MORPHOLOGICAL AND HISTOCHEMICAL STUDIES ON BONE DIFFERENTIATION IN TRANSPLANTED DENTAL PULP TISSUES

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Summary

Transplanted pulpal tissues were composed of stromal and vascular components. The pulpal mesenchymal cells were found to differentiate into fibroblasts and osteoblasts in the presence of vascular components and an extracellular matrix. Both fibronectin and glycosaminoglycans appear to promote the proliferation of vascular endothelium, which may synthesize a growth factor or an active mitogen for mesenchymal cells. Histochernically, the activities of alkaline phosphatase and Ca$^{++}$-ATPase were localized on the plasma membrane of fibroblasts, osteoblasts and vascular components. These enzymes may participate in the accumulation of calcium and in the hormonal regulation of mesenchymal cells. It can be concluded that pulpocytes exhibit an important potentiality for differentiation into osteoblastic cells in the presence of vascular components and an extracellular matrix.

I. Introduction

The pulpal tissue consisting of stromal and vascular components shows an enormous capacity not only for self replication but also for differentiation into bone$^{1,2}$. The osteogenic precursor cells proliferate and are capable of inducing bone formation under the action of some inducing factors such as ion alteration, growth factor, high alkaline pH and hormone-like substances$^{3,4,5}$. The production of collagen and noncollagenous proteins by osteoblasts or osteogenic precursor cells may yield direct effects upon the promotion of bone formation$^6$. In addition to these factors, alkaline phosphatase activity exerts certain specific functions for the synthesis of different tissue components such as fibrous proteins (collagen) and glycosaminoglycan$^7$. In particular, the hydrolysis of ATP seems to be associated with calcification and calcium transport$^8,9$. Furthermore, both Ca$^{++}$-ATPase and alkaline phosphatase are unusually important enzymes in regulating the functions of bone cells$^{10}$.

As stated above, some inducing factors may function in the activation and differentiation of connective tissue cells into osteoblasts, even though the mechanism underlying these processes remains to be elucidated. The present work has been undertaken to determine whether extracellular matrices such as fibronectin and glycosaminoglycan result in morphological
differentiation of transplanted pulp tissues and whether the coexisting activities of alkaline phosphatase and Ca\textsuperscript{++}-ATPase affects the process of such differentiation.

II. Materials and Methods

Twenty white rabbits weighing approximately 1.5 kg were employed as experimental animals. These rabbits were fed on Oriental solid foods and given a sufficient supply of water. Under the present experimental conditions, these rabbits were fixed prone on the table and their incisors were removed under general anesthesia with intravenous injections of pentobarbital sodium (Nembutal, 0.5 mg/kg) through their auricular vein. Hard tissues were then removed from the incisors to isolate proper dental pulps. The pulps were sliced into 2 mm thick graft strips for autoplantation.

The proper dental pulps were grafted under the true capsules of kidneys from the same animals. After transplantation, the rabbits were sacrificed chronologically on the 2nd, 3rd, 4th, 5th and 7th days, and at 2 weeks and 1 month. The kidneys were simultaneously perfused with Karnovsky's fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution (pH 7.4) for light and electron microscopic studies. For light microscopy, the extirpated and transplanted pulp tissues were sliced and refixed in 10% neutral formalin in 0.1 M phosphate buffer solution (pH 7.4). The tissues were embedded in paraffin. Sliced paraffin sections were dewaxed, dehydrated and stained with hematoxylin-eosin, alcian blue (pH 2.5)-PAS and Azan-Mallory stains.

