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BMP-induced Bone and Cartilage Formation with Geometrically Different Cell Substrates; Topobiology of Bone Formation

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幾何学的な違いを有する細胞支持体上でのＢＭＰ誘導骨・軟骨形成
――― 骨形成のトポバイオロジー

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BACKGROUND AND INTRODUCTION

Principles of hard tissue reconstruction

In order to establish useful methods for reconstruction of hard tissues, we need to give systematic consideration to elucidating the biochemical principles of formation and rectivity of the tissues. Since the periodontal tissues include not only alveolar bone, the biochemical principles for oral hard tissues reconstruction are more complex than that for bone. We have already proposed that four important factors in bone formation: (1) cells directly involved in bone formation, (2) matrices produced by the cells, (3)mineral ions from body fluids, and (4) various regulators.[1)]

However, to discuss the reconstruction of oral hard tissue, category of 'matrices' must be enlarged to include artificial implants that interact with cells. Moreover, geometrical property of matrices and mechanical stress toward the peri-implant tissues must be considered.

Importance of geometry of matrices

The geometry of the solid substratum for anchorage-dependent cells has been suggested to play a crucial role in the cell-differentiation. However, appropriate model systems are required for this hypothesis to be verified.

Bone morphogenetic protein (BMP) is known as a growth and differentiation factor that stimulates immature mesenchymal cells to create a process similar to endochondral ossification when it is subcutaneously implanted with insoluble bone matrix as a carrier.[2,3)] This insoluble bone matrix (IBM) in a granular form of about 0.3-0.5 mm diameter has been most widely used as a carrier for BMP for ectopic bone formation. Recently we have pointed out that this carrier is not just a drug delivery system, but an important cell-substratum for differentiation, since bone formation occurs only on the surface of the carrier.[4)]

Thus, we have started a systematic study concerning the

importance of geometrical factors of BMP–carrier in the chondro and osteogenesis (5). We have devised and tested at least seven different carriers (artifical matrices) other than IBM, combined each with partially purified BMP obtained by three–step chomatographic method and implanted into rat subcutaneously (6,7,8).

In this paper we demonstracte that BMP is able to induce immature cells more effectively into becoming chondrogenic cell, or also directly into becoming osteogenic cells, depending upon geometrical nature of the carriers used for the implantation.

MATERIALS AND METHODS

Source of BMP

Partially purified BMP obtained by three–step chomatographic method as previously reported. Briefly, freshly obtained bovine metatarsal bones were processed to obtain bone powers (one kg per extraction), which were sieved selectively between 70 and 400um, decalcified in diluted HCl at a constant PH of 2.0 at 4℃ and extracted with 4M guanidine HCl / 0.05 M Tris–HCl (PH 7.4). A detailed decription of the procedure used for purification of the active fraction in the guanidine extract by an eight–step chromatographic procedure has been reported (1,8).

For all experiments in this study of new carriers, the BMP preparation was used after a three –step chromatographic procedure using hydroxyapatite (5X30CM, Apatite International Inc., Tokyo), a Heparin–Sepharose column (Pharmacia LKB, 2.2X141CM). This fraction was free of TGF–β1 and β2 and was designated S–300 BMP. The S–300 BMP preparations were pooled from at least five extractions to maintain constant quality. It was combined each with at least seven different carriers (artificial matrices) and implanted into rat subcutaneously.

Taxonomy of BMP–carriers These carriers were classified into three types:fibro–, particle– and block–types.

(1) Fiber–type carriers include fibrous collagen membrane (FCM) and fibrous glass membrane (FGM).

(2) Particle–type carriers include collagen beads (CBDS), solid particles of hydroxyapatite (SPHAP) and porous particles of hydroxyapatite (PPHAP).

(3) Block–type carriers include porous blocks of hydroxyapatite (PBHAP) and honeycomb–shaped hydroxyapatite (HCHAP).

