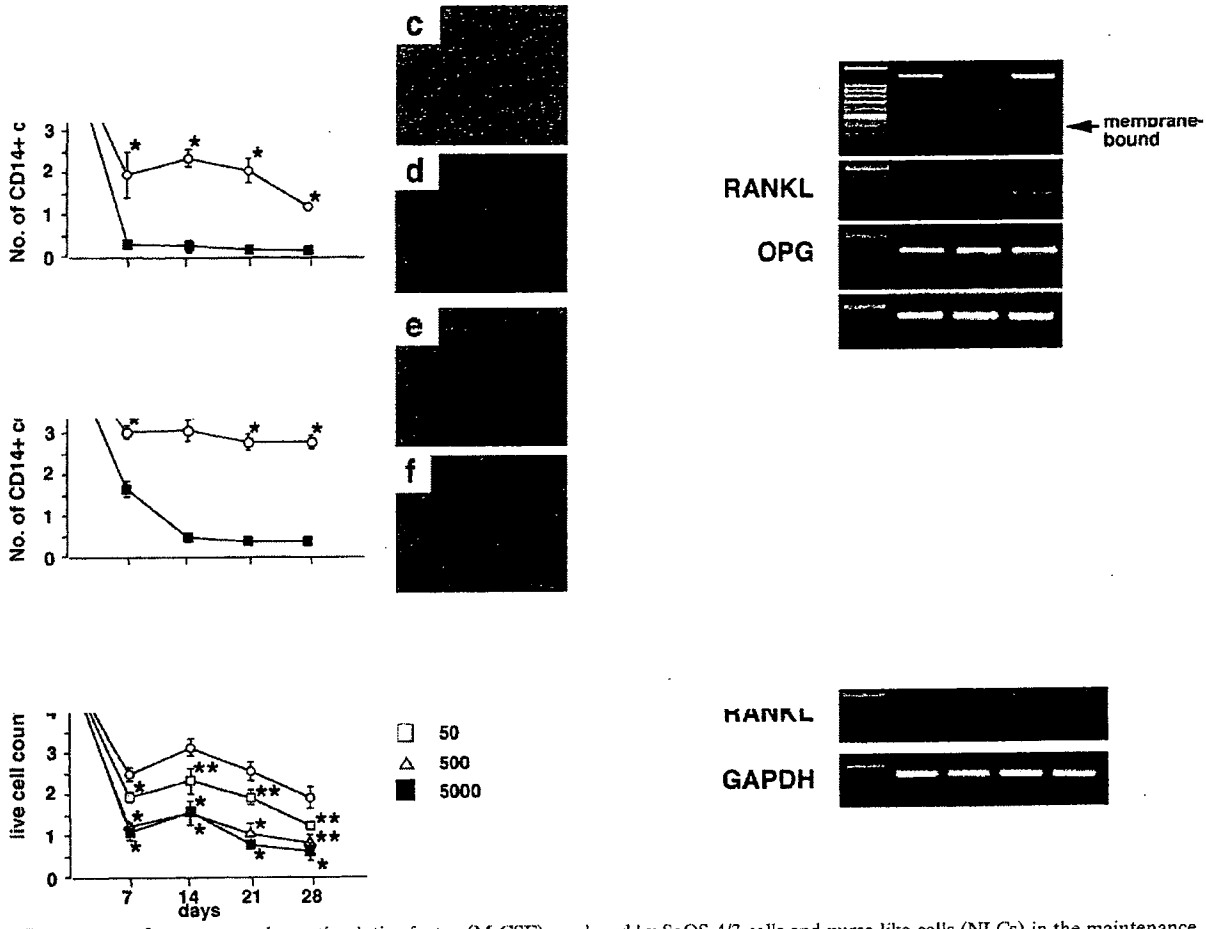

Figure 3. Importance of monocyte colony-stimulating factor (M-CSF) produced by SaOS-4/3 cells and nurse-like cells (NLCs) in the maintenance of CD14+ monocytes. **A,** Survival of CD14+ monocytes in cultures with or without NLCs or SaOS-4/3 cells. **a,** CD14+ monocytes were cultured with and without NLCs for 28 days, and the number of CD14+ monocytes recovered each week was counted. **b,** CD14+ monocytes were cultured with SaOS-4/3 cells in the presence and absence of parathyroid hormone (PTH; $10^{-8}M$), and the number of CD14+ monocytes recovered each week was counted. * = $P < 0.01$. **c** and **d,** Monocyte cultures with and without NLCs, respectively. **e** and **f,** Monocyte cultures with SaOS-4/3 cells in the presence and absence of PTH, respectively. (Original magnification × 40.) **B,** Total RNA was extracted from NLCs (lane 1) and from SaOS-4/3 cells treated for 3 days with (lane 2) or without (lane 3) PTH ($10^{-8}M$), and the expression of mRNA for M-CSF (secreted and membrane-bound forms), RANKL, osteoprotegerin (OPG), and GAPDH was analyzed by reverse transcription–polymerase chain reaction (RT-PCR). **C,** CD14+ monocytes were cocultured with NLCs in the presence of increasing concentrations of neutralizing antibodies (Ab) against human M-CSF, and the number of CD14+ monocytes recovered each week was counted. ★ = $P < 0.01$; ★★ = $P < 0.05$ versus cultures without antibodies. **D,** NLCs were treated with vehicle (control) (lane 1), PTH ($10^{-8}M$) (lane 2), prostaglandin E_2 ($10^{-6}M$) (lane 3), and 1α,25-dihydroxyvitamin D_3 ($10^{-8}M$) (lane 4). After 23 days, total RNA was extracted from NLCs, and the expression of mRNA for RANKL and GAPDH was analyzed by RT-PCR.

Osteoclast formation from CD14+ and NCD14+ monocytes in coculture with SaOS-4/3 cells. When NCD14+ monocytes were cocultured for 7 days with SaOS-4/3 cells, TRAP+ and VNR+ osteoclasts formed in the presence of PTH (Figure 2A). This osteoclast formation was completely inhibited by the addition of OPG. The mRNA for CTR, one of the most reliable signals for osteoclast differentiation, was detected in the coculture treated with PTH, but not in the coculture treated with PTH together with OPG (Figure 2B). Thus, NCD14+ monocytes differentiated into osteoclasts through their interaction with specialized stromal cells

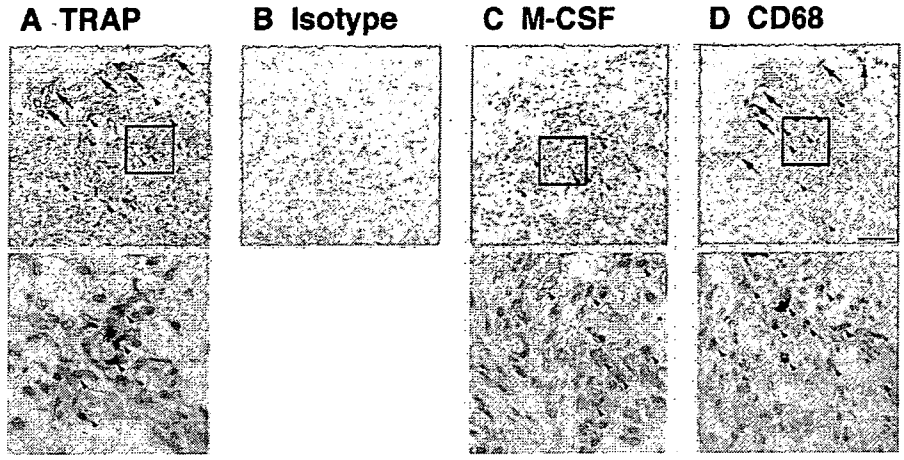

Figure 4. Localization of cells positive for tartrate-resistant acid phosphatase (TRAP), macrophage colony-stimulating factor (M-CSF), and CD68 in areas adjacent to the interface between bone and the progressive expansion of synovium in patients with rheumatoid arthritis. **A,** Some specimens stained positive for TRAP. **Arrows** indicate TRAP+ multinucleated cells; **arrowheads** indicate TRAP+ mononuclear cells. The other specimens were immunohistochemically stained with **B,** isotype control, **C,** antibodies against human M-CSF, and **D,** antibodies against CD68. **Arrowheads** in **C** indicate M-CSF+ synovial cells. **Arrowheads** in **D** indicate CD68+ monocytes; **arrows** in **D** indicate CD68+ multinucleated osteoclasts. Boxed areas in **A, C,** and **D** are shown at higher magnification underneath the respective panels (original magnification × 100). Bar = 100 μm.

that possess the ability to support osteoclast differentiation.

Involvement of M-CSF in the NLC-supported survival of CD14+ monocytes. When CD14+ monocytes were cultured without NLCs, almost all of the CD14+ monocytes disappeared within a week (Figure 3A, parts a and d). Approximately 40% of CD14+ monocytes survived for 4 weeks in coculture with NLCs (Figure 3A, parts a and c). SaOS-4/3 cells could not promote the survival of CD14+ monocytes in the absence of PTH (Figure 3A, parts b and f). However, treatment of the coculture with PTH enhanced the survival of CD14+ monocytes (Figure 3A, parts b and e). Furthermore, multinucleated cells were observed after a 4-week culture in the PTH-treated coculture (Figure 3A, part e). In contrast, multinucleated cells were not detected in cocultures with NLCs (Figure 3A, part c). These results suggested that PTH induced the expression of a cytokine(s) that promoted the survival of CD14+ monocytes and their resultant osteoclast formation.

