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Developmental Pattern and Histologic Structure at the Growing Sites of the Scales of *Polypterus senegalus*

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Abstract *The scales of Polypterus senegalus comprise ganoine (a true enamel), dentine, and bone and are an organ homologous to teeth. For a deeper understanding of the development and structure of teeth, it is important to recognize their evolutionary origin and history. Investigating the scales of living primitive fish can provide clues to the evolutionary history of teeth. This study describes the developmental pattern of odontogenic and osteogenic tissues, including cell differentiation and structure of the scales.*

Key words : Ganoine scale, Enamel, Dentine, Bone, Phylogeny

INTRODUCTION

Scales and teeth are considered to be evolutionarily derived from the dermal skeleton that appeared in the first jawless vertebrates, 500 million years ago[1~9]. The dermal armor was composed of external dentine tubercles, on which a hypermineralized tissue (enameloid or enamel) was observed in some taxa, and underlying bone-like tissues, so-called aspidin[4]. It is presumed that such dermal hard tissues functioned as a mechanical support, defensive outer shield, sensory organ, feeding organ, and/or reservoir of calcium and phosphorus[1~5]. The origin of the mineralization mechanisms for dentine and membrane bone is apparent in the early dermal skeleton associated with the onset of neural crest cell migration, probably primarily associated with the sensory system[4]. Living primitive fishes (Polypterid and Lepisosteida) have so-called ganoine scales, a tissue homologous to the dermal skeleton. Studies of the development and structure of these scales will provide useful information on the evolutionary processes of mineralization mechanisms as well as dentine and bone formation (e. g., which is the earliest hard tissue?). Our previous studies[10~12] revealed that ganoine matrix contains mammalian amelogenin-like proteins, suggesting that ganoine-forming cells have a preserved mammalian type of enamel proteins (the amino acid sequence of amelogenins is at least partially conserved). In the present study, the developmental pattern of dentine and bone and partial characterization of their matrices are discussed in connection with their evolutionary history.

MATERIALS AND METHODS

The ganoine scales were dissected from four *Polypterus senegalus* fish (50–150mm total length), anesthetized with cold water, and fixed in a mixture of 0.5% glutaraldehyde/4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 to 12 h and then in 2% osmium tetroxide for 2 h. Some specimens were decalcified with 4.25% EDTA for 2 to 3 weeks. Thses specimens were dehydrated through a graded ethanol series and embedded in Epon 812 resin and/or LRWhite.

The high-iron diamine thiocarbohydrazide-silver proteinate (HID–TCH–SP) staining technique was used to detect sulfated glycoconjugates in ganoine, dentine, and bone. This method was previously described in detail[13]. In brief, the specimens were placed in HID solution for 18 h, rinsed several times in distilled water, postfixed in 2% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.4), dehy-

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drated and embedded in Taab 812 resin. Ultrathin sections, cut with a diamond knife and floated onto stainless steel grids, were reacted with 2% thiocarbohydrazide, in 10% acetic acid for 15 min, rinsed in distilled water, and treated with 1% aqueous silver proteinate solution for 20 min in the dark. The ultrathin sections were observed without uranyl acetate and lead citrate staining.

The localization of amelogenins in the ganoine layer was examined using anti-bovine amelogenin antibodies (gift from Dr. H. Shimokawa, Tokyo Medical and Dental University) as previously described[10, 11].

RESULTS

1. Epidermal cell layer

The ganoine scales were composed of four layers : 1) epidermal cell layer, 2) ganoine layer, 3) dentine layer, and 4) bone layer (Figs. 1, 8). Several different types of cells were observed in the epidermal cell layer ; (1) large, ballooned cells in which the nucleus located in the center with few organelles (Figs. 2, 6), (2) inner most cells that differentiated into ganoine-forming cells (inner ganoine epithelium ; IGE), short columnar in shape (Fig. 3), and that secreted ganoine matrix on the previously deposited dentine matrix, but after maturation of the ganoine, however, became irregular-shaped or flattened (Figs. 2, 3, 5). (3) superficial cell layer covering the epithelium and containing a few goblet-like cells (Fig. 2), (4) mast cell-like cells which include many granules containing sulphated substances (Fig. 6). During its maturation, the ganoine membrane appeared between the IGE and mineralized ganoine (Figs. 2, 10). Blood vessels penetrated the dentine and ganoine layers and distributed into the epidermal cell layer (Fig. 4).

