GLYCOSAMINOGLYCAN DISTRIBUTION IN CHONDRO-OSTEOGENESIS INDUCED BY BONE MORPHOGENETIC PROTEIN IN MOUSE MUSCLE TISSUE WITH THE USE OF MONOCLONAL ANTIBODIES

HITOSHI NAKAMINE, AKIHIDE KAMEGAI, JUN-ICHI YASUDA,
NOBUTAKE KANEMATSU and MASAHICO MORI

*Department of Oral and Maxillofacial Surgery, Asahi University School of Dentistry,
1851-1 Hozumi, Motosu-gun, Gifu 501-02*

Received for publication February 1, 1994 and in revised form May 10,
1994 and re-revised form May 31, 1994

The process of heterotopic bone formation by bone morphogenetic protein (BMP) implanted in mouse muscle tissue was immunohistochemically studied in terms of the detection of glycosaminoglycans (GAGs) (chondroitin 4 sulfate, C4S; chondroitin 6 sulfate, C6S; keratan sulfate, KS; dermatan sulfate, DS; chondroitin sulfate, CS). Following BMP implantation into mouse muscle tissues, undifferentiated mesenchymal cells were found to infiltrate *in situ*, and C4S and KS were highly expressed at the initial phase. On day 5, undifferentiated cells expressed positive immunoreactivity for C4S, C6S, KS, CS, and DS, and chondroid matrices stained positively for C4S, C6S, KS, and DS, but weakly for CS. On day 7, they resemble hyalin cartilage tissues with positive reaction for all GAGs. In the cartilage cells, the expression of KS was particularly prominent. On day 14 to 21, chondro-osseous transformation was progressed with gradual calcification and with reduced stainings for all GAGs in their matrices. The calcified bone tissue indicated negative or trace positive reaction for GAGs. During BMP induced chondro-osteogenesis, the expression and accumulation of GAGs in the intercellular matrix play significant roles in chondrogenesis preceding endochondral ossification.

Heterotopic bone formation has been reported histopathologically in bone induction of salivary pleomorphic adenomas (30), mammary gland tumors (1), and calcifying epithelioma Malherbe (16, 36). Experimental bone induction has likewise been described in urinary bladder transplantation (7, 11). The mechanism underlying bone induction is probably related to metaplastic processes from nonspecific mesenchymal cells.

Numerous reports have been made, indicating BMP induces heterotopic bone formation via chondrogenesis in mouse skeletal muscle tissues (14, 18, 21, 27, 31, 33, 34). Perivascular mesenchymal cells (31, 33), muscle sheath cells, and myoblasts (18, 21, 27, 34) have been suggested as progenitor cells. Histologically, the process of BMP-induced heterotopic bone formation has been recorded (14, 18, 33). Tissues implanted with BMP showed cell infiltrations from the mesenchyme into the implanted areas and the rapid differentiation of these cells into chond-

roidal cells. The intermediate stage of bone synthesis by BMP coincided with the development of chondrocytes. The final bone formation stage was characterized by endochondral ossification.

The organized components of chondro-osteoid matrix were primarily composed of GAGs, with the exception of collagen, and it has been reported that the structural components of cartilage tissues contain several types of GAGs (3, 5, 9, 20, 24, 29). GAGs have been reported to be greatly concerned with ossification cascade (14, 20, 22, 24). In view of these, distribution and localization of GAGs during the course of BMP induced chondro-osteogenesis are supposed to have great concern with cells migration, differentiation, and transformation, thus suggesting matrix macromolecules coordinately regulate bone formation. The aim of the present study was to evaluate the roles of GAGs in the process of BMP induced bone formation.

MATERIALS AND METHODS

BMP was prepared from fresh bovine cortical bones as an aggregate of several insoluble non-collagenous proteins according to Urist's methods (32). To ascertain the activity of the xenogeneic implant, samples were assayed for bone induction in implants of 5 mg crude BMP in thigh muscles of 10 mice. The incidence of bone induction in specimens at 2 week post-implantation was found to be 70% to 90%, according to roentgenographic examinations. The details of this method were described previously (12, 13).