We previously reported that treatment of SaOS-4/3 cells with PTH stimulated the expression of mRNA for both the membrane-associated and secreted forms of M-CSF (21). RT-PCR analysis showed that NLCs constitutively expressed mRNA for the secreted form of M-CSF and for OPG, but not for RANKL (Figure 3B). In contrast, neither M-CSF mRNA nor RANKL mRNA was expressed in SaOS-4/3 cells in the absence of PTH, but the expression of mRNA for both the membrane-associated and secreted forms of M-CSF as well as for RANKL was induced in PTH-treated SaOS-4/3 cells (Figure 3B). OPG mRNA expression in SaOS-4/3 cells was not affected by PTH.

When cocultures of CD14+ monocytes and NLCs were treated with neutralizing antibodies against human M-CSF, the number of CD14+ monocytes that survived in the coculture decreased in a dose-dependent manner (Figure 3C). Higher concentrations of the antibody (500 and 5,000 ng/ml) almost completely blocked the survival of CD14+ monocytes in the coculture. In contrast to SaOS-4/3 cells, NLCs failed to express RANKL mRNA in response to PTH, PGE_2, or $1\alpha,25(OH)_2D_3$, which are known to stimulate RANKL expression in mouse osteoblasts/bone marrow stromal cells (Figure 3D). Osteoclasts were not formed in the coculture of NLCs and CD14+ monocytes, even in the presence of such RANKL-inducing factors (data not shown).

Histologic examination of sites of bone destruction in RA patients. We examined the distribution of CD68+ monocytes, TRAP+ osteoclasts, and M-CSF+

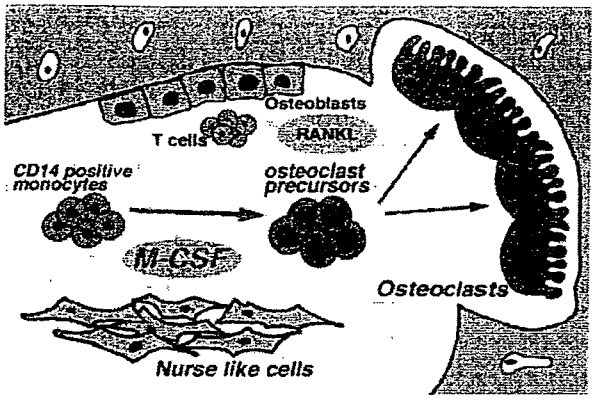

Figure 5. Possible role of nurse-like cells (NLCs) in bone destruction during progressive synovial expansion in rheumatoid arthritis (RA). NLCs show characteristics of fibroblast-like synoviocytes. NLCs support the survival of CD14+ monocytes as osteoclast precursors through the production of macrophage colony-stimulating factor (M-CSF). The resultant osteoclast precursors differentiate into osteoclasts that resorb bone in response to RANKL produced by osteoblasts and activated T cells. Thus, fibroblast-like synoviocytes present in RA synovium play important roles in RA-induced bone destruction by maintaining CD14+ monocytes that do not lose the capacity to differentiate into osteoclasts.

stromal cells in areas of progressive synovial expansion into bone in RA patients (Figure 4). TRAP+ multinucleated cells were detected along the surface of the bone and TRAP+ mononuclear cells were also detected in the pannus surrounding the damaged bone (Figure 4A). M-CSF+ cells were present in fibroblast-like synoviocytes surrounding areas of bone destruction (Figure 4C). CD68+ multinucleated cells and monocytes were detected at the bone–pannus interface and in the pannus surrounding the damaged bone (Figure 4D). Consistent with previous studies (12), we found that some multinucleated osteoclasts were positive for CD68. Thus, CD68+ monocytes and M-CSF+ synoviocytes were colocalized in areas adjacent to destroyed bone in RA patients.

DISCUSSION

In the present study, we demonstrated that NLCs support the survival of monocytes for a long period of time and that the resultant monocytes differentiate into osteoclasts in the presence of M-CSF together with RANKL or TNFα. Moreover, we revealed that M-CSF is one of the key molecules needed for NLCs to maintain monocytes without losing their capacity to differentiate into osteoclasts. CD68+ monocytes and M-CSF+ synoviocytes were colocalized in areas adjacent to destroyed bone in RA patients. These results suggest that fibroblast-like synoviocytes play an important role in bone destruction by continuously providing osteoclast precursors (Figure 5).

We compared the capacity of 2 types of cells, CD14+ and NCD14+ monocytes, to differentiate into osteoclasts. Consistent with the findings of previous studies (24–26), CD14+ monocytes differentiated into mature osteoclasts when stimulated with M-CSF and RANKL/TNFα. Mature osteoclasts were also generated from NCD14+ monocytes by treatment with M-CSF plus either RANKL or TNFα. NCD14+ monocytes expressed mRNA for RANK and TNFRI, suggesting that these cells could respond to RANKL and TNFα. SaOS-4/3 cells supported the differentiation of NCD14+ monocytes into osteoclasts in cocultures treated with PTH. Thus, NCD14+ monocytes interacted normally with stromal cells to receive RANKL and M-CSF signals. These results suggest that NCD14+ monocytes fulfill a phenotype of the osteoclast precursor.

M-CSF is known to induce the proliferation and maturation of monocyte/macrophages. SaOS-4/3 cells maintained CD14+ monocytes in the presence, but not the absence, of PTH. SaOS-4/3 cells produced M-CSF in response to PTH. NLCs constitutively expressed mRNA for the secreted type of M-CSF, and neutralizing antibodies against M-CSF strongly inhibited the NLC-supported survival of CD14+ monocytes. Using the mice transgenic for human TNFα as a model of erosive arthritis, Li et al (27) demonstrated that RANK signaling is not required for an increase in osteoclast precursors but is essential for osteoclast formation. Consistent with their finding, NLCs were able to support osteoclast precursors in the absence of RANKL. The monocyte-supporting activity found in NLCs appears to be a dominant trait of synovial cells. In fact, CD68+ monocytes and M-CSF+ synoviocytes were colocalized adjacent to areas of destroyed bone in RA patients. These results suggest that M-CSF produced by synoviocytes is involved not only in the formation of osteoclasts, but also in the maintenance of osteoclast precursors in the vicinity of RA-induced bone loss (Figure 5).

It is well known that M-CSF is synthesized by mesenchymal cells, including fibroblasts and osteoblasts (28–30). Therefore, we examined whether fibroblastic stromal cells other than SaOS-4/3 cells and NLCs could support the survival of CD14+ monocytes. Human skin fibroblasts derived from healthy volunteer donors and from human osteosarcoma MG63 cells supported the survival of CD14+ monocytes for 4 weeks, as the NLCs

had done (data not shown). These CD14+ monocytes obtained from cocultures with skin fibroblasts or MG63 cells differentiated into TRAP+ multinucleated cells in the presence of RANKL and M-CSF. These results support the conclusion that M-CSF produced by NLCs plays an essential role in the survival of osteoclast precursors for a long culture period.

The formation of osteoclasts from NCD14+ monocytes was observed in cocultures with SaOS-4/3 cells treated with PTH, but not those treated with NLCs. SaOS-4/3 cells expressed RANKL upon treatment with PTH, but NLCs could not express RANKL mRNA in response to any osteotropic factors. NLCs established from different patients all supported the survival of CD14+ monocytes (16), but none of them supported osteoclast formation in the presence of any osteotropic factors (data not shown). In contrast, several groups of researchers have reported that RA synovial fibroblasts support osteoclast formation in cocultures with peripheral blood mononuclear cells (11,26). Takayanagi et al (26) and Shigeyama et al (12) showed that RA synovial fibroblasts express RANKL in the presence of $1\alpha,25(OH)_2D_3$. Gravallese et al (10) reported that the expression of RANKL in synovial fibroblasts is difficult to detect in prolonged cultures. This assertion may support our findings concerning the characteristics of NLCs.

We previously demonstrated that NLCs may play an important role in disease pathogenesis by producing large amounts of cytokines and maintaining infiltrating lymphocytes (16,31). Burger et al (18,32) reported that blood-derived NLCs protect B cells from apoptosis through the production of stromal cell–derived factor 1. They also described the supporting mechanism, pseudoemperipolesis, in detail. Using Transwell culture plates, we confirmed that CD14+ monocytes survived for 4 weeks in the coculture with NLCs, even in the absence of the direct contact with NLCs. However, when those cells were further cultured with RANKL plus M-CSF, only a few osteoclasts were formed (data not shown). When cultured with M-CSF, CD14+ monocytes survived for 4 weeks, but could not differentiate into osteoclasts in the presence of M-CSF plus RANKL (data not shown). These results suggest that another unidentified stromal factor(s) is required to maintain CD14+ monocytes for a long period without the loss of their capacity to differentiate into osteoclasts.