For immunohistochemical observations, deparaffinized sections were treated as follows. All the sections were treated with 0.3% H\textsubscript{2}O\textsubscript{2} solution diluted in methanol for 30 minutes to inhibit the activity of endogeneous peroxidase and then washed in phosphate buffer solution (PBS) for 15 minutes and treated with 0.1% protease (Sigma Chem. Co. U.S.A.) dissolved in PBS for 10 minutes at room temperature to expose antigens. Subsequently, the sections were rinsed in PBS (pH 7.4) at 4°C for 15 minutes and incubated first with primary anti-rabbit fibronectin goat serum (dilution 1:200 Cappel Pro. P.A.U.S.A.) for 1 hour in a humid chamber at room temperature. After 15-minute washing in PBS, the sections were incubated with horseradish peroxidase anti-goat IgG (dilution 1:50, DAKO Inc.) for 30 minutes at room temperature and then washed in PBS for 15 minutes. The visualization reagent used was 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chem. Co. U.S.A.) in 0.05 M Tris-HCl buffer solution (pH 7.6) containing 0.005% H\textsubscript{2}O\textsubscript{2}. The stained sections were washed with water. Finally the sections were counterstained with Mayer's hematoxylin and mounted in permount\textsuperscript{11}.

For electron microscopy, transplanted tissues were fixed in 0.1 M cacodylate buffered 2.5% glutaraldehyde solution (pH 7.4) and were decalcified in 2% aqueous EDTA for 5 days. The decalcified tissue pieces were then rinsed in 0.1 M cacodylate buffer (pH 7.4) and postfixfixed in 0.1 M cacodylate buffered 1% osmium tetroxide (pH 7.4). To determine the localization of proteins including polypeptide, a fixative containing tannic acid was employed.

The particular fixative contained 2% tannic acid, 4% formaldehyde, 1.7% Na\textsubscript{2} SO\textsubscript{4} and 1/14 M veronal-acetate buffer. Tissues were fixed at pH 6.8, 4°C, for 1.5 hours in this
fixative and washed in a rinsing solution containing 8% saccharose and 1/14 M veronal-acetate buffer (pH 6.8) at 4°C for 1 hour.

For histochemical studies, 20 µm thick sections were cut with a microslicer (DOSAKA, Kyoto) and incubated in the following media to detect alkaline phosphatase (ALP) and calcium dependent adenosine triphosphatase (Ca++-ATPase) activity. After incubation, the tissue specimens were washed with the same buffer and the samples were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 hour at room temperature. These sections were dehydrated and embedded in epon resin. Thin sections of selected areas were cut on an Ivan Sorvall MT-5000 ultra-microtome using a diamond knife and doubly stained with a saturated aqueous solution of uranyl acetate followed by lead citrate and observed with a Hitachi H-800 electron microscope.

For enzyme histochemical studies, the following incubation media were employed:

1. Alkaline phosphatase (ALP)

The incubation medium used was made up of 0.2 M Tris-HCl buffer 5.6 ml (pH 8.5), 0.1 M sodium β-glycerophosphate 8.0 ml, 0.015 M magnesium sulfate 10.4 ml, 0.5% lead citrate 16 ml and sucrose 3.2 gm and was adjusted to pH 9.2. To assess the specificity of the enzyme activity, the following control procedures were performed in the same sections,

a) deletion of the substrate,

b) addition of 2.5 mM levamisole to inhibit the activity of non-specific alkaline phosphatase. Sections were incubated in the above solution for 15 minutes at room temperature.

2. Ca++-ATPase

Three solutions A, B and C were prepared; solution (basic) A contained 250 mM glycine-0.1 mM KOH buffer (pH 9.0), 3 mM ATP, 10 mM CaCl₂, 2.5 mM levamisol (KW-2-LE-T, Kyowa) and 4.0 mM lead citrate; solution B was the one in which the 10 mM CaCl₂ in A was replaced by 1 mM CaCl₂; and solution C contained an inhibitory additive of 50 mM quercetin together with all the components in basic solution A.

Sliced sections were incubated in solution A, B or C for 30 minutes at room temperature for electron microscopic studies.