Thirty-five mice (male ICR mouse; 5-week-old) were divided into 7 groups, as 1, 3, 5, 7, 14, 21, and 42 days, and each group consisted of 5 animals. Animals were treated with 5 mg of BMP implanted into the murine thigh muscles. Following roentgenographic examination, the thigh muscle tissues with chondro-

osseous tissues were removed surgically and fixed in 20% formalin adjusted by magnesium carbonate to pH 7.5. Tissue specimens were decalcified in 5% ethylenediaminetetraacetic acid-ethanol solution adjusted by 5 M citric acid to pH 7.2 and for 1 week at 4°C, and embedded in paraffin wax. Serial paraffin sections were cut at a thickness of 4 μ m and stained with hematoxylin and eosin (H-E), PAS, toluidin blue, and GAGs; CS, C4S, C6S, KS, and DS. Immunohistochemical methods for glycosaminoglycans (Tables 1-3): The avidin biotin peroxidase complex (ABC) method was used. Deparaffinized sections were treated for 30 min at 37°C with the following chondroitinase ABC (Seikagaku Kogyo Co., LTD) for CS and C6S, chondroitinase AC II (Seikagaku Kogyo Co., LTD) for C4S, and chondroitinase B (Seikagaku Kogyo Co., LTD) for DS. After rinsing, sections were treated with methanol solution containing 0.3% H₂O₂ for 30 min to block the activity of endogenous peroxidase. Following treatment with normal rabbit serum for 30 min, sections were reacted with antiproteoglycan-OS mouse (1:100, Seikagaku Kogyo Co., LTD), antiproteoglycan-4S mouse (1:100, Seikagaku Kogyo Co., LTD), antichondroitin-6S mouse (1:100, Seikagaku Kogyo Co., LTD), antiproteoglycan-DS mouse (1:100, Seikagaku Kogyo Co., LTD) and antiproteoglycan-KS mouse (1:100, Seikagaku Kogyo Co., LTD) respectively for 1 hr. Sections were rinsed in phosphate buffered saline (PBS) at pH 7.4 and then treated with biotinylated affinity-isolated mouse-immunoglobulin (1:200, Dakopatts, Denmark) for 30 min followed by ABC complex (1:200) for 30 min. For the visualization of the antigen-antibody complex, sections were immersed in 0.03% 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.005% H₂O₂ for 5 min.

TABLE 1. *Monoclonal antibodies to glycosaminoglycans*

monoclonal antibodies	working dilution
1-B-5 (antiOS)	1:100
3-B-3 (anti6S)	1:100
2-B-6 (anti4S)	1:100
5-D-4 (antiKS)	1:100

TABLE 2. *Enzyme treatment*

enzyme	identified
Chondroitinase ABC	CS C6S
Chondroitinase ACII	C4S
Chondroitinase B	DS

TABLE 3. *Immunohistochemical method*

1. Deparaffinization
2. Digestion with enzyme, see Table 2.
3. Inactivation of endogenous peroxidase: methanol with 0.3% H₂O₂, 30 min.
4. Background blocking: normal rabbit serum, 1:20, 30 min.
5. 1st layer: 1 h, see Table 1.
6. 2nd layer: Biotinylated affinity-isolated rabbit IgG to mouse IgG 1:200, 30 min. (Dakopatts, Denmark)
7. AB complex: 1:100, 30 min. (Dakopatts, Denmark)
8. Visualization of peroxidase activity by incubation for 5 min in 0.03% 3,3'-diaminobenzidine tetrahydrochloride/0.05 M Tris buffer solution (pH 7.6) containing 0.005% H₂O₂

FIGS. 1A-C. BMP implanted tissue of the 1st stage (3 days post-implantation). Proliferated undifferentiated mesenchymal cells infiltrated between implanted BMP (BMP) and muscle tissue. Hematoxylin and eosin, original magnification $\times 100$ B. Tissue section of the 2nd stage (7 days post-implantation). Chondroidally changed tissue was visualized. Hematoxylin and eosin, original magnification $\times 100$ C. Tissue section of the 3rd stage. Bone tissue was being formed via endochondral ossification. Hematoxylin and eosin, original magnification $\times 100$

RESULTS

Histopathologic Findings

The process of BMP induced heterotopic bone formation was divided into 3 stages; The 1st stage was characterized by the infiltration of undifferentiated mesenchymal cells (Fig. 1A), the 2nd stage represented chondroidal changes occurring rapidly in fibrous tissue (Fig. 1B), and in the 3rd stage the chondroidally changed tissues were transformed into bone tissues (Fig. 1C). On day 21, bone marrow tissue was formed. On day 42, the induced bone tissues was gradually resorbed with the appearance of numerous osteoclasts.