Histologic examinations showed that numerous M-CSF+ stromal cells were detected in the vicinity of damaged bone in RA patients. Consistent with our results, it was previously shown that RA synovial fibroblasts expressed M-CSF (33) and that the concentration of M-CSF in synovial fluid was higher in RA patients than in patients with osteoarthritis (34). Moreover, a number of CD68+ cells and TRAP+ cells were colocalized in areas adjacent to the same sites of destroyed bone in RA patients. These results suggest that synoviocytes appear to participate in the recruitment and maintenance of osteoclast precursors in the vicinity of RA-induced bone loss (Figure 5).

In conclusion, our study suggests that NLCs are involved in RA-induced bone destruction by maintaining osteoclast precursors in areas of progressive synovial expansion. M-CSF constitutively produced by synovial fibroblasts is essential in the maintenance of osteoclast precursors in the vicinity of damaged bone. Therefore, blocking of the M-CSF pathway as well as the RANKL pathway may be a therapeutic target for the prevention of bone destruction caused by synovial expansion in patients with RA.

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REVIEW

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Bone tissue engineering with porous hydroxyapatite ceramics

Abstract The main principle of bone tissue engineering strategy is to use an osteoconductive porous scaffold in combination with osteoinductive molecules or osteogenic cells. The requirements for a scaffold in bone regeneration are: (1) biocompatibility, (2) osteoconductivity, (3) interconnected porous structure, (4) appropriate mechanical strength, and (5) biodegradability. We recently developed a fully interconnected porous hydroxyapatite (IP-CHA) by adopting the "form-gel" technique. IP-CHA has a three-dimensional structure with spherical pores of uniform size that are interconnected by window-like holes; the material also demonstrated adequate compression strength. In animal experiments, IP-CHA showed superior osteoconduction, with the majority of pores filled with newly formed bone. The interconnected porous structure facilitates bone tissue engineering by allowing the introduction of bone cells, osteotropic agents, or vasculature into the pores. In this article, we review the accumulated data on bone tissue engineering using the novel scaffold, focusing especially on new techniques in combination with bone morphogenetic protein (BMP) or mesenchymal stem cells.

Key words Bone · Hydroxyapatite ceramics · Tissue engineering · Mesenchymal cell

Introduction

Bone tissue has a vigorous potential for regeneration in itself, as observed in fracture healing. However, to reconstruct a large bony defect or to treat poor bone-healing conditions, bone grafts are required. Up to the present, autogenous bone grafting has been the gold standard because of its obvious advantages in osteogenic capacity, osteoconduction, mechanical properties, and the lack of adverse immunological response. On the other hand, autogenous bone grafting techniques have some limitations,[1] such as the requirement of additional surgery for harvesting, the availability of grafts of sufficient size and shape, and the risk of donor site morbidity,[2–4] which may include fracture, long-lasting pain, nerve damage, and infection. Therefore, in the field of orthopedics or craniofacial surgery, many kinds of biomaterials have been developed as bone substitutes, such as ceramics, polymers, metals, and organic or non-organic bone substitutes.[5–9]

Among them, hydroxyapatite (HA) ceramics have been used extensively as a substitute in bone grafts,[10–14] because the crystalline phase of natural bone is basically HA. The ceramics are available as dense or porous types and as granules or blocks. Different pore sizes, porosities, and strengths are also available. However, recent clinical evidence has revealed that the pores are not replaced with new bone for a substantial period of time, probably due to the limited interconnection of the pores.[15] We recently developed a fully interconnected porous form of hydroxyapatite ceramic (IP-CHA) to overcome this disadvantage.[16] Another problem of HA is that it is osteoconductive, but not osteoinductive. If the ceramics themselves possessed bone-forming activity, their clinical applications would be greatly expanded. With these facts in mind, we have tried to develop a new bone tissue engineering system using IP-CHA in combination with bone morphogenetic protein (BMP), mesenchymal stem cells from bone marrow, and vascular prefabrication.

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Interconnected porous hydroxyapatite ceramics

We have developed a fully interconnected porous HA ceramic (IP-CHA, NEOBONE): porosity 75%, average pore size 150 μm, and average interpore connections 40 μm, by adopting the "foam-gel" technique (Fig. 1).[16] This approach

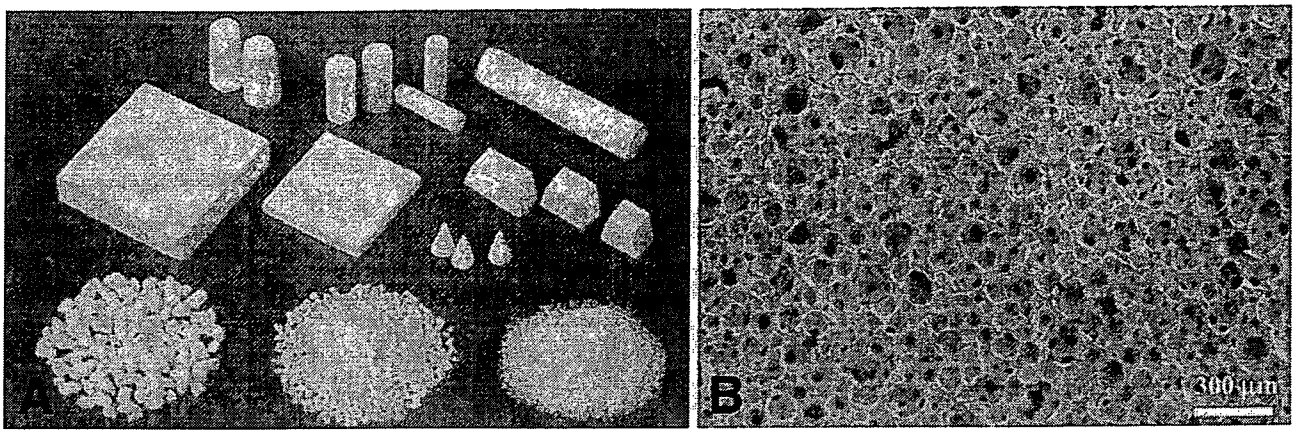

Fig. 1A,B. Macroscopic and microscopic photographs of interconnected porous hydroxyapatite (IP-CHA, NEOBONE). **A** The different types of IP-CHA. The materials were manufactured by Toshiba Ceramics. **B** Scanning electron microscopy (SEM) showed the microstructures of IP-CHA. Spherical pores (100–200 μm in diameter) were divided by thin walls and interconnected by interpores (10–80 μm in diameter)

involves a crosslinking polymerization step that gelatinizes the foam-like CHA slurry in a rapid manner, thus promoting the formation of an interconnected porous structure. The wall surface of IP-CHA is very smooth and HA particles are aligned closely to one another and bound tightly.

In IP-CHA, the majority of the interpore connections ranged from 10–80 μm in diameter, with a maximum peak at about 40 μm, which would theoretically be permissive to cell migration or tissue invasion from pore to pore.[17] The compression strength of IP-CHA is 12 MPa, while the compression strength of cancellous bone is 1–12 MPa.[18]

Osteoconduction in vivo

We histologically analyzed bone ingrowth in cylindrical blocks (6 mm in diameter) of IP-CHA using the rabbit femoral condyle model.[16,19] Within 6 weeks after implantation of IP-CHA, mature bone ingrowth was seen in all the pores throughout the block. In the pores, bone, bone marrow formation through interpore connections with osteoblastic rimming, and vessels were all observed. We also examined the sequential change in the compression strength of the IP-CHA implanted in rabbit femoral condyle. The initial compressive strength of IP-CHA was approximately 10–12 MPa. The implanted IP-CHA steadily increased its compressive strength with time until 9 weeks after implantation, finally reaching a value of about 30 MPa.[16,19]

Bone tissue engineering with bone morphogenetic protein

Bone morphogenetic protein (BMP) is a biologically active molecule capable of inducing new bone formation.[20,21] We have analyzed the efficacy of IP-CHA as a delivery system for recombinant human BMP-2 (rhBMP-2). We combined two biomaterials to construct a carrier/scaffold system for rhBMP-2: IP-CHA and a synthetic biodegradable polymer poly D,L-lactic acid–polyethyleneglycol block co-polymer (PLA-PEG).[22,23] We used a rabbit radius model to evaluate the bone-regenerating activity of rhBMP-2/PLA-PEG/IP-CHA composite. At 8 weeks after implantation, all bone defects in groups treated with 5 μg of rhBMP-2 were completely repaired with sufficient strength (Fig. 2).[24] Using this carrier scaffold system, we reduced the amount of rhBMP-2 necessary for such results to about a tenth of the amount needed in previous studies. Enhancement of bone formation is probably due to the superior osteoconduction ability of IP-CHA and the optimal drug delivery system provided by PLA-PEG. The synthetic biodegradable polymer PLA-PEG/IP-CHA composite is an excellent carrier/scaffold delivery system for rhBMP-2, and strongly encourages the clinical effects of rhBMP-2 in bone tissue regeneration.