III. Results

The transplanted pulp tissues contained star- and spindle-shaped proper pulpocytes with a PAS reactive cytoplasm, vascular components and alcian blue reactive extracellular matrix (Fig. 1). On the second day, the grafts were surrounded by connective tissues which were relatively cellular. In these tissues a large number of enlarged vessels were filled with erythrocytes, neutrophils and eosinophils (Fig. 2-a). Small hemorrhage spots were detected around dilated blood vessels (Fig. 2-a). In addition to these components, star- and spindle-shaped fibroblasts were provided with an elongated PAS reactive cytoplasm in the perivascular spaces (Fig. 2-b). On the third day, granular PAS reactive substances were recognized in clusters of mesenchymal cells in association with collagenous fibers proliferated in pericellular areas (Fig. 3). In the course of such a process, vascular components and alcian blue reactive sub-
Fig. 1. Alcian blue reactive stromal substance is noted in the intercellular spaces of pulpocytes. Alcian blue-PAS stain, × 100.

Fig. 2-a. A number of enlarged blood vessels (V) are filled with erythrocytes, neutrophils and eosinophils. Hematoxylin-eosin stain, × 200.

Fig. 2-b. Enlarged fibroblastic cells exhibit PAS reactive cytoplasm in the edematous perivascular stroma at 2 days after transplantation. Alcian blue-PAS stain, × 200.

Fig. 3. PAS reactive osteoid tissues are noted involving degenerated mesenchymal cells in vascular stroma at 4 days after transplantation. Alcian blue-PAS stain, × 100.

Fig. 4. Differentiated osteoblastic cells are arranged linearly around bony trabeculae at 5 days after transplantation. Hematoxylin-eosin stain, × 200.
Fig. 5. Light microscopical localization of fibronectin is noted in the endothelial and related regions of newly formed blood vessels and pericellular spaces at 3 days after transplantation. Fibronectin immunohistochemistry stain by the indirect method and hematoxylin stain, × 200.

Fig. 6. Light microscopical weak localization of fibronectin is found in the endothelium of blood vessels around osseous tissues at 7 days after transplantation. Fibronectin immunohistochemistry stain by the indirect method and hematoxylin stain, × 200.

stances were decreased in amount. After the fifth day, the transformation of fibroblasts into osteoblasts occurred promptly. Transformed fibroblasts with an eccentric round nucleus and a cytoplasm containing pale areas were distributed around the osseous tissues (Fig. 4).

After the seventh day, the grafts were virtually transformed into laminated osseous tissues, around which a lining of osteoblasts was detected.

Immunohistochemically, fibronectin was localized in the endothelial cells together with their basement membrane in the pulpal tissues. On the second and third days, more intense reaction products were demonstrated in the endothelium and its basement membrane in newly formed blood vessels and fibroblasts in the pericellular spaces (Fig. 5). After the fifth day, fibronectin was weakly localized in the endothelium including the perivascular spaces, in parallel with the maturation of blood vessels and bone formation (Fig. 6).

Electron microscopically, in transplanted pulps, pulpcytes were provided with an enlarged cytoplasm containing numerous mitochondria, elements of smooth and rough endoplasmic reticulum and a few elements of Golgi complex (Fig. 7). In the spaces surrounding these mesenchymal cells, mature capillaries and arterioles were encountered, which contained a large number of platelets in their lumen. Small amounts of collagen fibrils and amorphous materials were likewise noted in the intercellular spaces. On the second day, dilated capillaries were increased in number and proliferated fibroblasts were found to contain increased amounts of ribosomes, smooth and rough endoplasmic reticulum elements in the cytoplasm.

On the third and fourth days, the increased numbers of fibroblastic cells revealed dilatations of rough endoplasmic reticulum elements, and a few bundles of short parallel threads were occasionally identified within saccules in the cytoplasm (Fig. 8). Electron dense materials of granular appearances were found in the cytoplasm of such mesenchymal cells (Fig. 9). At this stage, proliferated capillaries contained large numbers of platelets and were associated with non-fibrillar amorphous materials and increased striated collagen fibrils in the perivascular spaces.

The mesenchymal cells presumed to be preosteoblasts contained oval nuclei and nucleoli. In their enlarged cytoplasms, well developed rough endoplasmic reticulum elements and mitochondria were seen.