RESULTS AND DISCUSSIONS

Results of FGM and PPHAP

Each BMP–combined carrier include unique pattern of bone and cartilage formation. To give typical examples in our study, partially purified BMP was combined with a fibrous glass membrane (FGM, with a fiber diameter of 1 μm and thickness of 0.6 μm) and porous particles of hydroxyapatite (9) (PPHAP, Nippon Steel Works, with a particle size of 0.3–5 mm and a pore size of 150 μm) and implanted subcutaneously into the backs of rats. These two

geometrically different solid-state carriers induced tissues in quite different manners. FGM/BMP implants induced cartilage formation within the entire inner area of the membrane accompanied by a small amount of bone formation on the surface of the membrane at two weeks after implantation, and zones of immature cells, developing and hypertrophic chondrocytes and bone formation were clearly observed. In contrast, PPHAP/BMP implants induced bone that directly followed fibrous tissue formation within the pores of PPHAP without any detectable cartilage formation.

Analysis of biochemical markers revealed that the type II collagen content in FGM/BMP was six times higher than that in IBM/BMP at from 1 to 2 weeks, while there was no detectable type II collagen in PPHAP/BMP even at 1 week after implantation. In addition, type X collagen was detected in BMP/FGM at 2 weeks. Definite biochemical evidence of bone formation in PPHAP/BMP was provided by the increase of osteocalcin contents. In short, FCM induced bone and cartilage independently, and PPHAP induced only bone, while FGM induced only cartilage inside the membrane accompanied by bone formation on the membrane surface.

Interpretation

It was explained that PPHAP create a space sufficient for vasculature, while the tight network of FGM (1 μm exclusion size) provides immature cells with space for penetrating into the membrane, but not for vascular formation.

Furthermore, FGM with a pore size of less than 0.6 μm did not induce bone or cartilage at all, which indicated that both immature cells and vasculature could not enter into this FGM with a smaller pore size. These results were interpreted by geometrical factor of the carriers which control the vascularization associated with the implants.

CONCLUSION

The results clearly indicate that BMP is able to induce not only cartilage formation but also direct bone formation, depending upon geometrical nature of the carriers used for the implantation.

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Expression of Extracellular Matrix Glycoprotein participates to Chondrogenesis in Salivary Pleomorphic Adenoma and Mixed Tumor of Skin

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細胞外基質蛋白の出現と唾液腺多形性腺腫と皮膚混合腫瘍における軟骨形成の関係

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Introduction

Salivary pleomorphic adenomas and mixed tumors of the skin showed histologically resemblance that both tumors consist of epithelial luminal cells of tubulo−ductal structure and modified myoepithelial cells. It has been described that modified myoepithelial cells irrespective to varying types and cell shapes participate chondrogenesis, and that they showed positive immunostaining for MoAb K8.12, vimentin, S−100 protein and neuron specific enolase(NSE), and rarely positive for glial fibrillary acidic protein(GFAP)(1,3,4,6) and PGP 9.5. Recently, bone morphogenetic protein(BMP), potent bone including factor, has shown that immunohistochemical expression of BMP was positive in the outer tumor cells of tubulo−ductal structure and modified myoepithelial cells and chondroid changed cells. In general aspects of chondrogenesis in both tumor lesions, hyalinous tissue and chondroidally changed tissue are the result of secondary alterations induced by modified myoepithelial cells, that synthesis of glycosaminoglycans for chondroitin 4 −sulfate proteoglycan(C4SPG), chondroitin 6−sulfate PG(C6PG), keratan sulfate PG(KSPG) and dermatan sulfate PG(DSPG)(6).

Materials and Methods

A total of 20 cases of pleomorphic adenoma of the salivary glands and 20 cases of mixed tumor of the skin, chondroid syringoma were evaluated. The tumor specimens were fixed into 10% formalin and embedded in paraffin. Paraffin sections of 4um were made for histologic orientation with HE staining, and for immunohistochemistry with extracellular matrix glycoproteins, laminin, fibronectin and tenascin.