The detailed findings were as follows; 1) The 1st stage: on day 1 post BMP implantation, undifferentiated cells rapidly infiltrated around the BMP implants and migrated to fibroblast-like cells. The initial tissue response to BMP started with cell growth *in situ*. 2) The 2nd stage: This stage was further subdivided into an early transformation stage on day 4 to 5, and the stage of chondroidal change which began approximately on day 7. In tissue sections on day 5, a rapid transformation into chondroidal cells was displayed in the fibrous tissues. The tissue component consisted of BMP masses, chondroidally changed tissues with hyalin cartilage and fibroblast-like cells exhibiting a marked proliferation potentiality. The BMP-induced osteogenesis was characterized by the

presence of chondroidal tissues on day 4 to 6. Such a chondrocytotic migration at this stage was an onset of bone induction. Tissue sections from day 7 revealed an increase in degree of cartilage formation. 3) The 3rd stage (bone formation stage): In sections on day 14, chondro-osseous structures were noted which were intermediate form between chondroidally changed tissues and calcified bone tissues. However, the center of the BMP implanted foci was not transformed to fibrous or osteo-chondroidal tissues; osteogenesis proceeded in the periphery or outer zone of chondroidally changed tissues. Histologically, the architecture of BMP induced bone could be divided into 1) an outer lamellated zone, 2) an osteo-chondroidal zone or a hyalin cartilage zone in the intermediate region, 3) an amorphous matrix of cartilage in an inner zone, and 4) a homogeneous, acellular region as a center of the mass. Most of the central regions was composed of homogeneous and acellular structures showing diffuse stainings for toluidin blue but exhibiting negative PAS reaction. The calcified bone revealed a lamellated structure with a fibrous capsule adjacent to muscle tissues. The osteo-chondroidal structures stained markedly with toluidin blue and slightly for PAS. Cartilage cells showed histological similarity to those of the transforming zone in epiphyseal cartilage. The hyalin cartilage zone contained well developed cartilage cells and stained homogeneously with hematoxylin and eosin, and toluidin blue but

TABLE 4. Glycosaminoglycans staining pattern of chondro-osteogenesis induced by BMP

	CS	C4S	C6S	KS	DS
The 1st Stage (1 to 3 days)					
Undifferentiated mesenchymal cells	0	+4	0	0	0
BMP implanted tissue ground substances	0	+4	±	±	0
Infiltrating cells, in BMP implanted area	0	0	0	+4	0
The 2nd Stage					
Early Transformed Stage (4 to 7 days)					
Transforming fibrous cells	0	+2	+2	+4	+2
Chondroidally changed cells	+1	+2	+2	+4	+3
Chondroidal matrices	±	+2	+2	+1	+2
Chondroidally Changed Stage (7 days)					
Chondroidally changed cells	±	0/+1	0/+2	+4	+3
Chondroid matrices	0/+2	+3	+2	+1	+2
The 3rd stage (14 to 21 days)					
Chondroidal cells	±/+2	+1	+1	0/+3	+1
Chondroidal matrices	0/+3	0/+3	0/+2	0/+2	+1/+4
Calcifying tissue	0	0	+1	0	0/+2

0: negative ±: trace +1: slight +2: mild +3: moderate +4: strong

FIGS. 2A-C. BMP implanted tissue on day 3. Border zones between muscle tissue and BMP implanted tissue stained conspicuously for C4S (m: muscle tissue, BMP: BMP implanted). B. BMP implanted tissue on day 3 serial section of (A). BMP implanted tissue showed homogeneous and intense staining for C4S. C. BMP implanted tissue on day 1 post implantation. Infiltrating cells showed an abundant staining reaction for KS. Bars A, B=100 μ m C=50 μ m

without positive PAS reaction.

Glycosaminoglycans Immunohistochemistry (Table 4)

1) The 1st stage: Homogeneous materials including BMP masses and undifferentiated mesenchymal cells infiltrating in the BMP implanted area stained strongly only for C4S (Figs. 2A, B). The infiltration area exhibited a limited number of cells that stained strongly with KS (Fig. 2C).

