HETEROTOPIC BONE FORMATION IN TUMOR STROMAL TISSUE —IMMUNOHISTOCHEMICAL CONSIDERATIONS—

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Ectopic and heterotopic bone formation rarely occur in stromal tissues of various epithelial tumors. In the present study, ectopic bone forming tumors; calcifying epithelioma Malherbe (6), renal cell carcinoma (1), thyroid adenocarcinoma (1) and ovary carcinoma (1) were examined. The process of ectopic bone formation could be classified into 1) bone formation related to epithelial tissue as found in calcifying epithelioma of Malherbe, 2) bone formation from perivascular smooth muscle, and 3) that due to metaplasia occurring in stromal connective tissue of epithelial tumors. Immunohistochemical detection of proteoglycans (PG) was made in bone-forming matrix areas, and these areas were positive for chondroitin 4 and 6 sulfate, and dermatan and heparan sulfate PGs. Immunohistochemically, fibronectin, collagen III and bone sialoprotein as matrix proteins, and carbonic anhydrase as calcification marker were tested and they appeared in these pre-calcifying tissues.

It is a well-known phenomenon that heterotopic or metaplastic bone formation occurs in extraskelatal tissues. In general, extraskelatal tissue bone formation can be divided into primary or heterotopic and secondary or metaplastic. In the case of heterotopic ossification, there are no preceding lesions, whereas in metaplastic bone formation, there are preexisting lesions. Examples of heterotopic ossification are osteoma cutis (2, 11, 14), Albright's syndrome ossification (3), calcifying epithelioma Malherbe (16), stromal ossification in tumors of the ovary, kidney and breast (1, 8-10), and also experimental bone induction in transplanted bladder mucosa (6, 12, 15).

It was reported earlier that the biological mechanism of bone formation in calcifying epithelioma Malherbe was related to lectin-binding materials accumulated in pre-ossifying zones as well as positive staining of carbonic anhydrase activity in the same areas (16). With regard to ossification and matrix formation, proteoglycans and bone

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sialoprotein accumulation might be one of the important factors in initial mineralization (13, 18, 23). Bone matrix is composed of proteoglycans (PGs), such as chondroitin sulfate PG (CSPG), dermatan sulfate PG (DSPG), heparan sulfate PG (HSPG), and keratan sulfate PG (KSPG) (5), and they are also contained in blood vessel walls, and smooth muscles (4, 17, 18), cartilage matrix (81, 23, 24), and so on (7, 13). Identification of these PGs has already been described immunohistochemically by the use of specific monoclonal antibodies accompanied with enzymatic digestion (5). Immunohistochemical evaluation of PGs and bone sialoprotein in bone and cartilage tissues is of importance to understand the mechanism of ossification or calcification in bone-forming sites.

We examined immunohistochemically stromal ossification areas in various types of epithelial tumors using PGs, fibronectin, collagen III, bone sialoprotein, and carbonic anhydrase.

MATERIALS AND METHODS

Materials were selected from epithelial tumors that had already been shown to exhibit bone formation histologically or sometimes radiographically. A total of 9 cases of tumors with ossification and a case of calcification were used (Table 1). Tissue specimens fixed with 10% formalin were embedded in paraffin and 4 μ m serial sections were made for pathological examination with H&E stain, as well as for detection of PGs, fibronectin, collagen III, bone sialoprotein, and carbonic anhydrase I.

Immunohistochemical Methods

Proteoglycans (PGs):

Immunohistochemical method for detection of PGs was that described by Couchman *et al.* (5). Monoclonal antibodies (MoAbs) 1-B-5 (antiOS, antiPG Di-0; SEIKAGAKU KOGYO CO., LTD, Tokyo, Japan) 1 : 100, 3-B-3 (anti6s, antiPG Di-6; SEIKAGAKU KOGYO CO., LTD, Tokyo, Japan) 1 : 100, and 9-A-2 (anti4S, antiPGi-4; SEIKAGAKU KOGYO CO., LTD, Tokyo, Japan) 1 : 100 were used in combination with chondroitinase ABC, chondroitinase AC II, and chondroitinase B predigestions (Table 2). Deparaffinized sections were firstly incubated with above enzymes (2.5 μ /ml, 2.5 μ /ml, and 0.05 μ /ml, respectively; SEIKAGAKU KOGYO CO., LTD, Tokyo, Japan) for 1 hr at 37°C. After enzymatic pretreatments, the sections were immersed with normal goat serum and 0.01 M PBS (pH 7.2) containing

TABLE 1. *Heterotopic bone formation in tumor*

tumor	No. of case
Calcifying Epithelioma	6
Renal Cell Carcinoma	1
Thyroid Adenocarcinoma	1
Ovary Carcinoma	1
total	9