2. Dentine and ganoine

The scales developed by the addition of newly-formed dentine matrix followed by ganoine matrix in the margins (Fig. 7). The ganoine-forming cells were arranged in a line on the newly-formed dentine (Fig. 7a). Although the dentine deposition always preceded ganoine deposition, ganoine forming-cells came into apposition with the lateral surface and began to secrete ganoine matrix when dentine was formed in certain thickness and width (Fig. 7b), which temporarily interfered with further lateral dentine development. Accordingly, at this stage, the ganoine had a hook shape (Fig. 7). After this ganoine was completely matured, however, the ganoine-forming cells moved away, and newly-differentiated dentine forming cells adjacent to the hook-shaped matured ganoine began to secrete new dentine matrix. Dentine-forming cells sometimes extended cytoplasmic processes into the dentine (Fig. 7b). The repeating processes of dentine and ganoine formation are shown in Figs. 9a–c (see figure legends).

3. Bone

In the scales of *Polypterus senegalus*, which are more than 10mm in length, there were at least two distinct layers of bone tissues : one composed of parallel-fibered matrix, the other composed of woven-fibered matrix, in which large vascular cavities were usually present (Figs. 1, 3). The formation of parallel-fibered bone was not observed, because it is formed at an early stage of scale ontogeny. Woven-fibered bone was additionally deposited by the flattened cells facing the surface of the bone (Fig. 5, arrow).

4. Partial characterization of ganoine, dentine, and bone matrices

Sulphated glycoconjugates were preferentially detected over the dentine matrix (Fig. 10) but there were also few located over the parallel-fibered bone matrix (Fig. 11). Large amounts of sulphated substances were present over the matrix around osteocyte-like cells (Fig. 12). Specific immuno-gold labeling for mammalian amelogenins was observed over the ganoine layer (Fig. 13).

DISCUSSION

As a part of a series of phylogenic evolutional studies, we previously reported the developmental patterns and composition of tooth hard tissues of lower vertebrates[10~15]. Aprismatic enamel of amphibians and reptiles contains no sulphated glycoconjugates but the enameloid (tissue closer to the dentine) covering the tooth tip of fish includes sulphated glycoconjugates. Furthermore, sulphated substances are present in the surface layer of mammalian prismatic enamel, suggesting that for prismatic enamel formation, certain sulphated materials are prerequisite and might be a phylogenetically advanced character. Thus, comparative histology and embryology can provide useful clues on the evolutionary proc-