2) The 2nd stage: On day 5 chondroidally changed cells and infiltrated mesenchymal or fibrous cells stained markedly for KS, whereas the chondroid

matrix was stained slightly for KS (Figs. 3A, B). These cells revealed mild immunoreactivity for C6S (Fig. 3C), C4S (Fig. 3D), and DS. In tissue sections on day 7, cartilage tissue formation was more advanced (Fig. 4A). In the hyalin cartilage, cartilage cells were strongly reactive for KS staining (Fig. 4B), and moderately positive for DS staining (Fig. 4C); whereas the cartilage matrix was not so vividly reactive for both KS and DS (Figs. 4B, C). In the same specimen, only a trace staining for CS was seen in cartilage cells (Fig. 4D). C4S and DS were specifically reacted in the car-

FIGS. 3A-D. BMP implanted tissue on day 5.

A. Fibrous and chondroidally metaplastic cells stained abundantly for KS. B. Chondroidally changed tissue and implanted BMP. Most chondroidally changed cells showed high KS staining. C. Similar region as in (A). Matrix in chondroidally metaplastic tissue stained mildly for C6S. D. Similar region as seen in (B). Matrices in chondroidally changed tissue stained mildly for C4S. Bars=100 μ m

FIGS. 4A-F. BMP implanted tissue changed to hyalin cartilage tissue on day 7. A. Chondroidally changed tissue. H&E staining. B. Serial section of (A). Chondrocytes showed abundant staining for KS; whereas, matrices stained slightly for KS. C. Serial section of (A) and (B). Chondrocytes stained positive reaction for DS and the surrounding matrices stained mildly. D. Early forming cartilage tissue stained weakly for CS in restricted areas. E. Developing cartilage matrices and associated fibrous tissue stained positive for C4S. F. Matrices of chondroidally changed tissue stained positively for DS. Bars = 100 μ m

FIGS. 5A-F. Chondroidally changed tissue on day 14. A. Toluidin blue staining. Chondroidally changed cells gradually transformed to osseous tissue as in the outer zone which is an area of calcified bone formation. B. Cartilage matrices showed moderately positive staining for C4S. C. Chondroidally changed tissue usually showed slight or mild reaction for C6S. D. Developing cartilage tissue stained for CS at moderate levels; whereas, the peripheral matrices were unstained. E. Matrix forming zone near chondroidally changed tissue showed the highest DS staining. F. Cartilage cells in chondroidally changed tissue stained positively for KS, and the matrix forming zone stained weakly. Bars A=250 μ m B-E=100 μ m F=50 μ m

tilage matrix (Figs. 4E, F), while the reaction products for C6S were mild in the matrix.

3) The 3rd stage: The chondro-osteoid tissues were characterized by the presence of particular histochemical staining patterns for GAGs during BMP-osteogenesis in the muscle tissues. The cartilage cells, undergoing the calcifying process, stained strongly with toluidin blue (Fig. 5A) and PAS. The induced cartilage tissues exhibited positive GAGs staining for cells or matrix on day 14 (Figs. 5B-F); highly accumulated of DS, C4S, and CS in the extracellular matrix, and KS and CS in chondrocytes. In particular, the staining for DS was markedly expressed in the calcification front (Fig. 5E). In serial sections stained for toluidin blue and PAS, the homogeneous

acellular matrix representing a matrix forming zone for cartilage, stained moderately for DS (Fig. 5E), mildly for KS (Fig. 5F) and weakly for C6S and C4S. In sections demonstrating the processes of bone formation, the zones of cartilage tissues, osteoid formation zones and zones of calcified bone could clearly be differentiated (Fig. 5E). The KS staining of cartilage cells was vivid in the proliferating zone, less marked in the cartilage matrix, and negative in the osteoid tissues (Fig. 5F). In serial sections the DS staining was positive in the cartilage matrix of the chondro-osseous transforming zone, whereas it was negative in the osteoid tissues (Fig. 6A). The staining reaction for C6S was diffusely distributed at the border between chondroidal and osseous structures (Fig. 6B).

FIGS. 6A, B. Chondroidally changed tissue on day 14. A. Cartilage matrices and matrix forming cartilage showed moderate staining for DS. B. Border tissue from chondroid matrix formation stained positively for C6S. Bars = 50 μ m

DISCUSSION

Classic histochemical observations on proteoglycans (PGs) or GAGs have been made using alcian blue stainings in combination with specific enzyme digestions (5, 9), and monoclonal antibodies (MoAbs) specific for epitope(s) of GAGs combined with enzyme digestion techniques have recently been in general employed as well (2, 4, 17, 19, 22, 23, 26, 28, 29). These investigations have provided significant supplemental information to previously identified PGs and GAGs by biochemical evaluations. In addition the antigenicity of the matrices in cartilage and mineralized tissues has been increasingly clarified, using specific antibodies (2, 3, 29).