Results

Laminin: Basement membrane of gland structure stained positively for laminin, and some of intercalated duct cells was also positive. Fibrillar modified myoepithelial cells in hyalinous structure expressed positive staining to laminin, but no staining in hyalinous matrix. Fibronectin: Some of acinar cells and duct cells, not all, expressed condensed reaction for fibronectin. In pleomorphic adenoma, limited tumor cells have shown strong reaction, but in skin mixed tumor modified myoepithelial cells are routinely stained for fibronectin. Fibrillary cells in hyalinous tissue showed marked fibronectin staining, however hyalin matrix showed no reaction product. There are slight and moderate staining in chondroid matrix, but not in nuclear area(2). Tenascin: Tenascin is expressed in epithelial-mesenchymal interface including basement membrane in large excretory duct of the salivary glands and eccrin glands. No tenascin immunostaining was found in luminal tumor cells of both tumor lesions, but tenascin was positive in modified myoepithelial cells, hyalinous tissue and prechondroid tissue with slightly reaction and chondroid matrix with constantly moderate.

Discussion

It is generally accepted that matrix production from progenitor cells was an initiating progress of the histogenesis of chondro-osseous tissue, and chondrogenesis appeared in pleomorphic adenoma of the salivary glands and mixed tumor of the eccrin sweat glands was probably the same mechanism. On this regards, it has previously described that the biosynthesis of GGs by modified myoepithelial cells and plasmacytoid cells in both tumors was assessed by PGs immunohistochemical technique(6). The cells in hyalinous tissue and chondroidally changed tissue showed almost PGs deposition, C4SPG, C6SPG, KSPG and DSPG. Those finding suggest that modified myoepithelial cells irrespective with their cell shapes and locations probably produced several GGs into hyalinous structure and such hyalinous tissue was gradually changed to chondroid matrix. The processes of those chondroidally alteration were related to express of BMP and S-100 protein as significant modulate calcium signaling in situ(1, 3, 4). In the present study, extra cellular matrix glycoproteins, laminin, fibronectin and tenascin developed modified myoepithelial cells, particularly strong expressed in fibrillary myoepithelial cells. Distribotion of laminin, fibronectin(2) and tenascin was a little different in tumor parenchyme, matrix forming process was more pronounced in immunostaining of fibronectin and tenascin, and fibrillary modified myoepithelial cells were more abundant in laminin staining. The possible histogenetic

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origin of chondrogenesis in both tumor lesions is various types of modified myoepithelial cells, and they produced GGs as already reported, as well as biosynthesis of extra cellular matrix proteins as laminin, fibronectin and tenascin was strongly effected to chondroid matrix formation into tumor tissue.

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Immunohistochemistry in Extracellular Matrix

	BMP	laminin	fibronectin	tenascin	GGs
Epithelial tumor cells					
luminal cells	−	−	−～+	−	−
outer cells	+	−	−～+	−	+
modified					
myoepithelial cells	+	+	+	+	+
plasmacytoid cells	+	−～+	+	+	+
Hyalinous tissue	−	−	−	−～+	−～+
Chondroid tissue					
chondroid matrix	−	−	+	+	+
chondrocytes	+	−	−	−	−

Osteogenic Capacity of Bovine Bone Morphogenetic Protein (bBMP) Bound to Bioceramic Materials: Experimental Observations and Clinical Applications

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BMP 复合生物陶瓷成骨性的实验研究和临床应用

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INTRODUCTION

In 1986, Urist reported successful repair of human bone defects by using human BMP. So far, the small quantities of human BMP available, its high price and the rapid absorption of implants of BMP alone in the body preclude its effectiveness as a practical adjunct to clinical treatment. Therefore, a non-human BMP delivery system is essential for clinical applications. There are several reports in the literature which describe observations of the bone-inducing ability of the bovine BMP complexand the different results obtained are dependent upon the delivery system used . In the present report, we have used multipore bioactive glass ceramic (BGC) and multipore ceramic bovine bone (CBB) compounded with bovine BMP (bBMP) in order to evaluate their osteogenic ability in experimental and clinical situations. Successful repair of jaw defects enhanced by implants of the bBMP complex was shown in this report.