Bone tissue engineering with mesenchymal stem cells

We investigated whether IP-CHA can be utilized as a scaffold for cell-based bone tissue engineering using the rat subcutaneous model of Ohgushi et al.[25,26] We used bone-derived mesenchymal stem cells as a source of bone-producing cells. Bone marrow cells were collected from the femur of rats and were cultivated in minimal essential medium supplemented with 15% fetal bovine serum. IP-CHA discs ($R = 5$ mm, $h = 2$ mm) were soaked in the cell suspension overnight and further cultured in the same medium with β-glycerophosphate, ascorbic acid, and dexamethasone for 14 days. The discs were then implanted into the subcutaneous tissue of rats and harvested 2–8 weeks after implantation. All the implants showed bone formation inside the pore areas as evidenced by decalcified histological sections and microcomputed tomography images (Fig. 3).[27,28] The

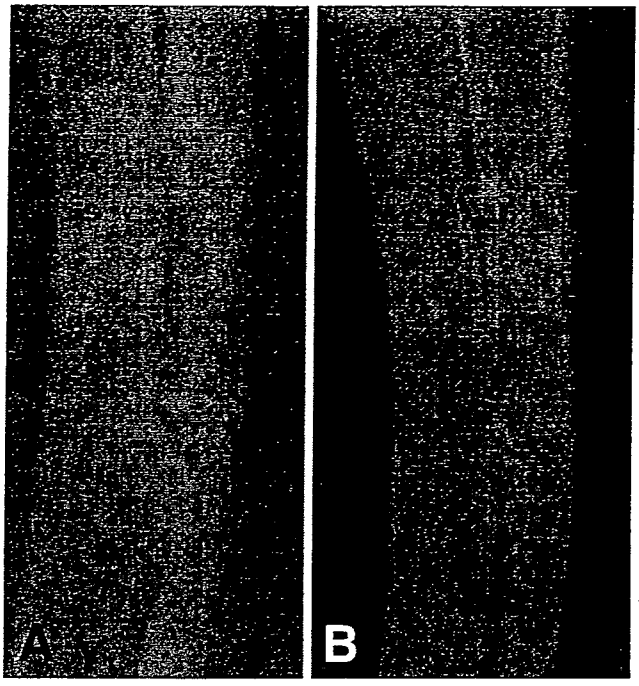

Fig. 2A,B. Soft X-ray photographs of the healing of a bone defect in a rabbit radius. **A** The IP-CHA-alone group at 8 weeks after implantation. *Radiolucent lines* are clearly visible between the IP-CHA and host bone, and the radiodensity of IP-CHA did not increase. **B** The recombinant human bone morphogenetic protein 2 (rhBMP-2, 5μg)/IP-CHA group at 8 weeks after implantation. Bony unions were observed at the junction sites and the radiodensity of IP-CHA increased

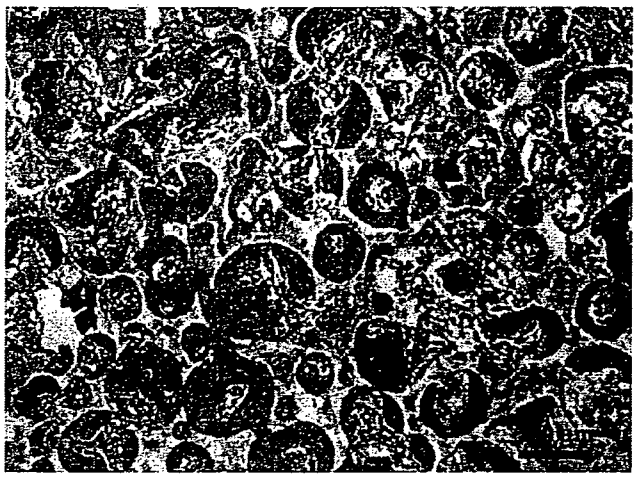

Fig. 3. New bone formation within the IP-CHA with bone marrow-derived mesenchymal stem cells. The IP-CHA was recovered from rat subcutaneous tissue 4 weeks after implantation. Most of the pores were filled with newly formed bone (H.E. staining)

bone volume increased over time. At 8 weeks after implantation, extensive bone volume was detected not only in the surface pore areas but also in the center pore areas of the implants. The combination of IP-CHA and mesenchymal cells could be used as an excellent bone graft substitute because of its mechanical properties and capability of inducing bone formation.

Bone tissue engineering with vascular prefabrication

A vascular network and sufficient blood supply are essential for bone regeneration. Introduction of vasculature into porous implants is another important aspect in regeneration for larger bone defects. We investigated the possibility of integrating IP-CHA with a capillary vessel network via insertion of a vascular pedicle to determine whether this procedure enhanced new bone formation in tissue engineering. This kind of approach to support blood supply generation in porous biomaterials has also been reported by others using subcutaneous tissue and muscle flaps.[29,30] IP-CHA loaded with 10μg of rhBMP-2 was implanted subcutaneously into rat groin with insertion of superficial inferior epigastric vessels. At 3 weeks, IP-CHA/BMP composite with vascular insertion exhibited abundant new bone formation in the pores of the deep portion close to the inserted vessels (Fig. 4).[19,31] This novel system of integrating a vascular network with IP-CHA is considered a useful technique for bone tissue engineering.

Application for cartilage repair

Several investigators have reported on the repair of articular cartilage defects using diverse approaches such as gene-enhanced techniques, direct injection of growth factors, and in vitro cell expansion,[32–34] but the repair remains a major obstacle in tissue engineering. We developed a new technology for articular cartilage repair, consisting of a triple composite of rhBMP-2, PLA-PEG polymer and IP-CHA, to induce regeneration of both subchondral bone and articular cartilage. Full-thickness cartilage defects in the rabbit were filled with the rhBMP-2 (20μg)/PLA-PEG/IP-CHA composite. One week after implantation, a vigorous repair had occurred in the subchondral defect and an agglomeration of mesenchymal cells, migrated from the surrounded bone marrow via the interconnecting pores of the IP-CHA, was detected. At 6 weeks, subchondral defects were completely repaired by subchondral bone and articular cartilage covered the bone (Fig. 5).[35] The regenerated cartilage manifested a hyaline-like appearance, with a columnar organization of chondrocytes and a mature matrix. This novel cell-free technology using the triple composite of rhBMP-2, PLA-PEG, and IP-CHA could mark a new development in the field of articular cartilage repair.

Conclusion and perspectives

The three-dimensional fully interconnected porous structure of IP-CHA encourages bone ingrowth in the material

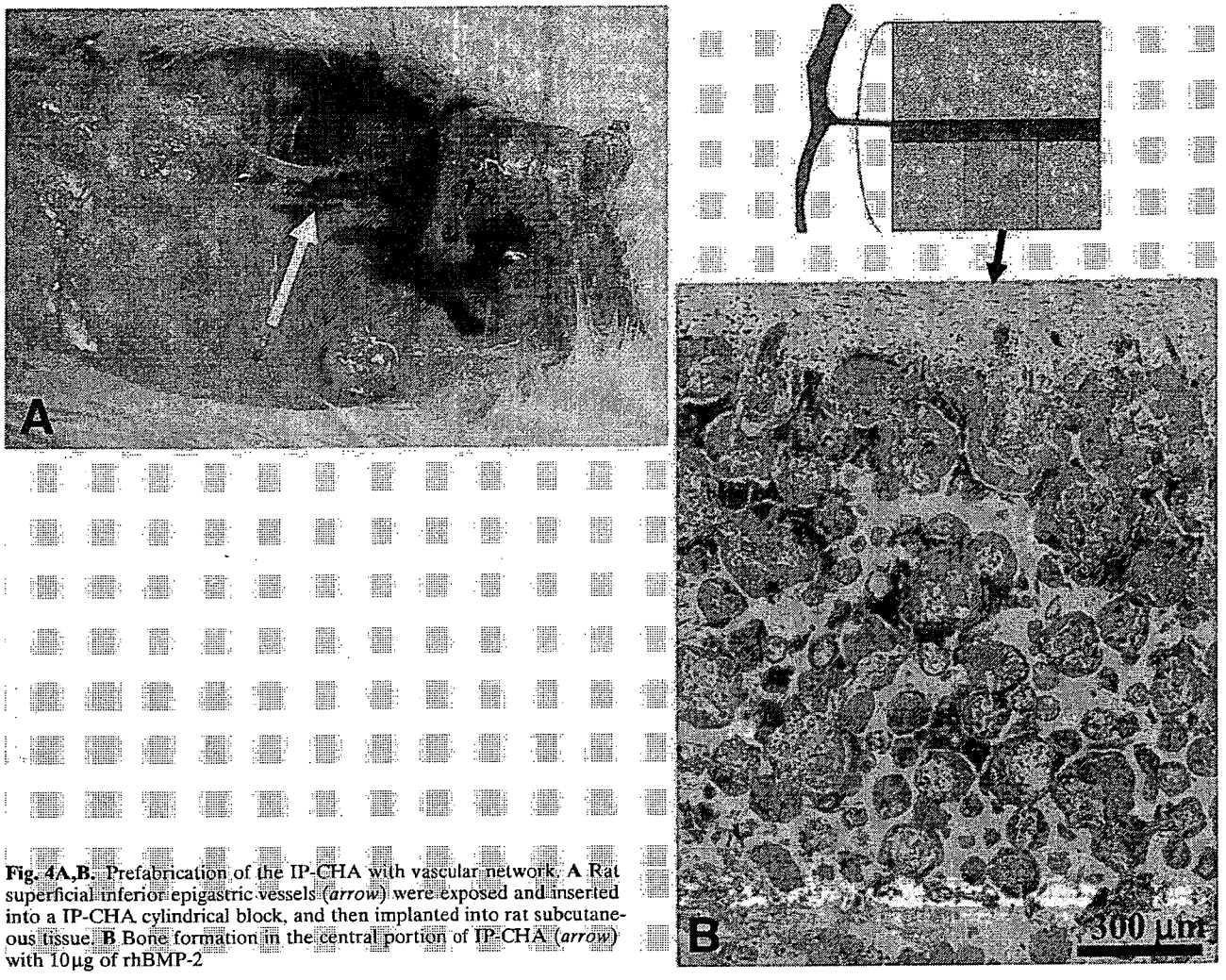

Fig. 4A,B. Prefabrication of the IP-CHA with vascular network. **A** Rat superficial inferior epigastric vessels (*arrow*) were exposed and inserted into a IP-CHA cylindrical block, and then implanted into rat subcutaneous tissue. **B** Bone formation in the central portion of IP-CHA (*arrow*) with 10μg of rhBMP-2