TABLE 2. *Assay method for each of the chondroitin sulfates*

Enzyme	Antibody	Proteoglycan
Chondroitinase ABC	1B5 (anti0S)	Chondroitin
	3B3 (anti6S)	Chondroitin 6S (CS6-PG)
	9A2 (anti4S)	Chondroitin 4S (CS4-PG)
		Dermatan Sulfate (DSPG)
Chondroitinase AC II	9A2 (anti4S)	Chondroitin 4S (CS4-PG)
Chondroitinase B	9A2 (anti4S)	Dermatan sulfate (DSPG)

0.3% H_2O_2 for 20 min in order to block nonspecific peroxidase. The ABC method for identifying PGs was used. With the use of the 3 MoAbs combined with the 3 enzymatic predigestion procedures, immunohistochemical identification of chondroitin PG (CPG), chondroitin 4S and 6S PG (C4SPG, C6SPG), dermatan sulfate PG (DSPG), keratan sulfate PG (KSPG), and heparan sulfate (HSPG) was accomplished. Fibronectin and Carbonic anhydrase I:

The PAP method, using anti-human fibronectin (1 : 100 dilution; Dakopatts, Copenhagen, Denmark) and anti-human erythrocyte carbonic anhydrase I (CA I) (1 : 100 dilution; Green Cross, Kobe, Japan) as the first antibody, was used to detect fibronectin and CA I.

Collagen III:

Antibody to calf skin type III procollagen was raised in guinea pigs (22, 27). Deparaffinized sections were incubated in a 0.025% solution of protease type VII (Sigma, St. Louis, USA) in phosphate buffered saline, pH 7.3 (PBS) for 20 min at room temperature. They were then treated with methanol solution containing 0.3% H_2O_2 in order to inactivate endogenous peroxidase. Following treatment with normal rabbit serum (1 : 20; Wheaton, USA) for 30 min, the sections were reacted with the type III procollagen antibody (1 : 100) for 1 hr, rinsed with PBS, and then treated with horseradish peroxidase-conjugated rabbit anti guinea pig IgG (1 : 40; Dakopatts, Denmark) for 30 min. For visualization of the antigen-antibody complex, the sections were immersed in 0.03% 3, 3'-diaminobenzidine tetrahydrochloride solution containing H_2O_2 for 5 min.

Bone sialoprotein:

Mouse monoclonal antibody to bone sialoprotein was used by the ABC method. Bone sialoprotein, which contains sialic acid but no phosphorous, was isolated from the rat mandible (20). The antibody was raised against the bone sialoprotein using a similar protocol (21). The molecular mass of this sialoprotein was 59,000 dalton by SDS-polyacrylamide gel electrophoresis.

Controls: Primary polyclonal or monoclonal antibodies were substituted by normal rabbit serum or normal mouse serum, respectively. These controls were negative.

RESULTS

Calcifying epithelioma Malherbe

Six cases of bone-forming calcifying epitheliomas were examined, and 3 of them showed positive staining for PGs.

Ossification occurred adjacent to or within the areas of shadow cells (Figs. 1A and 2A). The border zone between newly formed bone tissue and connective tissue contained eosinophilic amorphous material (Fig. 1A), as well as collagenous fibers and osteoblast-like cells (Fig. 2A). Immunodepositions denoting PGs were found in border zones adjacent to newly formed bone: C4SPG, C6SPG, and DSPG were strongly stained (Figs. 1B and 1C), and collagenous fibers near bone-forming areas showed positive staining for C4SPG and DSPG (Fig. 1B) but were negative for CPG and HSPG. Connective tissue near the border zone stained positively for carbonic anhydrase I, collagen III and fibronectin (Figs. 2B, 2C, and 2D).

Renal cell carcinoma

The tissue specimens of clear cell carcinoma of the kidney were divided into 3 parts, and all three parts showed intense ossification in tumor stromal tissue as well as in perivascular tissue. Ossification always started within the stromal connective tissue; however immunohistochemically detectable PGs were not found in stromal tissue. There was evidence of marked proliferation of fibrous tissue adjacent to newly forming bones (Fig. 3A). This fibrous tissue and areas showing transformation to bone structures stained intensely for C4SPG and DSPG, and slightly for C6SPG (Fig. 3B). In more mature bone tissue areas (Fig. 4A), the fibrous tissue stained less intensely for PGs compared with those of proliferating fibrous tissue, and newly formed bone was strongly positive for C4SPG and moderately to slightly so for C6SPG and DSPG (Fig. 4B).