MATERIALS AND METHODS

Bovine BMP (bBMP) was extracted and purified according to the procedures previously reported.

Porous bioactive glass ceramic (BGC) particles (pore size 50–120 um; sintered temperature, 950℃ ; ball–shaped, diameter 0.5–2.0mm: supplied by Hua Xi Medical University, Chendu, China) were used. The composition of this BGC by chemical analysis is: P O –SiO –CaO–MgO.Ceramic bovine bone (CBB) was prepared from bovine rib bone according to previously reported procedures .

Preparation of bBMP complex:

The extracted bBMP was dissolved in 4M guanidine hydro–chloride and dialyzed against a large quantity of water, together with BGC or CBB in the dialysis tube. The weight ratio of bBMP / BGC or CBB was about 1:25. After lyophilization, the complex was sterilized with ethylene–oxide gasn.The bone–inducing ability of bBMP–BGC and bBMP–CBB (bioassay):

To investigate osteogenicity of bBMP–BGC and bBMP–CBB, 12 two months adult SD rats were divided into two groups. Group I: bBMP–BGC was implanted into the femoral muscle pouches of six rats, whereas BGC alone was implanted in the contralateral side in the same rats as controls. Group II: bBMP–CBB and CBB alone were implanted into the femoral muscle pouches of the other six rats, using identical methods to those used in Group I. The implants were left in situ for six weeks and then excised, together with the surrounding soft tissues, fixed in 10% formalin for 24 hr, decalcified in hydrochloric acid, embedded in paraffin wax, sectioned at 6um and stained with haematoxylin and eosin for histological examination.

Clinical applications of the bBMP complex:

Eleven patients with intraosseous cysts of the oral region were treated in the Department of Maxillofacial Surgery at the Stomatological College, Xi'an and constituted a consecutive series. Of the 11 cases (see table 1), seven were radicular cysts and four were median maxillary cysts. The size of the cysts was between 1.5–3.0 cm. Of the three control cases, one was implanted with BGC alone, one with CBB alone and the third case was treated without any implant. Eight cysts were placed in the experimental group and were treated with bBMP–complexed bioceramic implants.

Roentgenographic and clinical examinations were carried out at one, three, five, six and 12 months postoperation .

RESULTS

Experimental Animal Procedures:

No new bone formation was found in any of the control groups of rats six

weeks postoperatively. The implants of both BGC and CBB were surrounded by a thin condensed layer of fibrous tissue containing occasional fibroblasts. The tissues identified within the interior pores of BGC or CBB implants were loosely scattered collagen fibres and fibroblastic cells in an irregular arrangement.

In Group I, the newly formed bone tissues were closely connected with the surface of the BGC particles(Fig 1). Usually a dense layer of bone of variable thickness arranged into concentric lamellae with osteocyte lacunae was found surrounding the external surface of the implant. Substantial amounts of new bone were detected developing in isolated foci within the centrally located pores. Newly formed bone tissue was detected throughout the full thickness of the implants of bBMP– BGC and the pores in the central area of the ceramic contained a delicate reticular network of bone(Fig 2). In Group II, newly formed bone was deposited directly onto the surface of the bBMP–CBB implants and often appeared to extend directly toward more central regions. No individual osteogenic foci were detected in the central regions of CBB implants. No obvious signs of resorption of either BGC or CBB by osteoclasts were evident at six weeks postoperation. Tissue around the bBMP– BGC and bBMP–CBB implants was free of obvious inflammatory cells although a mild chronic inflammatory reaction was sometimes present associated with the implants of BGC and CBB.