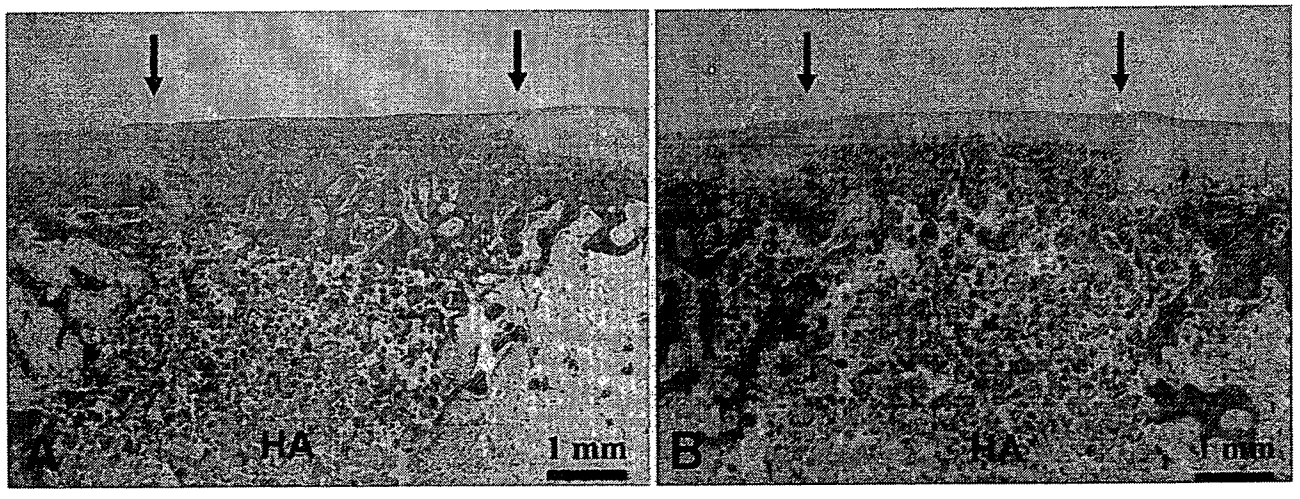

Fig. 5A,B. Articular cartilage repair by the BMP/poly D,L,-lactic acid–polyethylenglycol (PLA-PEG)/IP-CHA composite. *Arrows* indicate the margins of the defect; *HA*, IP-CHA (H.E. staining). **A** PLA-PEG/IP-CHA group at 6 weeks after implantation. Subchondral bone was regenerated, but no articular cartilage was observed. **B** BMP/PLA-PEG/IP-CHA group at 6 weeks after implantation. Well-organized hyaline-like cartilage covering the regenerated subchondral bone was observed

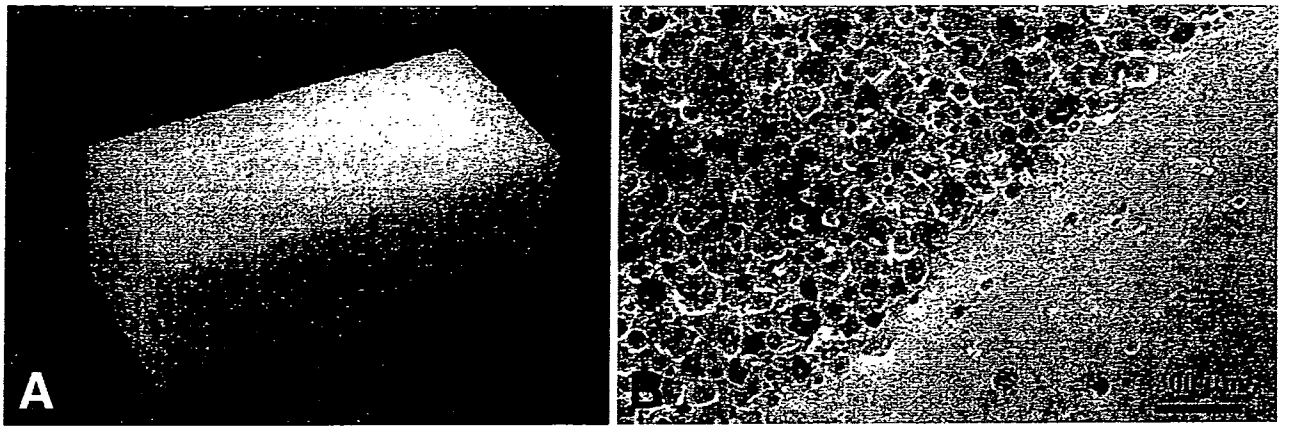

Fig. 6. Macroscopic (**A**) and microscopic (SEM) (**B**) photographs of the solid/interconnected porous HA composite

and eventually leads to good incorporation of the material into host bone. The synthetic scaffold can be prefabricated into specific sizes and shapes to match bone defects, and even into a composite with the solid form of HA in order to reinforce its initial mechanical strength (Fig. 6). We believe that IP-CHA itself is an excellent bone substitute for filling bone defects and should be considered as an alternative to autogenous bone. In addition, IP-CHA could serve as a good scaffold for cytokine-based or cell-based tissue-engineered bone and cartilage. In fact, we have been successful in bone tissue engineering using rhBMP-2, mesenchymal cells, or vasculature in animals. IP-CHA can be also applied to articular cartilage repair. IP-CHA is now commercially available, and we have applied IP-CHA as a bone substitute for the treatment of more than 50 patients with benign bone tumors or nonunion of a fracture, and obtained some favorable clinical results. Recently, we started a clinical study with the combination of IP-CHA and autologous mesenchymal cells for bone tissue repair. Additional studies with larger animals including dogs or monkeys and precise clinical evaluation are necessary, but we believe that bone tissue engineering with IP-CHA offers new approaches to the treatment of patients requiring skeletal reconstruction.

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骨吸収・骨形成の相互作用

BMPシグナルと骨形成・骨吸収

Role of BMP signals in bone formation and resorption

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骨芽細胞特異的プロモーターを用いた種々の遺伝子改変マウスが作製・解析され，骨形成因子(BMP)の骨形成・維持における機能がここ数年の間に明らかにされつつある．骨芽細胞特異的にBMPシグナルを抑制すると骨芽細胞の機能が障害され，骨量が減少することから，BMPシグナルは生後の骨質の維持に不可欠であることが明らかにされた．一方で細胞培養系において，BMPは破骨細胞を刺激することが報告されている．BMPの骨に対する作用は骨形成・骨吸収の両面から理解する必要がある．

key words

骨形成因子(BMP)
遺伝子改変マウス
骨形成
骨吸収
noggin
内軟骨性骨化

はじめに

骨形成因子(bone morphogenetic proteins：BMPs)は，皮下に移植した場合に異所性に骨・軟骨形成を誘導する物質として1965年にUristによって同定された[1]．その発見の経緯から，BMPは骨形成において重要な役割を果たしていると40年前から考えられ，また難治性骨折や骨欠損といった疾患において治癒を促進する治療薬としての役割が期待されてきた．昨今，骨形成・骨吸収を制御する分子メカニズムが詳細に解析され，それに参画する物質が明らかにされてきたが，BMPsはこれらの研究においても相互作用やそのシグナル伝達の関係を調べるべき候補となり，あるいは実験のコントロールとして使われてきた．しかし意外にも，BMPsの生体の骨形成における機能や，骨折治癒に対する明確な作用が明らかになったのはここ数年のことである．本稿では主に遺伝子改変マウスを用いて明らかにされたBMPの骨形成・骨吸収における役割について概説する．