The present immunohistochemical study for the identification of C6S, C4S, KS, and DS during BMP-induced osteogenesis has elucidated the following significant features. First, BMP-induced bone was synthesized via chondroidal osteogenesis, and secondly, the tissues of BMP-induced chondro-osteogenesis expressed GAGs both in cartilage cells and matrices. Thus, GAGs were found to occur specifically in the 1st stage of the BMP-implanted area. Fibrous or metaplastic ossification was reported to occur in connective tissue cells as seen in tumor stromal ossification (15, 16, 18, 21) or experimental bone induction by urinary bladder transplantation (7, 11). It is significant that BMP does not directly induce the replacement of bone tissues, but BMP directly induces the proliferation of neighboring undifferentiated mesenchyme cells, and these cells then induce chondroidal metaplasia. In mammals, heterotopic cartilage formation was found in salivary pleomorphic adenomas (30, 37), adenocarcinoma (6), and skin mixed tumors (38), with the expression of BMP in modified myoepithelial cells.

Specific PGs or GAGs could be identified by means of MoAbs accompanied by specific enzyme (chondroitinase ABC, chondroitinase AC II, and chondroitinase B) digestion techniques (2). The structures, functions, and biological significance of GAGs have widely been reviewed in that CS is usually contained in cartilage tissues and blood vessel walls, DS in tendon and skin, heparan sulfate in the cell surface and basement membrane, and KS in the corneal stroma (10). In arterial tissues, CS was identified as a primary structural component (17); however, KS was identified as a major component of the atherosclerotic lesion of the aortas in pigeons (25). In mammals, KS has been reported as a major component of the cornea matrix and of cartilage tissues biochemically (9). From an immunohistochemical and ultrastructural

standpoint, the synthesis of cartilage matrix and the role of GAGs have been investigated in epiphyseal cartilage (29), articular cartilage calcification processing of type II collagen (23). Mark *et al.* (19) reported 5 kinds of MoAbs used to detect the CS groups involved in regulated changes during embryogenesis. These authors noted that the staining with MC21C antibody (IgM, 6S segment) decreased in intensity in morphogenesis of the bone. Mark *et al.* (19) have likewise noted that aggregation of C4S and C6S in odontogenesis are associated with developmental regulation and play a role in odontogenesis in mouse tooth germs. In the initial phase of BMP chondro-osteogenesis, GAGs expression was evident in terms of pronounced accumulation of C4S. The positive staining for C4S was found to be a direct response to chondroid matrix synthesis.

The distribution pattern of GAGs in the 2nd stage resembled that of chondroidally changed cells in salivary pleomorphic adenomas, where a structural similarity has been noted to hyalin cartilage (26). In the BMP-induced chondrogenesis, GAGs expression was predominantly KS and DS, slightly for C6S and C4S, and negative for CS. During BMP-induced chondro-osteogenesis, the most intense expression was found in KS and it was confined to cartilage cells; whereas the DS expression was distributed in the cartilage matrix. With few exceptions, the immunohistochemical localizations of KS and DS were different in chondroidally changed tissues in early developmental stages. The GAGs-synthesizing phase was the most significant feature of the cartilage formation and cartilage matrix synthesis in the BMP-induced chondro-osteogenesis. From cartilage tissues to bone formation via endochondral ossification, the staining for KS and DS gradually declined in intensity and was finally rendered negative with the formation of bone tissues. It has been reported that GAGs regulate that growth or deposition of hydroxyapatite and calcium phosphate formation (3, 8). In this regard, Takagi *et al.* (28), using 4 MoAbs and 1 PoAbs, reported immunohistochemical reactions illustrating definite differences between GAGs of predentin and dentin. These authors demonstrated a selective decrease in staining intensity or molecular alteration of GAGs during dentinogenesis and showed mineralization to be associated with enzymatic degradations of GAGs, main matrix substances. From the results obtained in the present study, it is stated conclusively that proteoglycan synthesis by mesenchymal and chondroidally changed cells for the production of ground substances could be involved in BMP-induced chondro-osteogenesis.