Clinical Procedures:

All patients treated with the BMP– complex demonstrated the roentgenographic evidence of bone repair to the surgical defects. Follow– up examinations at 3 years after the operation showed no swelling or other abnormalities.

Overall healing time of the intrabony defects of the oral cysts for the eight bBMP treated patients averaged 3.7 months (range 3.0–4.5 months); for the three patients in the control group, the healing time ranged from six to eight months.

The eight patients received bBMP in a dose of 15– 20 mg incorporated with BGC or CBB (bBMP / BGC = 1 / 25). Clinical observations indicated that the bBMP–BGC or bBMP–CBB were well tolerated and the dose of the bBMP–complex was suitable. Except the light swelling of three patients' local wound area in the first three days postimplantation (the swelling disappeared immediately after the third day postimplantation), no patients developed problems with wound healing. There was no clinical or laboratory evidence of excessive hyperaemia or of any immune reactions (fever, leucocytosis, eosinophilia or urticaria) during the healing period. Roentenography showed that implanted

granules of bBMP–BGC and bBMP– CBB became fused together two months after operation. The newly formed bone appeared to develop from the peripheral regions of the host defects. Three months after implantation, bone formation was well advanced and the implant area was almost completely closed(Fig 3) . Four–months after implantation, the intraosseous defects were completely healed(Fig 4). In comparison, defects in all three control patients from the same time period showed only minimum closure . Between six and eight months after implantation, no obvious signs of resorption of the implanted BGC and CBB of both tested and control groups were evident.

DISCUSSIONS

In recent years, many kinds of synthetic biomaterials for bone surgery have been developed and extensive basic research on their properties has been performed . Clinical applications have also been successfully achieved, mainly in orthopedic and oral surgery. Much attention has been paid to calcium phosphate materials. Our results demonstrated that complexed implants of BMP and bioceramic are available for the clinical treatment of bone defects.

Before the clinical application, the bone induction of bBMP complex was observed in animals. The result showed that BGC and CBB provided significant bone inducing ability when they were complexed with bBMP. The surfaces of BGC and CBB were surrounded by new bone, which developed as ingrowths into the center of BGC and CBB. Immune rejection of the synthetic bone grafts was not seen.

Our cases also suggest that bBMP–bioceramic grafts could be used for repair of human bone defects. The synthetic grafts implanted in these patients with bony defects of oral cysts contained BMP in doses of about 15–20 mg. After the operation within two–three months, roentgenographic examination showed new bone formation; by four to five months postoperation, the bonedefects had healed. Compared to the control group, new bone was rapidly induced by BMP–complexed implants in the treatment group. The results showed that the bBMP–BGC and bBMP–CBB implants were an effective delivery system, permitting the differentiation of new bone over an extended period. This may be effected by the relatively slow release of BMP from the complex, even though bBMP was present in small doses.

Klawitter and Hulbert determined that approximately 100 um was the minimum pore size for effective bone ingrowth. Most synthetic porous implant

materials in use have a random pore size of 100–500 um. In an investigation of hydroxyapatite pore sizes, Kawamura et al. reported that 90 um and 200 um pore sizes of BMP / HAP appeared to show much more bone formation than larger pore sizes. Our work shows that a pore size of BGC of 50–120 um was suitable for the new bone ingrowth in the deeper central–regions of the implants. The pore size of CBB appeared to be adequate for bone ingrowth, but was more brittle mechanically than BGC. In addition, if the problems of the resorption of BGC and CBB could be solved, bBMP–BGC and bBMP–CBB could be produced in large quantities and would be an excellent implant material for clinical application.