BMPシグナル伝達

BMPの細胞内へのシグナル伝達はⅠ型およびⅡ型のセリン/スレオニンキナーゼ型受容体を通して行われる[2](図1)．TGF-β(transforming growth factor-β)やBMPリガンドが結合することにより，Ⅱ型受容体はⅠ型受容体をリン酸化する．BMP IA型受容体[BMPR-IA or ALK (activin receptor-like kinase)-3]，BMP IB型受容体(ALK-6)，そしてALK-2がBMPシグナルを伝達する．TGF-β/BMP受容体の下流にはSmadと呼ばれる一群の蛋白質が存在し，細胞内シグナルを伝達する．哺乳類では8種のSmadが同定され，3つのサブグループに分類される．特異型Smad (R-Smad) はⅠ型受容体によってリン酸化される．Smad1，Smad5，そしてSmad8はBMPシグナルを伝達し，一方，Smad2およびSmad3はTGF-βシグナルを伝達する．リン酸化を受けた特異型Smadは共有型Smad (Co-Smad)であるSmad4と多量体を形成し，核内へ移行して種々の転写因子と相互作用して標的となる遺伝子の転写を直接的あるいは間接的に制

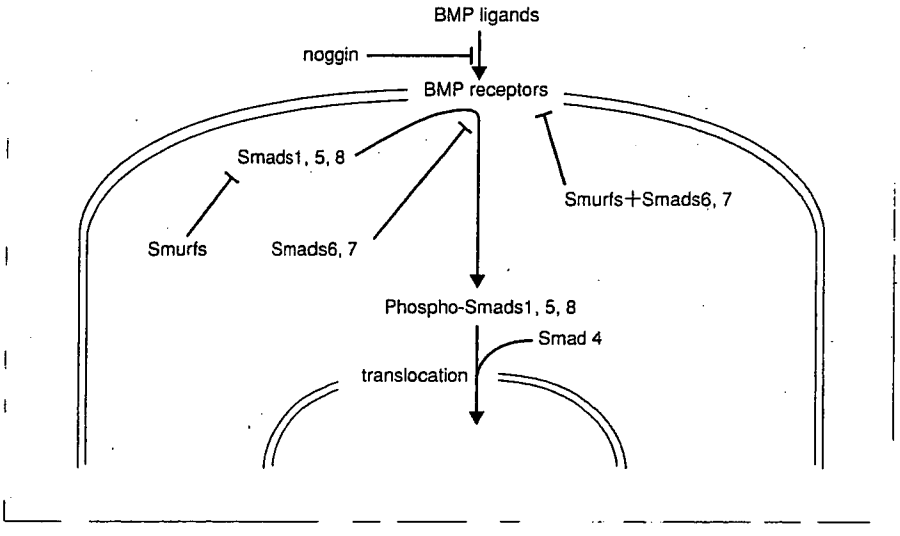

図1　BMP シグナル伝達

BMP シグナルは BMP 受容体を介して主に Smad 蛋白で伝達される．この過程は各段階で調節を受ける．細胞外では noggin，gremlin などがアンタゴニストとして働く．細胞内では抑制型 Smad (I-Smad) である Smad6，Smad7が特異型 Smad と I 型受容体との結合を競合し，特異型 Smad のリン酸化を抑制する．さらに Smurf を介した Smad と BMP 受容体のユビキチン化と蛋白分解が報告されている．

御する．

BMP シグナル伝達は各段階で調節を受ける．細胞外ではいくつかの分子がアンタゴニストとして働く．Noggin は主に軟骨で発現し，BMP に結合して BMP が受容体に作用することを阻害する[3]．また，細胞内では抑制型 Smad (I-Smad) である Smad6と Smad7 が特異型 Smad と I 型受容体との結合を競合し，特異型 Smad のリン酸化を抑制する．Smad7が BMP と TGF-β の両方のシグナル伝達を阻害するのに対し，Smad6は BMP シグナルを主に阻害するようである．さらに，ユビキチン化と蛋白分解による Smad シグナル伝達の阻害が報告されている．Smurf1 (Smad ubiquitin regulatory factor-1) と Smurf2は Smad1と Smad5の分解を誘導する．さらに，Smurf1は Smad6や Smad7と相互作用することで I 型受容体と結合し，これらの受容体をユビキチン化・蛋白分解へと誘導することが明らかにされた[4]．一方，Smurf1のノックアウトマウスの解析から，Smurf1は MEKK2の分解誘導によって JNK (c-Jun N-terminal kinase) シグナルを抑制することが示され，BMP シグナルの抑制効果はこの経路を阻害することによると報告されている[5]．

BMP ノックアウトマウス

Urist による BMPs の発見後しばらくして遺伝子クローニング[6]と蛋白抽出[7][8]が行われた．そして，BMPs は多くのメンバーからなるファミリーを形成し，TGF-β スーパーファミリーに属することが明らかにされた．BMP ファミリーには growth and differentiation factor サブファミリーのメンバーも含まれる．生体での BMP の生理作用を調べるために，BMP を欠失したマウスが作製・解析された．*Bmp2*，*Bmp4* は着床後の発生初期の胎児に発現するために，受精後9日ごろに致死となり，骨格形成が始まる12.5日目以降の解析は不可能であった．BMP I A 型受容体や II 型受容体のノックアウトマウスも，中胚葉の形成不全により9日ごろに致死となった．その

表　骨芽細胞特異的BMPシグナル抑制マウス

promoter	transgenic/knockout	文献
osteocalcin	noggin transgenic mice	11, 12
osteocalcin	gremlin transgenic mice	14
Col1a1	dominant negative *BMPR IA* transgenic mice	9
Col1a1	*Smurf1* transgenic mice	15
osteocalcin	*BMPR IA* conditional knockout mice	10

Col1a1：Ⅰ型コラーゲンα1鎖遺伝子，
Col1a2：XI型コラーゲンα2鎖遺伝子，
Col2a1：Ⅱ型コラーゲン遺伝子，
BMPR IA：BMP ⅠA型受容体遺伝子，
BMPR IB：BMP ⅠB型受容体遺伝子

致死の原因は，BMPシグナル伝達が障害されたためと考えられた．一方，*Bmp5*，*Bmp6*，*Bmp7*そして*Gdf5*のノックアウトマウスは生き残り，骨格コンポーネントの数や形の異常を呈した．BMP ⅠB型受容体のノックアウトマウスもほぼ同様の形態異常を呈した．これらのノックアウトマウスの解析から明らかにされたことはBMPが骨格形成のパターニングに関与していることであり，その後の骨形成おける作用はあまり明らかにされなかった．このマイルドな表現型の異常は，同じ組織に発現する他のBMPファミリーメンバーやその受容体が，欠失したBMPや受容体の機能を代償したためと推測されている．この考えは*Bmp5*と*Bmp7*の両方を欠失したダブルノックアウトマウスが発生初期に致死となることから証明された．また，BMPは骨格に限らず広範な組織で発現するため，ノックアウトマウスでは腎臓や眼など種々の組織で異常が認められる．

骨芽細胞特異的BMPシグナル抑制マウス

そこで，数年前から組織特異的にBMPシグナルを抑制したトランスジェニックマウスが作製・解析されるようになった(表)．骨組織特異的発現を得るために，Ⅰ型コラーゲンα1鎖遺伝子(*Col1a1*)プロモーターの骨特異的シスエレメントあるいはオステオカルシンプロモーターが用いられた．*Col1a1*プロモーターを用いてdominant negative BMPR IBを発現させ，骨芽細胞においてBMPシグナルを抑制したトランスジェニックマウスでは，生後において骨量の低下と骨形成速度の低下を認める一方，骨芽細胞数と破骨細胞数はともに不変であった[9]．これにより，BMPシグナルが生後の骨量の維持に必要であることが判明する一方，骨芽細胞数が減少しなかったことからBMPは骨芽細胞の機能に必要であると考えられた．骨芽細胞数が不変であったことは，培養細胞の実験において，recombinant BMPs添加が未分化な細胞を骨芽細胞の系譜へ強力にリクルートすると考えられてきたことからは意外な結果であった．同様に，オステオカルシンプロモーターCreトランスジェニックマウスを用いて骨芽細胞においてBMPR IAを欠失させBMPシグナルを抑制したコンディショナルノックアウトマウスにおいても，生後の骨量の低下と骨形成速度の低下を認める一方で，骨芽細胞数と破骨細胞数はともに不変であった[10]．しかし，このBMPR IAコンディショナルノックアウトマウスでは，生後10ヵ月あたりから骨量の増加と破骨細胞機能の低下を認めた．その理由として，骨芽細胞の機能低下が破骨細胞の支持機能の低下につながっている可能性が推測されている．

BMPのアンタゴニストであるnogginは結晶構造解析と機能解析から，BMPを極めて特異的に阻害することが明らかにされた[3]．このため，種々の組織においてnogginを過剰発現させBMPシグナルを抑制したモデルマウスが作製されている．オステオカル

シンプロモーターを用いて noggin を過剰発現させたマウスにおいても生後における骨量の低下と骨形成速度の低下を認める一方，骨芽細胞数と破骨細胞数はともに不変であった[11)12)]．Noggin トランスジェニックマウスの骨変化が軽微である理由としては，オステオカルシンプロモーターが骨芽細胞の中でも限られた成熟した細胞にのみ発現しているためと推測されている[12)]．それに比して，*Col1a1* プロモーターは骨芽細胞において遺伝子を強力に発現させることで知られている[13)]．また，noggin とは別の BMP アンタゴニストである gremlin をオステオカルシンプロモーターを用いて骨芽細胞に過剰発現させ，BMP シグナルを抑制したマウスでは，骨量の低下，骨形成速度の低下と，骨芽細胞数および破骨細胞数の低下も認めた[14)]．Smurf1 を *Col1a1* プロモーターで過剰発現させたトランスジェニックマウスでは，骨量の低下，骨形成速度の低下，骨芽細胞数の減少が認められ，骨芽細胞の増殖と分化の両方が阻害されていた[15)]．