There were many areas showing various degrees of perivascular bone formation. Staining for PGs in perivascular smooth muscle was most prominent for DSPG, slight for C6SPG and C4SPG (Figs. 5A, 5B, 5C), and negative for KSPG. Calcified or mature bone tissue showed variable deposition of PGs, but staining intensities were slight, whereas proliferating fibrous tissue adjacent to newly formed bone stained intensely for DSPG. No specific distribution of fibronectin or collagen III was found in stromal connective tissue or in ossification areas. Bone sialoprotein was detected in matrix forming structure, which coincided with the presence of PGs there (Fig. 5D).

Irregularly sized trabecular bones were usually found in the stromal tissue or renal carcinomas. Staining reactions for PGs were confined to immature or bone-forming matrix tissue (Fig. 6D), and were absent in connective tissue fibers and calcified bones. Staining reactions of C6SPG, C4SPG, and DSPG were abundant in the former tissues (Figs. 6A, 6B, and C). Areas positive for bone sialoprotein coincided

FIGS. 1A-C. Calcifying epithelioma Malherbe with ossification. $\times 80$

FIG. 1A. H&E staining. Ossification occurred adjacent to shadow cells, and periphery of newly bone formation show eosinophilic zones and contains osteoclastic cells.

FIG. 1B. Immunostaining of chondroitin 4 sulfate proteoglycan (C4SPG). Peripheral zones of newly bone formations stain positively, connective tissue fibers in stromal tissue also stain with C4SPG.

FIG. 1C. Immunostaining of dermatan sulfate proteoglycan (DSPG). Bone forming zones at the periphery of newly formed bone structures stain intensely.

FIGS. 2A-D. Calcifying epithelioma Malherbe with ossification. $\times 70$

FIG. 2A. Histologic aspects reveal bone formation, shadow cells and connective tissue stroma.

Active bone formation area shows presence of osteoblastic cells (H&E).

FIG. 2B. Carbonic anhydrase I. Border zone of ossification indicates positive reaction.

FIG. 2C. Collagen III staining. Stromal tissue (S) entirely stains with collagen III, and periphery of bone formation areas shows more intense staining (arrows).

FIG. 2D. Fibronectin staining. Similar finding as seen in collagen III staining is found.

with those positive for PGs (Fig. 6E).

Thyroid adenocarcinoma

Papillary carcinoma showed a large amount of bone formation in the tumor stroma. Perivascular smooth muscle stained intensely for C6SPG and slightly for CPG. Connective tissue fibers adjacent to or near the bone formation showed positive reactions for CPG, C4SPG, C6SPG and DSPG. The periphery of newly formed bone tissue stained for C4SPG intensely. No staining reaction for KSPG and HSPG was found in connective tissue or immature bone. Small follicles of the tumor indicated positive staining for C6SPG in limited parts. The border zone between newly forming bone and connective tissue indicated positive reaction of fibronectin, and collagen III.

FIG. 3A. Bone tissue transformation from fibrous stromal tissue in renal carcinoma. Bone tissue is irregularly stained with hematoxylin. $\times 40$

FIG. 3B. Dermatan sulfate proteoglycan (DSPG) staining. Fibrous connective tissue (F), pre-existing fibers to bone formation tissue indicate strongly positive staining to DSPG. $\times 40$

Ovarian carcinoma

Ovarian osteogenesis was found in the corpus albicans. Staining reactions for all PGs examined were absent in the bone structures. The bone tissue contained basophilic amorphous material and eosinophilic mature type of lamellar bone.

Tonsillar calcification

Calcified materials were located in large necrotic areas and then dystrophic calcification. Almost all of the PGs were detected in the periphery of necrotic masses; the highest staining intensity was given by DSPG, strong reactions occurred for C4SPG, C6SPG, and KSPG, and HSPG was only slight stained. Amorphous material in the central necrosis stained for DSPG at a slight-to-moderate level.

FIG. 4A. Bone forming area of renal cell carcinoma. There are irregular sized bone tissue in the stroma (H&E). $\times 40$

FIG. 4B. Dermatan sulfate proteoglycan (DSPG) stain. Uncalcified or newly forming bones stain with contain DSPG (arrow), and calcified bones stain slightly. $\times 40$

DISCUSSION

Bone formation is dependent on local pH, concentrations of calcium,

FIG. 5A. Perivascular smooth muscle ossification in stromal tissue of renal carcinoma. There are numerous dilated vessels and irregular trabecular bone formations (H&E). $\times 32$

FIG. 5B. Chondroitin 4 sulfate proteoglycan (C4SPG) staining. Certain areas of bone structure stain markedly whereas other bony areas and perivascular structures stain slightly. $\times 32$

FIG. 5C. Dermatan sulfate proteoglycan (DSPG) staining. Non-calcified bone forming area, perivascular smooth muscle zone and immature bone tissue stain abundantly. $\times 32$

FIG. 5D. Bone sialoprotein immunostaining. Slightly positive deposition is coincided with strongly positive staining area with DSPG. $\times 32$

FIGS. 6A-E. Stromal metaplastic ossification in renal carcinoma.