After the fifth day, mature osteoblasts involved well developed elements of rough endoplasmic reticulum and eccentric enlarged nuclei (Fig. 10). In the present observations on the tissue specimens fixed with the solution containing tannic acid, a positive reaction was obtained in the osseous tissues composed of collagen fibrils and other polypeptides. Collagen fibrils were well stained and their electron opacity was different from other ultrastructures, revealing their periodic striations (Fig. 10 inset).
Fig. 7. Ultrastructure of a pulpocyte in the transplanted pulpal tissues. The pulpocyte shows an enlarged cytoplasm containing small numbers of elements of Golgi complex (G) and smooth and rough endoplasmic reticulum (ER) together with numerous mitochondria.

Uranyl acetate and lead citrate stain, $\times$ 4,000.

Fig. 8. Ultrastructure of fibroblastic cell in the graft after 3 days. a. The cytoplasm containing abundant elements of rough endoplasmic reticulum (ER) and ribosomes is close to collagen fibrils.
(Cf) and vascular endothelium (E).

Uranyl acetate and lead citrate stain, × 9,100. b. A few bundles of parallel short threads are discerned within sacculles in the cytoplasm of a fibroblastic cell (arrow head). Uranyl acetate and lead citrate stain, × 12,000.

**Fig. 9.** Ultrastructure of a mesenchymal cell in the graft after 4 days. Variously sized granular electron dense bodies (DB) are noted in the cytoplasm of the cell. Uranyl acetate and lead citrate stain, × 9,100.

**Fig. 10.** Ultrastructure of an osteoblast (Ob) around osteoid tissues in the graft after 5 days. The cytoplasm of the cell contains an abundance of rough endoplasmic reticulum elements and an eccentric nucleus and is located in close proximity to collagen fibrils (Cf, inset) and matrix vesicles (Mv). Tannic acid stain, × 3,100. × 12,400 (inset).

According to the present enzyme histochemical studies on the transplanted pulp tissues, the activity of alkaline phosphatase was weakly demonstrated on the plasma membranes of stroma cells and vascular endothelium at early stages.

After the third and fourth days, however, the reaction products of alkaline phosphatase activity were localized markedly on the plasma membranes of fibroblasts and vascular basement membrane, together with lysosomes, elements of endoplasmic reticulum and Golgi complex in the cytoplasm of fibroblastic cells and pericellular calcium like dense bodies of various sizes were noted in the intercellular spaces (Fig. 11-a, b). In the extracellular spaces, the alkaline phosphatase activity was localized in the matrix vesicles. In both the fibroblastic and osteoblastic cells, however, such enzyme activity was abolished by the deletion of the substrate and suppressed appreciably by the addition of levamisole, which is known to be a potent inhibitor of the activity of non-specific alkaline phosphatase.

In the transplanted pulp tissues, the activity of Ca++-ATPase could not be demonstrated in the stroma cells and vascular components at early stages.

After the third day, however, the activity of Ca++-ATPase was demonstrated on the plasma membrane of the mesenchymal, osteoblastic and vascular endothelial cells, the basement membrane of the blood vessels and matrix vesicles (Fig. 12). Quercetin markedly inhibited the activity of Ca++-ATPase not only in the mesenchymal cells but also in the osteoblasts, matrix vesicles, vascular endothelial cells and basement membrane.