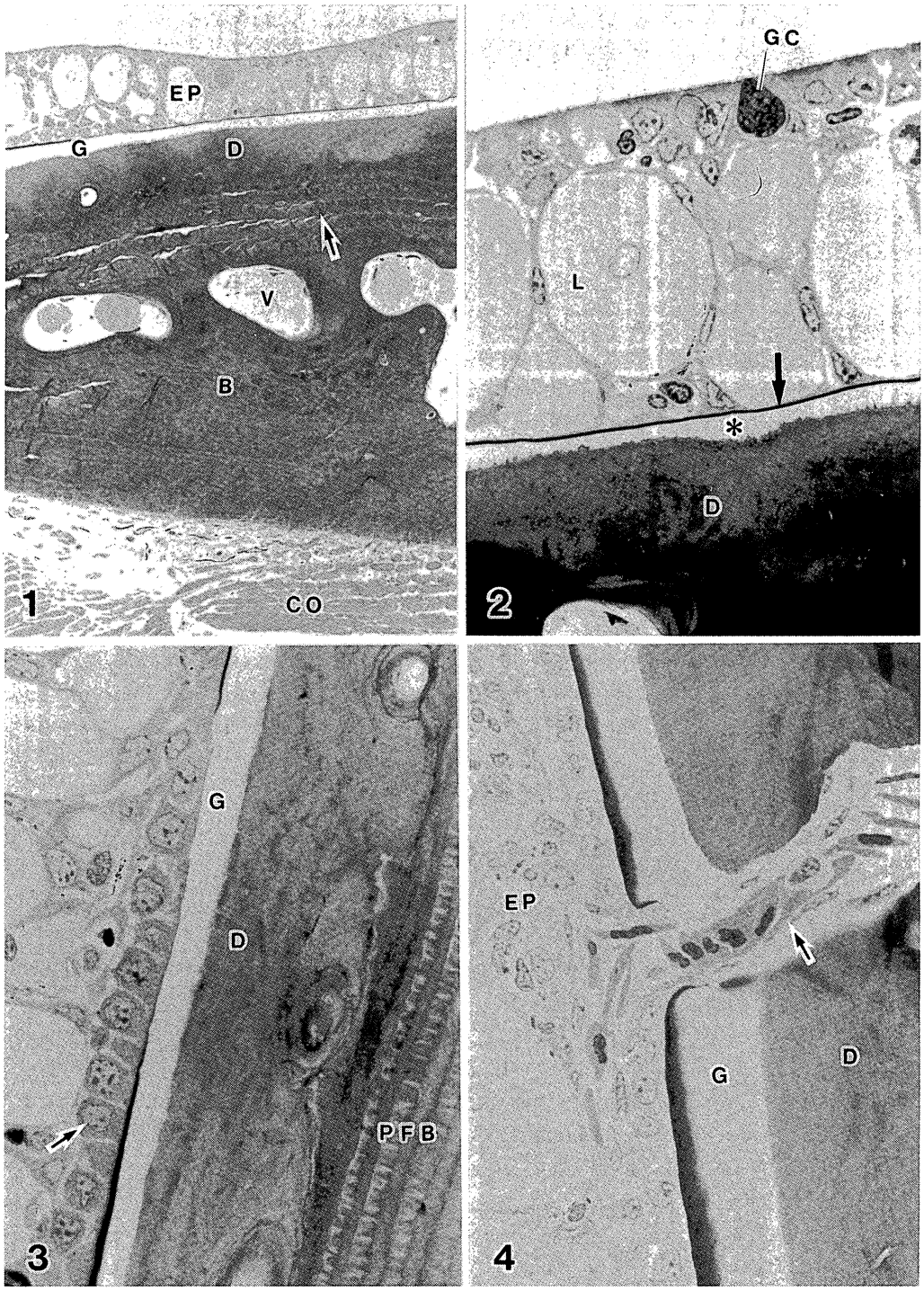

Fig. 1. Photomicrograph showing the ganoine scales. Arrow indicates parallel-fibered bone. EP : epidermis, G : ganoine layer, D : dentine, B : bone, V : vascular cavity, CO : collagen bundle ×200

Fig. 2. A higher magnified picture of the epidermal cell and ganoine layers. The empty space (asterisk) indicates a matured ganoine layer dissolved by decalcification. Arrow indicates ganoine membrane. D : dentine, L : large, ballooned cell, GC : goblet cell ×400

Fig. 3. Ganoine forming cells (arrow) are short columnar in shape. D : dentine, G : ganoine layer (dissolved by decalcification), PFB : parallel-fibered bone ×400

Fig. 4. A blood vessel (arrow) penetrating the dentine (D) and ganoine (G) layers and invading into the epidermis (EP). ×400