FIG. 6A. Chondroitin 6 sulfate proteoglycan (C6SPG) staining. $\times 80$

FIG. 6B. Chondroitin 4 sulfate proteoglycan (C4SPG) staining. $\times 80$

FIG. 6C. Dermatan sulfate proteoglycan (DSPG) staining. Newly forming bone structures stain markedly with C6S6PG, C4S4PG and DSPG. $\times 80$

FIG. 6D. Dermatan sulfate proteoglycan (DSPG) staining. Bone forming zone at periphery of bone tissue and irregularly matrix forming area stain markedly. $\times 32$

FIG. 6E. Bone sialoprotein staining. Bone matrix forming area shows positive staining. $\times 32$

phosphorus, and citrate ions, vitamin D-binding proteins, calcium-binding proteins, presence of alkaline phosphatase and carbonic anhydrase activities, and certain inducing factors. These conditions might participate in either normal bone formation in skeletal tissue or heterotopic ossification. Bone induction is known to occur in transplanted bladder epithelium, and epithelial structures and basement membrane are probably involved actively in this induction (6, 12, 15).

In the case of ossification in calcifying epitheliomas, Burgdorf and Nasemann (2) found 35 cutaneous osteomas of which 7 were classified as calcifying epithelioma Malherbe. The mechanism of bone formation should be similar to that of bone induction in transplanted bladder epithelium. That is, the presence of shadow cells and then basement membrane might be necessary for production of important induction factors for bone formation, as well as lead to induction of osteoprogenitor cells from connective tissue. It was shown earlier that histogenesis of this type of heterotopic bone formation is associated with accumulation of lectin-binding materials (complex carbohydrates) and carbonic anhydrase activity, which promote initial calcification (16). The location of these lectin-binding sites coincides with that of PGs positive areas found in the present study. Also precalcified bone tissue particularly exhibited to PGs and bone sialoprotein, as found in the normal bone formation.

Metaplastic bone formation in connective tissue is a usual phenomenon as is stromal bone formation in epithelial tumors; *i.e.*, ectopic ossification in renal cell carcinomas (8, 9), mammary carcinomas (10), and serous ovarian cystadenocarcinomas (1). Urogenital transitional epithelium may have the potential for osteogenesis in subepithelial connective tissue, as in the case of experimental bone formation in transplanted bladder epithelium. However, it is likely that all heterotopic bone formation probably involves mataplasia of connective tissue elements. Transition from cartilaginous structure to bone tissue was not found in stromal bone formation.

The biologic mechanism of metaplasia in connective tissue and vascular smooth muscle tissue is unknown; however, certain tissue and cell circumstances, plus the simultaneous presence of biologically active induction factors, may increase the risk locally. If certain inducing factors are supplementally involved in vascular tissue of tumor stroma, mineralization and bone formation might occur in the vascular smooth muscles, and stromal inflammation seems to be an additional factor. Osteoblasts may develop from pre-existing mesenchymal cells to form a matrix that is then calcified through the action of enzymes like alkaline phosphatase and carbonic anhydrase. In the present results, non calcified bone tissue expected to undergo further ossification showed various degrees of staining for PGs. This was correct evidence as accumulations of PGs in bone matrix, as well as positive reaction.

No conspicuous evidence of bone formation from cartilaginous tissue was observed, except for salivary pleomorphic adenomas. Pleomorphic adenomas of salivary glands, mixed tumors of skin, and mammary carcinomas are composed of epithelial structures and modified myoepithelial cells. Areas of modified myoepithelial cells in these tumors sometimes transform to chondroidal or osteochondroidal cells. Without participation of modified or neoplastic myoepithelial cells in such tumors, no cartilaginous ossification might be exhibited (19, 25, 26). Matrix-forming area of chondroidal transformation in modified myoepithelial foci also displayed strong reactions for KSPG (25); however, in the present study, no expression of KSPG was observed in any type of stromal bone formation. It is recognized that two different processes of

bone formation were found in tumor tissues; one is chondrogenesis from modified myoepithelial cells in salivary pleomorphic adenomas and skin mixed tumors which indicating KSPG, and second is metaplastic ossification from stromal connective tissue or perivascular smooth muscle tissue which showing certain PGs excluding KSPG.

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