REFERENCES

- Ashley, D. J. B.: Evan's historical appearance of tumors of the mammary glands, *Tumors of the Mammary Grands*, C Livingstone, 1978, pp. 372-409.
- Coughman, J. R., Caterson, B., Christner, J. E. and Baker, J. R.: Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissue. *Nature* 307; 650-652, 1984.
- Dziewiatkowski, D. D. and Majznerski, L. L.: Role of proteoglycans in endochondral ossification: Inhibition of calcification. *Calcif. Tissue Int.* 37; 560-564, 1985.
- Funderbruch, J. L., Caterson, B. and Conrad, G. W.: Distribution of proteoglycans antigenically related to corneal keratan sulfate proteoglycan. *J. Biochem.* 262; 1634-1640, 1987.
- Hasty, K. A., Smith, G. N. Jr. and Kang, A. H.: Histochemical identification of sulfation position in chondroitin sulfate in various cartilages. *J. Histochem. Cytochem.* 31; 1367-1374, 1983.
- Hatakeyama, S., Ohara-Nemoto, Y., Kyakumoto, S. and Satoh, M.: Expression of bone morphogenetic protein in human adenocarcinoma cell line. *Biochem. Biophys. Res. Commun.* 190; 695-701, 1993.
- Huggins, C. B.: The formation of bone under the influence of epithelium of the urinary tract. *Arch. Surg.* 22; 377-408, 1931.
- Hunter, G. K., Wong, K. S. and Kim, J. J.: Binding calcium to glycosaminoglycans: An equilibrium dialysis study. *Arch. Biochem. Biophys.* 206; 161-167, 1988.
- Ippolito, E., Pedrini, V. A. and Pedrini-Mille, A.: Histochemical properties cartilage proteoglycans. *J. Histochem. Cytochem.* 31; 53-61, 1983.
- Iozzo, R. V.: Proteoglycan: Structure, function, and role in neoplasia. *Lab. Invest.* 53; 373-396, 1985.
- Kagawa, S.: Enzyme histochemistry of bone induction by urinary bladder epithelium. *J. Histochem. Cytochem.* 13; 255-264, 1965.
- Kamegai, A., Muramatsu, Y., Tanabe, T., Mori, M. and Inoue, S.: Immunohistochemical demonstration of S-100 protein in cartilage tissue induced by electric stimulation or by bone morphogenetic protein. *Acta Histochem. Cytochem.* 23; 209-218, 1990.
- Kamegai, A., Tanabe, T., Nagahara, K., Kumasa, S. and Mori, M.: Pathologic and enzyme histochemical studies on bone formation induced by bone morphogenetic protein in mouse muscle tissue. *Acta Histochem.* 89; 25-35, 1990.
- Kamegai, A., Tanabe, T., Shimamura, N., Kumasa, S., Yamada, K. and Mori, M.: Immunohistochemical and enzyme histochemical studies in bone formation induced by bone morphogenetic protein (BMP) in mouse muscle tissue. In "Mechanisms and Phylogeny of Mineralization in Biological Systems", ed. by S. Suga and H. Nakahara, Springer-Verlag, Tokyo, 1991, pp. 111-115.
- Kumasa, S., Mori, H., Mori, M., Shibutani, T., Iwayama, Y., Tsujimura, T., Ohnishi, T., Araki, N., Nakata, M. and Kurisu, K.: Heterotopic bone formation in tumor stromal tissue. —Immunohistochemical considerations—. *Acta Histochem. Cytochem.* 23; 427-439, 1990.
- Kumasa, S., Mori, H., Tsujimura, T. and Mori, M.: Calcifying epithelioma of Malherbe with ossification. Special reference to lectin binding and immunohistochemistry of ossified sites. *J. Cutan. Pathol.* 14; 181-187, 1987.
- Lark, M. W., Yeo, T-K, Mar, H., Lara, S., Hellström, I., Hellström, K. E. and Wight, T. N.: Arterial chondroitin sulfate proteoglycan: Localization with a monoclonal antibody. *J. Histochem. Cytochem.* 36; 1211-1221, 1988.
- Mahy, P. R. and Urist, M. R.: Experimental heterotopic bone formation induced by bone morphogenetic protein and recombinant human interleukin-1B. *Clin. Orthop.* 237; 236-244, 1988.
- Mark, M. P., Baker, J. R., Kimata, K. and Ruch, J-V: Regulated change in chondroitin sulfation during embryogenesis: An immunohistochemical approach. *Dev. Biol.* 34; 191-204, 1990.
- Nathanson, M. A.: Proteoglycan synthesis by skeletal muscle undergoing bone matrix-directed transformation into cartilage in vitro. *J. Biol. Chem.* 258; 10325-10334, 1983.
- Nathanson, M. A.: Bone matrix-directed chondrogenesis of muscle in vitro. *Clin. Orthop.* 200; 142-158, 1985.
- Poole, A. R., Webber, C., Pidoux, I., Choi, H. and Rosenberg, I. C.: Localization of a dermatan sulfate proteoglycan (DS-PG II) in cartilage and the presence of an immunologically related species in other tissues. *J. Histochem. Cytochem.* 34; 619-625, 1986.
- Ratcliffe, A., Fryer, P. R. and Hardingham, T. E.: The distribution of aggregating proteoglycans in articular cartilage. *J. Histochem. Cytochem.* 32; 193-201, 1984.
- Reddi, A. H., Hascall, V. C. and Hascall, G. K.: Changes in proteoglycan types during matrix-induced cartilage and bone development. *J. Biol. Chem.* 253; 2429-2436, 1978.
- Robbins, R. A., Wagner, W. D., Sawyer, L. M. and Caterson, B.: Immunolocalization of proteoglycan types in aortas of pigeons with spontaneous or direct induced atherosclerosis. *Am. J. Pathol.* 134; 615-626, 1989.
- Sibutani, T., Iwayama, Y., Tsuboe, M., Ando, C., Yamada, K. and Mori, M.: Immunohistochemical localization of glycosaminoglycans with the use of monoclonal antibodies in salivary pleomorphic adenoma. *Anticancer Res.* 10; 1533-1542, 1990.
- Syftestad, G. T. and Urist, M. R.: Growth hormone dependent matrix-induced heterotopic bone formation. *Proc. Soc. Exper. Biol. Med.* 163; 411-415, 1980.
- Takagi, M., Hishikawa, H., Hosokawa, Y., Kawai, A. and Rahemtulla, F.: Immunohistochemical localization of glycosaminoglycans and proteoglycans in pre-dentin and dentin of rat incisors. *J. Histochem. Cytochem.* 38; 319-324, 1990.
- Takagi, M., Parmley, R. T. and Denys, F. R.: Ultrastructural cytochemistry and immunocytochemistry