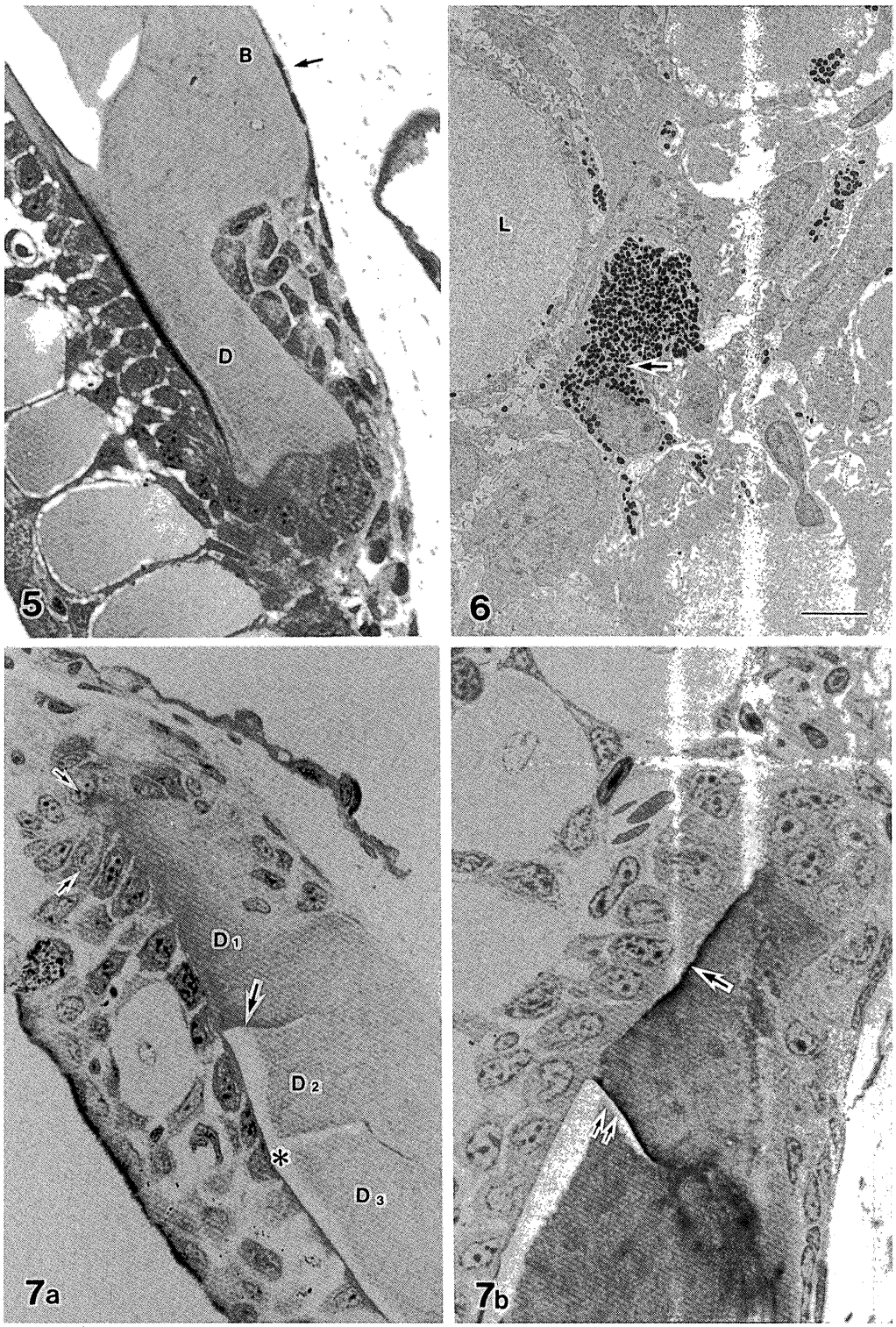

Fig. 5. Basal bone layer (B) is covered by flattened osteoblast-like cells (arrow). D : dentine ×400

Fig. 6. Electron micrograph showing mast cell-like cell (arrow) in the epidermal cell layer. L : large ballooned cell Bar=5um

Fig. 7. **a**. Growing site of the scales. D1 indicates newly forming dentine. D2 and D3 show previously deposited dentine. Note that the empty space (asterisk), representing the ganoine layer dissolved by decalcification, is shaped like hook (large arrow). When a certain thickness of dentine is formed, ganoine-forming cells (small arrows) face the dentine surface. **b**. Initial ganoine matrix begins to secrete on the dentine (arrow). Note that dentine-forming cells extend their cytoplasmic processes into the dentine matrix. Double arrows indicate a hook-shaped ganoine layer. ×400