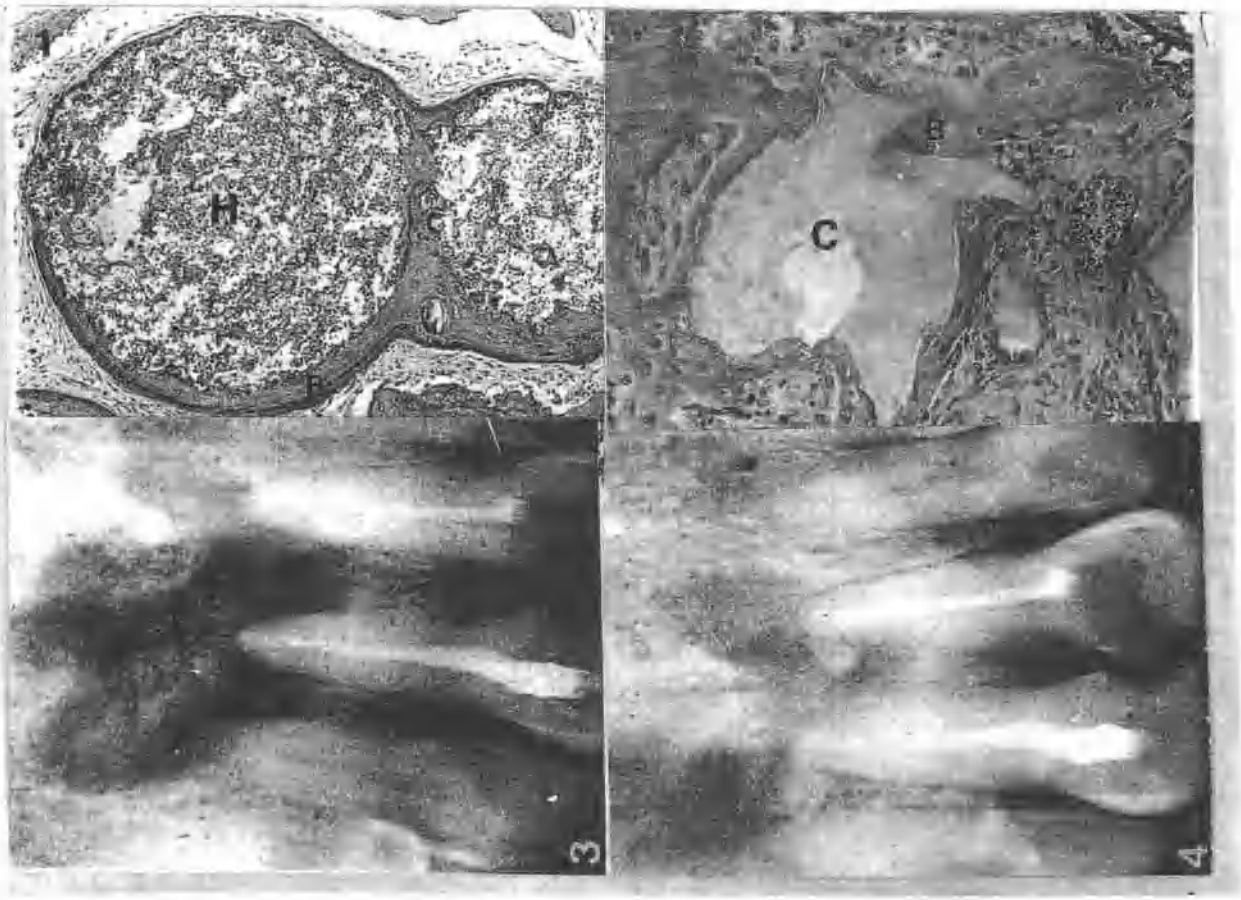
ILLUSTRATION

Fig 1. Four weeks after implantation of BMP / BGC, new bone was found on the surface of BGC, H:HAP, B: new bone, H–E, × 10.

Fig 2. Four weeks after implantation of BMP / BGC, new bone was observed around BGC, C:BGC, B:new bone, H–E, × 10.

Fig 3. Three months after implantation bone formation was well advanced .

Fig 4. Six months after implantation the intraosseous defect was conpletely healed.



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