- of proteoglycans associated with epiphyseal cartilage calcification. *J. Histochem. Cytochem.* 31; 1089-1100, 1983.
30. Thackray, A. C. and Lucas, R. B.: Tumors of the major salivary glands; Pleomorphic adenoma. In "Atlas of Tumor Pathology, 2nd series, Fascicle 10", ed. by A. C. Thackray and R. B. Lucas, AFIP, Washington, 1974, pp. 16-39.
31. Urist, M. R.: Surface-decalcified allogenic bone (SDAB) implants. *Clin. Orthop.* 56; 37-50, 1968.
32. Urist, M. R., Chang, J. J., Lietze, A., Huo, Y. K., Brownell, A. G. and DeLange, R. J.: Preparation and bioassay of bone morphogenetic protein and polypeptide fragment. *Methods Enzymol.* 146; 294-312, 1987.
33. Urist, M. R., DeLange, R. L. and Finerman, G. A. M.: Bone cell differentiation and growth factors. *Science* 220; 680-686, 1983.
34. Urist, M. R., Terashima, Y., Nakagawa, M. and Stomas, C.: Cartilage tissue differentiation from mesenchymal cells derived from mature muscle in tissue culture. *In Vitro* 14; 697-706, 1978.
35. Vertel, B. M., Morrel, J. J. and Barkman, L. L.: Immunofluorescence on cartilage matrix synthesis. The synthesis of link protein, chondroitin sulfate proteoglycan monomer and type II collagen. *Exp. Cell Res.* 158; 423-432, 1985.
36. Wiederserg, H.: Das Epithelioma Calcificans. *Dermatol. Monatsschr.* 157; 867-883, 1971.
37. Yang, L. J., Jin, Y., Nakamine, H., Sumitomo, S., Kamegai, A. and Mori, M.: An immunohistochemical study of bone morphogenetic protein (BMP) in pleomorphic adenoma of salivary gland. *Virchows Arch. A* 422; 439-443, 1993.
38. Yang, L. J., Nakamine, H., Kamegai, A., Sumitomo, S. and Mori, M.: Immunohistochemical evaluation of bone morphogenetic protein (BMP) in mixed tumor of skin. *J. Dermatol. Sci.* 1994, in press.