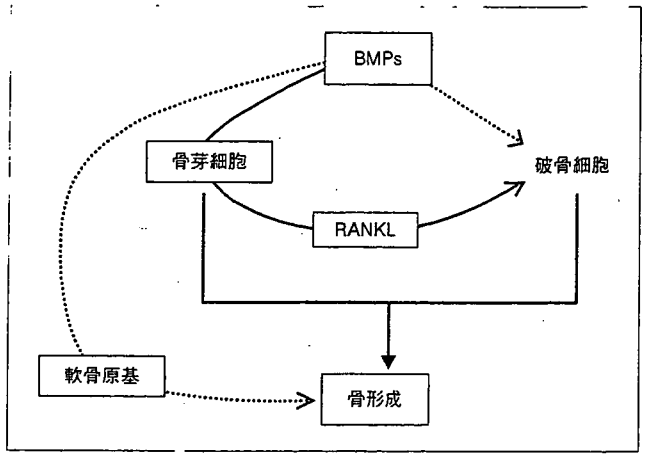

図2　骨形成に対する BMP の作用
骨組織において BMPs は骨芽細胞だけでなく，破骨細胞にも作用し得る．これには RANKL を介する経路と直接破骨細胞へ作用する経路があると考えられる．また，BMPs は軟骨原基へ作用する結果，骨形成にも影響する．

破骨細胞に対する BMP の作用

BMP は異所性骨化を誘導し得るという現象とその名前から，BMP の骨芽細胞に対する作用に注目が集まるが，BMP は破骨細胞を刺激することが培養系において示されている（図2）．骨芽細胞様細胞と破骨細胞前駆細胞との共培養において，noggin を添加すると骨芽細胞分化が抑えられるとともに破骨細胞形成も抑制されることが報告されている[16)]．Noggin によって骨芽細胞の RANKL (receptor activator of NF-κB ligand) の発現量が低下するため，この系では骨芽細胞による破骨細胞支持能が阻害されたために破骨細胞数が減少したと考えられている．その一方で破骨細胞は BMP 受容体を発現し，BMPs は破骨細胞を直接刺激することが報告されている[17)-19)]．M-CSF と RANKL によってマクロファージから誘導される破骨細胞への分化を，BMPs が促進することが示された[18)]．また，TGF-β にも破骨細胞に直接作用して，分化を促進する作用があることが示された[20)]．I 型コラーゲンプロモーターを用いて noggin を骨特異的に発現させたトランスジェニックマウスを作製したところ，胎児期後期から生後早期にかけて破骨細胞数が減少して骨量が増加すること，BMP4を過剰発現させた場合は胎児期後期に破骨細胞数が増加して骨量が減少することを見い出した[21)]．

内軟骨性骨化と BMP

膜性骨化に対し，内軟骨性骨化では一旦軟骨で骨格が形成され，それを鋳型に骨に置き換わる．四肢の骨格は内軟骨性骨化の過程を経て形成される．したがって原基である軟骨の性状が，その後の骨形成に大きな影響を与えるであろうことが予想される．BMP は軟骨細胞に対して増殖作用がある．我々が作製した BMP を軟骨特異的に過剰発現させたトランスジェニックマウスでは大きな軟骨原基が形成される[22)23)]．このトランスジェニックマウスの骨を調べたところ，骨幅は広く，骨梁が増大しており，BMP の軟骨細胞に対する作用が間接的に骨形成を制御していることがわかった[21)]．同様に，

軟骨特異的にnogginやSmad6を過剰発現させてBMPシグナルを抑制したトランスジェニックマウスでは骨量が減少していた．特にSmad6トランスジェニックマウスの骨組織から破骨細胞を培養したところ正常であったので，このマウスでは軟骨原基における細胞肥大化の遅れが骨量の減少につながったと考えた[24]．

おわりに

BMPsは種々の組織に発現および機能し，その標的細胞は多岐にわたる．生体の骨組織においても，BMPは骨芽細胞のみでなく破骨細胞に対しても直接作用することは十分に考えられる．実験的骨折モデルなどに対しBMPを投与する場合，状況によっては骨吸収を起こすこともみられる．また内軟骨性骨化の経過から，軟骨細胞に対するBMPsの強力な作用が骨形成に大きな影響を及ぼす．BMPが有する作用は骨形成のみでなく，骨吸収に対する作用，そして軟骨細胞に対する作用を含め総合的に理解する必要がある(図2)．

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シンポジウム　各種人工骨の臨床応用と問題点

連通気孔構造を有するハイドロキシアパタイト人工骨の臨床応用
—物理学的特性・臨床的特徴・問題点—*

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はじめに

ハイドロキシアパタイト(hydroxyapatite: HA)をはじめとするリン酸カルシウムセラミックスは骨と化学的に結合するバイオアクティブセラミックスであり，その優れた生体親和性，骨伝導能から人工骨として最も適していると考えられている．緻密体および多孔体HAは1980年代より骨補填剤として広く臨床使用されてきた[1)-7)]．特に多孔体HA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，長期の臨床症例の解析から気孔内への新生骨侵入は数ミリ程度に限られることが明らかになってきた[1)]．このような新生骨の侵入していない気孔は強度面で不利であり，多孔体HA移植後2，3年経過してから骨折した症例も報告されている[2),5),7)]．これまでの多孔体HAの気孔構造はいわゆる“軽石状”であり，骨髄腔がすべて連続している海綿骨の骨梁構造とは似て非なるものであった．このような“マクロポア”の構造を制御して，気孔と気孔を組織侵入に十分なサイズの気孔間連通孔でつなげることができれば，多孔体HAの気孔内のbone ingrowthは当然，改善されると考えられる．われわれが物質・材料研究機構(田中順三主任研究員，菊池正紀研究員，生駒俊之研究員)，東芝セラミックス(株)，(株)エム・エム・ティーと共同で開発した連通多孔体ハイドロキシアパタイトセラミックス(interconnected porous calcium hydroxyapatite ceramics: IP-CHA，ネオボーン®，東芝セラミックス(株)製)は，気孔間の連通性に主眼をおいて開発され深部の気孔にまで新生骨が侵入しうる合成多孔体HA骨補填材である[8)]．その特徴は，ほぼすべての気孔が大きい径の気孔間連通孔でつながり組織の侵入が可能であること，そして術前加工性や術中操作性に問題のない強度を同時に実現したことである．

Key words: Hydroxyapatite, Bone substitute, Interconnected porous structure, Tissue engineering, Bone regeneration

*Clinical Application of the Interconnected Porous HA Ceramics: Its Nature and the Point of Issue

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連通多孔体ハイドロキシアパタイトセラミックスの三次元構造

IP-CHAの最大の特徴はその三次元構造にある(図1)．ほぼ球形の気孔が隣接し，気孔間連通孔で互いに連絡する構造になっている．骨補填材として臨床用に開発したネオボーン®の気孔率は75%で，現在市販されている多孔体セラミック人工骨で最も高い気孔率を有する[9)]．走査電子顕微鏡像による検討では，ネオボーン®の気孔は比較的均一なサイズで直径が150-200 μmであり，気孔の内壁には気孔間連通孔が開いており，隣接する気孔と交通している．この気孔間連通孔の直径は90%以上が10-80 μm(平均約40 μm)であり，1つの気孔に通常4-5個以上の連通孔が存在する．高温で焼成されているためHAの粒子が互いに密に結合しており，気孔

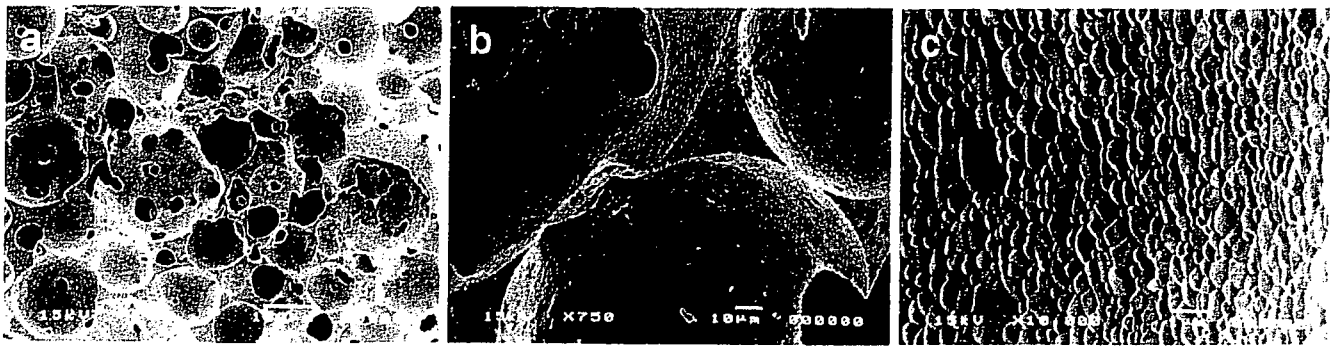

図1 IP-CHA75/ネオボーン®の走査電子顕微鏡像. (a)気孔はほぼ球形で互いに密に隣接しており, (b)気孔壁には多数の気孔間連通孔が観察される. (c)壁の表面はスムーズでHA粒子が密に結合している.

