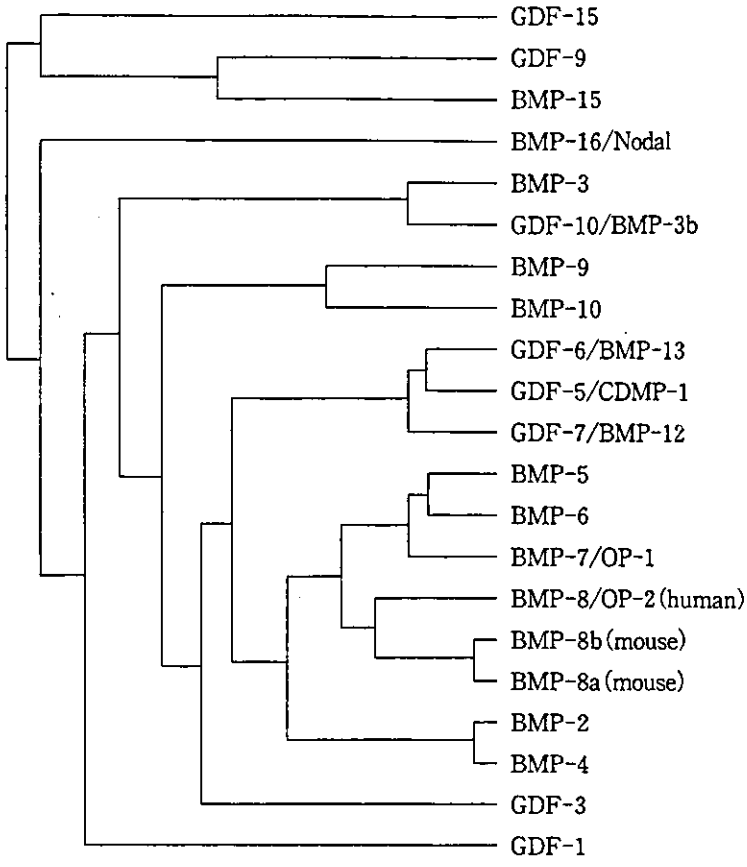

図1 BMPサブファミリーの系統樹(文献[3]より改変)
BMP: bone morphogenetic protein, GDF: growth differentiation factor, OP: osteogenic protein, CDMP: cartilage-derived morphogenetic protein

このシグナル伝達系は細胞内外で調節されている．細胞外には，Noggin，Chordin，Follistatin，DANファミリー蛋白などのBMPに結合するアンタゴニストが存在している[4]．レセプターレベルでは，おとりレセプターとして考えられているBAMBIの発現によりBMPシグナルを抑制することが報告されている[5]．また，抑制型SmadであるSmad6/7がI型レセプターに結合し，特異型Smadの活性化を抑制している．特に抑制型SmadはBMPによりその発現が誘導されることから，BMPは自らのシグナルで自己のシグナルを調節していると考えられる[6]．細胞質内では，特異型Smadを分解するSmurf-1が存在し，特異型Smadの量を調節してBMPシグナルを負に制御している[7]．また，抑制型Smadが特異的Smadと共有型Smadの複合体形成を阻害しているという報告もある．一方，核内ではTobやc-skiなどの転写因子やp300/cAMP-response element binding protein(CREB) binding protein(CBP)などの共役因子がSmadと結合してBMP応答遺伝子の転写を制御している．

BMPシグナル伝達系は，他のサイトカインシグナル伝達系や細胞内シグナル伝達系とクロストークしている．EGFやFGFのシグナルを司るmitogen activated protein(MAP)キナーゼシグナル伝達系[8]や細胞運動などのシグナルを司るRho-Rhoキナーゼ伝達系などのシグナル伝達系[9]がBMPシグナルを修飾している．最近の報告では，β-cateninを細胞内伝達分子としてもつWntシグナル伝達系とBMPシグナルとのクロストークも指摘されており[10]，BMPシグナルの作用とその機序は複雑なメカニズムによって制御されていると推測されている．