**IV. Discussion**

During the course of bone induction, responding cells differentiating into osteoblasts have been presumed to be derived from perivascular connective tissue cells in transplanted tissues\(^{14,15}\). A fibroblast growth factor emanating from endothelial cells and their basement membrane could stimulate not only perivascular stromal cells but the proliferations of adjacent endothelial cells\(^ {16}\). In the early stages of transplantation, fibronectin and glycosaminoglycans could be detected in the vascular endothelium and interstitial tissues of the transplanted pulp tissues examined in the present study. Fibronectin is known to facilitate the attachment of endothelial cells to collagen. The morphological detection of fibronectin is useful for clarifying the mechanism of capillarization, since fibronectin may play a crucially important role in the process of neovascularization. In addition, fibronectin has an affinity for binding collagen fibrils
Fig. 11. Electron microscopical localization of the activity of alkaline phosphatase in the graft after 4 days. a. Alkaline phosphatase activity is revealed on the plasma membrane of a fibroblastic mesenchymal cell, matrix vesicles (Mv) and pericellular calcium-like dense bodies (Os). Uranyl acetate stain, × 3,500. b. The localization of alkaline phosphatase activity is noted in elements of Golgi complex (G) and lysosomes of a fibroblastic mesenchymal cell. Alkaline phosphatase and uranyl acetate stains, × 12,000.
and glycosaminoglycan and activates the function of mesenchymal cells including fibroblasts. Sato et al. reported that extracellular matrix consisting of fibronectin and glycosaminoglycan was synthesized by endothelial cells and could regulate the growth of endothelial cells.

Damaged pulpal tissues are believed to acquire embryonal potency and the pulpal mesenchymal cells appear to differentiate into odontoblasts in the presence of extracellular matrix. In vitro, the extracellular matrix of embryonic teeth was shown to be capable of inducing mesenchymal cells to differentiate into odontoblasts.

The extracellular matrix is believed to influence cell growth, attachment, migration and maturation. Recent biochemical and morphological studies have indicated that the extracellular matrix at various stages of angiogenesis could exert important effects upon endothelial cell growth and capillary morphogenesis. Endothelial cells can synthesize bone active mitogens and bone cells could be a target of endothelium derived growth factors. Thus, there appears to be a close relationship between the initiation of bone formation and vascularization. In keeping with this, not only an abundance of extracellular matrix but also an increase in the number of capillaries was noted in the present tissues during the early stages of transplantation.

A suitable level of oxygen brought about by an increase in the number of capillaries was conceived to be needed for the differentiation of mesenchymal cells originating from dental pulp cells.

In the present transplanted pulpal tissues, proliferated mesenchymal cells were found to contain abundant elements of rough endoplasmic reticulum and a few bundles of short threads within saccules in the cytoplasm, and the intracytoplasmic dense materials were noted in association with both the production of collagen fibrils and calcium deposition. Such findings appear to indicate that the endoplasmic reticulum elements were the site of the synthesis of collagen precursors presumed to be pro-α-chains. This procollagen seems to be converted into tropocollagen within the matrix and then polymerized into collagen fibrils, so as to be embedded in ossified tissues.

In addition to all these morphological findings, the electron dense reaction products of alkaline phosphatase and Ca++-ATPase activity were associated with the plasma membranes of the fibroblasts and osteoblasts and the matrix vesicles distributed in close proximity to osteoblasts in the present osteoid tissues. Simpson et al. reported that mRNA could lead to the sequential synthesis of complementary DNA codes for osteoblastic stimulating factors and the activity for production of alkaline phosphatase in a prostatic cancer cell line.

In view of these findings, the alkaline phosphatase may act so as to increase the phosphate concentration, facilitating hydroxyapatite formation and to perform certain specific functions in the synthesis of various tissue components such as fibrous proteins. On the other hand,
the activity of Ca\(^{++}\)-ATPase has been correlated with calcium pumps needed for the accumulation of calcium within cells and matrix vesicles\(^{16,27}\).

Alkaline phosphatase and ion-transporting ATPase activity in the present mesenchymal and osteoblast-like cells can be taken to regulate the composition of extracellular fluids and thus the processes of mineralization\(^{28,29}\).

The physiological properties of the plasma membrane of mesenchymal cells are believed to be concerned with calcium transport, electrical properties and hormonal responses\(^{30}\).

The activity of ATP-hydrolysis at alkaline pH is known to play an important role in calcium ion transport and calcification in hard tissues\(^{31}\).

In view of the present results and discussions, it seems probable that undifferentiated mesenchymal cells from rabbit incisor pulps may contribute not only to the metabolism of calcium phosphate but to the production of collagen. It may be concluded that these cells show an important potentiality for differentiation into osteogenic and presumably odontogenic cells in vascular environments and related extracellular matrix.

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References


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