表1 IP-CHAと市販多孔体HAの微細構造および圧縮強度の比較

	試作品			ネオボーン® IP-CHA75	既製多孔体HA
	IP-CHA57	IP-CHA63	IP-CHA69		
全気孔率(%)	57	63	69	75	47.6-64.5
平均気孔径(μm)	58.2	68.5	80.4	191	176-268
気孔間連通孔径(μm)	5	12	25	39	<1-22
実質有効気孔率(%)	40.0	50.2	65.3	67.3	2.5-36.7
圧縮強度(MPa)	68.6	39.8	25.1	12.0	8-60

壁の表面がスムーズである.

気孔率, 気孔径, 気孔間連通孔の最適化および既製品との比較

このIP-CHAの製造方法の特徴は, 起泡ゲル化技術を取り入れたことである[8]. 起泡ゲル化技術では, スラリーに起泡剤を加えて攪拌し起泡させたのち, ゲル化させ構造を固定するため, 攪拌・起泡過程での種々のパラメーターの調整により気孔および気孔間連通孔の制御が可能である. 前臨床の段階でプロトタイプとして作成したIP-CHA57(総気孔率57%), IP-CHA63(総気孔率63%), IP-CHA69(総気孔率69%), 臨床用に開発したIP-CHA75(ネオボーン®, 総気孔率75%), および既製の多孔体HA骨補填材の構造的・力学的特性を表1に示す. IP-CHA75(ネオボーン®)の平均気孔径は約190 μm, 平均気孔間連通孔径は約40 μmであるが, 気孔率を69%に下げたIP-CHA69の平均気孔径は通常のIP-CHAに比べ半分以下の80.4 μm, 気孔間連通孔径は約半分の25 μmに低下する. 水銀圧入ポロシメーターによる気孔間連通性についての評価では, 直径10 μm以上の気孔間連通孔でつながっている気孔による気孔率を「有効気孔率」とすると, IP-CHA75の有効気孔率が67.3%であったのに対し, IP-CHA69で65.3%, IP-CHA63で50.2%, IP-CHA57で40%と総気孔率が低下するにつれ有効気孔率も低下していた. 既製の多孔体HA人工骨の有効気孔率は2.5-36.7%となっており, IP-CHA, 特にIP-CHA75とIP-CHA69で気孔の連通性がきわめて高いことがわかる. 一方, 気孔率を下げるとIP-CHAの圧縮強度は高くなる傾向が見られたが, 気孔率の高いIP-CHA75でも12 MPa, IP-CHA69で25 MPa程度と海綿骨の2-8倍程度の強度があり, 従来の多孔体HA人工骨の中で中間的な値を示していた. IP-CHAがきわめて高い気孔率を有するにもかかわらず比較的高い圧縮強度を有するのは, 焼成度が高くHAの粒子が密に結合しており, 壁の構造が緻密体HAに類似するためと考えられる. これらの解析からIP-CHA75とIP-CHA69は, 既製品と比べ明らかに良好な気孔間連通性を示すと同時に, 既製品と同等の圧縮強度を持つことが解った.

IP-CHAの開発コンセプトは, 良好な気孔内への骨伝導能(bone ingrowth)を持つ骨補填材であるので, 深部気孔内への新生骨形成を評価するためにウサギ大腿骨顆部に径6 mmの円柱形ブロックを埋入し組織学的に検討したところ, IP-CHA75では気孔内の骨組織の

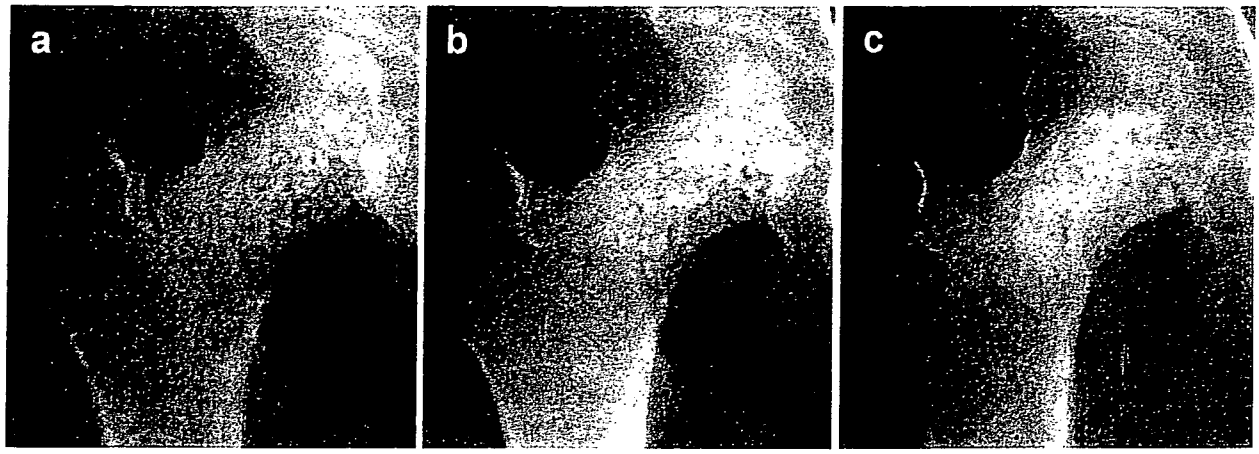

図2　ネオボーン®移植後のX線経過．18歳女性，大腿骨頚部骨巨細胞腫．(a)手術直後のX線像．(b)移植後2カ月でわずかに移植部のX線透過性が低下し，(c)6カ月では顆粒が融合し強い硬化像を示している．

侵入は良好で，6週間で円柱の中心部，すなわち表層から3 mmまでほぼすべての気孔内に成熟骨組織の侵入が見られたが，IP-CHA69では6週で表層から2 mmまでの気孔内にしか骨形成が見られず，中心部の深さ3 mmでは線維性結合織のみが形成されており，気孔率を下げると気孔内への骨組織侵入の速度が低下した．既製の多孔体HA骨補填材でも6週の時点で中心部の深さ3 mmではほとんど骨形成が見られなかった．IP-CHA75の初期強度は10-12 MPaであるが，骨内埋植後，インプラント中心部分の圧縮強度を計測すると，骨形成が進むにつれて上昇し，埋植後6週で20 MPa，9週で30 MPa以上の圧縮強度に達しており，移植後速やかに強度を獲得する．これらのことから，臨床で用いるには気孔率75%，気孔径150-200 μm，平均気孔間連通孔径40 μmのものが至適と考え骨補填材として臨床用に開発した．

IP-CHA/ネオボーン®の臨床的特徴

IP-CHA75/ネオボーン®を骨補填材として開発するにあたり行った臨床治験の結果[10]では，良性骨腫瘍および腫瘍類似疾患の手術において使用した場合，単純X線上，術後2-3カ月でネオボーン®移植部の骨硬化が出現し，6カ月ではほぼすべての症例で強い骨硬化に伴いネオボーン®顆粒間や骨との間が不明瞭になるという変化が観察された(図2)．このようなX線変化は従来の多孔体HA人工骨では通常平均術後12カ月ごろで見られるとされており，ネオボーン®のX線上の変化はきわめて速いと考えられるが，これはネオボーン®では顆粒間だけでなく気孔の内部にも骨形成が起こるためと考えられる．また，このようにX線上早期から硬化像が明らかとなることは，臨床医にとって荷重開始可能時期を推測するのに好都合である．強度については，手術中の操作には問題がなく，エアートームなどによる加工も容易であるが，ハンマーなどで衝撃を加えれば破損する．荷重部など強い力学的負荷のかかる部位での単独使用は難しく，適宜，金属製プレートなどの併用が必要である．

ネオボーン®の特徴を生かした適応拡大の試み

この20年間に種々のリン酸カルシウム系骨補填材が製品化されてきたが，臨床医にとっては選択肢が増え，歓迎すべきである一方，それぞれの製品の特徴をよく理解し，個々の症例の病態に合わせて正しい材料を選択することが要求される．たとえば，オスフェリオン®(オリンパス光学製)は，初期強度が低いものの，吸収されやすく自家骨に置換されやすい特徴を生かして，小児や関節軟骨下骨部の骨欠損に有利と考えられる．セメントのように硬化するリン酸カルシウムペーストのバイオペックス®(三菱マテリアル製)は，その操作性が魅力であるが，十分な強度を得るには操作に熟練を要するとともに，bone ingrowthは期待できないため，小皮切による低侵襲手術や上肢など非荷重部での使用に向いている．

ネオボーン®についても，その特性を理解し，適所に用いることが肝要である．既製の骨補填材と比較したネオボーン®の特徴は，①臨床的に速やかに移植部の骨と融合する，②深部気孔内骨組織侵入(bone ingrowth)が期待できる(ブロック体や特殊形状のインプラントを含めて)，③術前，術中の加工が容易である，④吸収はきわめて緩徐か，または吸収されない，⑤強