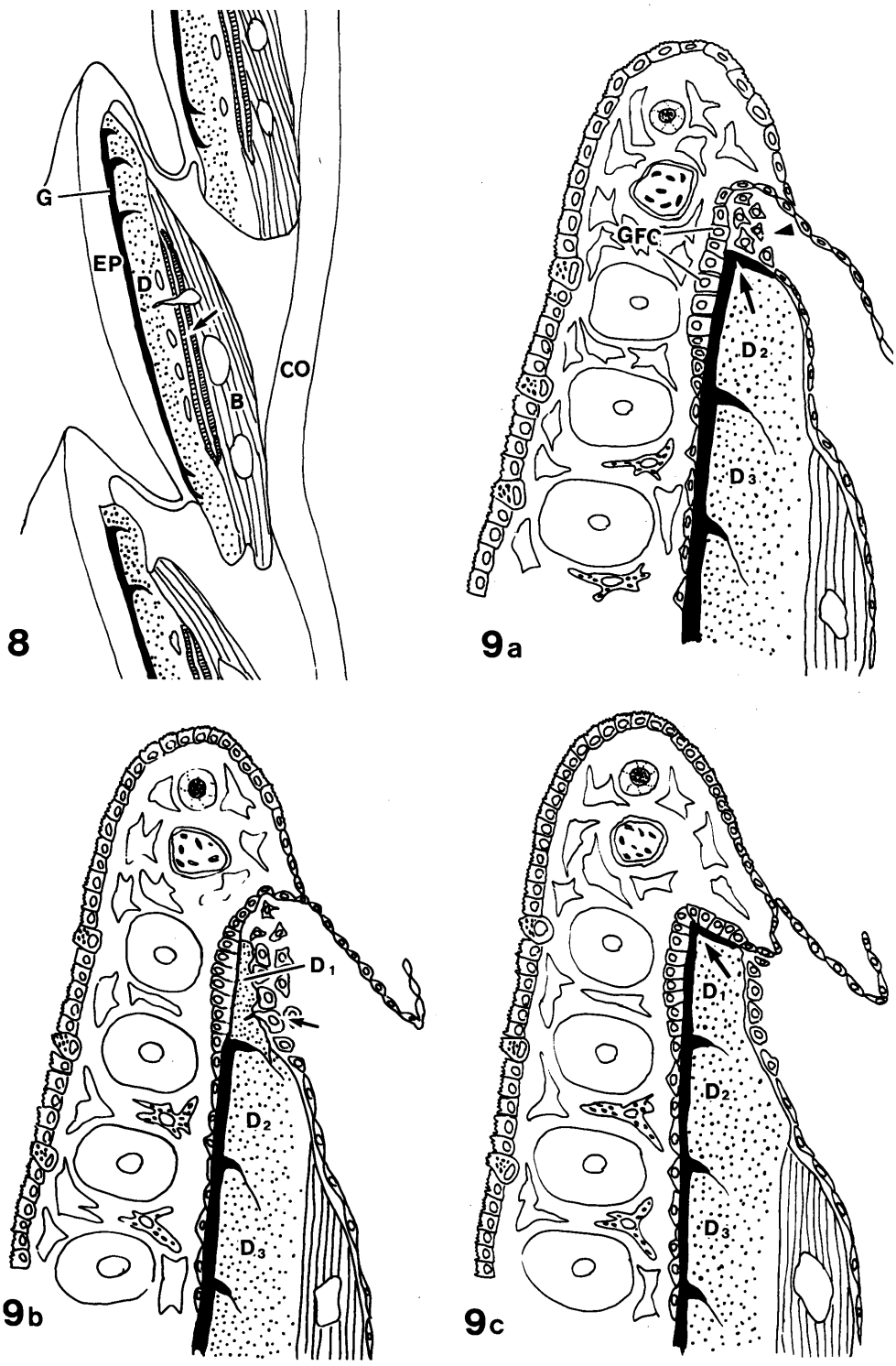

Fig. 8. Schema showing the structure of ganoine scales of *Polypterus senegalus*.
EP: epidermis, G : ganoine, D : dentine, B : bone (an arrow indicates parallel fibered bone), Co : Collagen bundle

Fig. 9. Schematic representation showing the developmental relation between dentine and ganoine at successive stages. **a**. After the hook-shaped ganoine layer (arrow) has been formed on the dentine (D2), the ganoine-forming cells separate from the surface of the ganoine. Undifferentiated connective tissue cells (arrowhead) then migrate there and/or under the inner most cells (ganoine forming cells : GFC) of the epidermis. **b**. The cells that differentiated into dentine-forming cells (arrow) begin to secrete new dentine matrix (D1). **c**. After a certain thickness of dentine (D1) is deposited, the lateral growing surface is covered by ganoine forming cells. They start to secrete ganoine matrix. Accordingly, the configuration of the ganoine matrix here has a hook-shape (arrow).

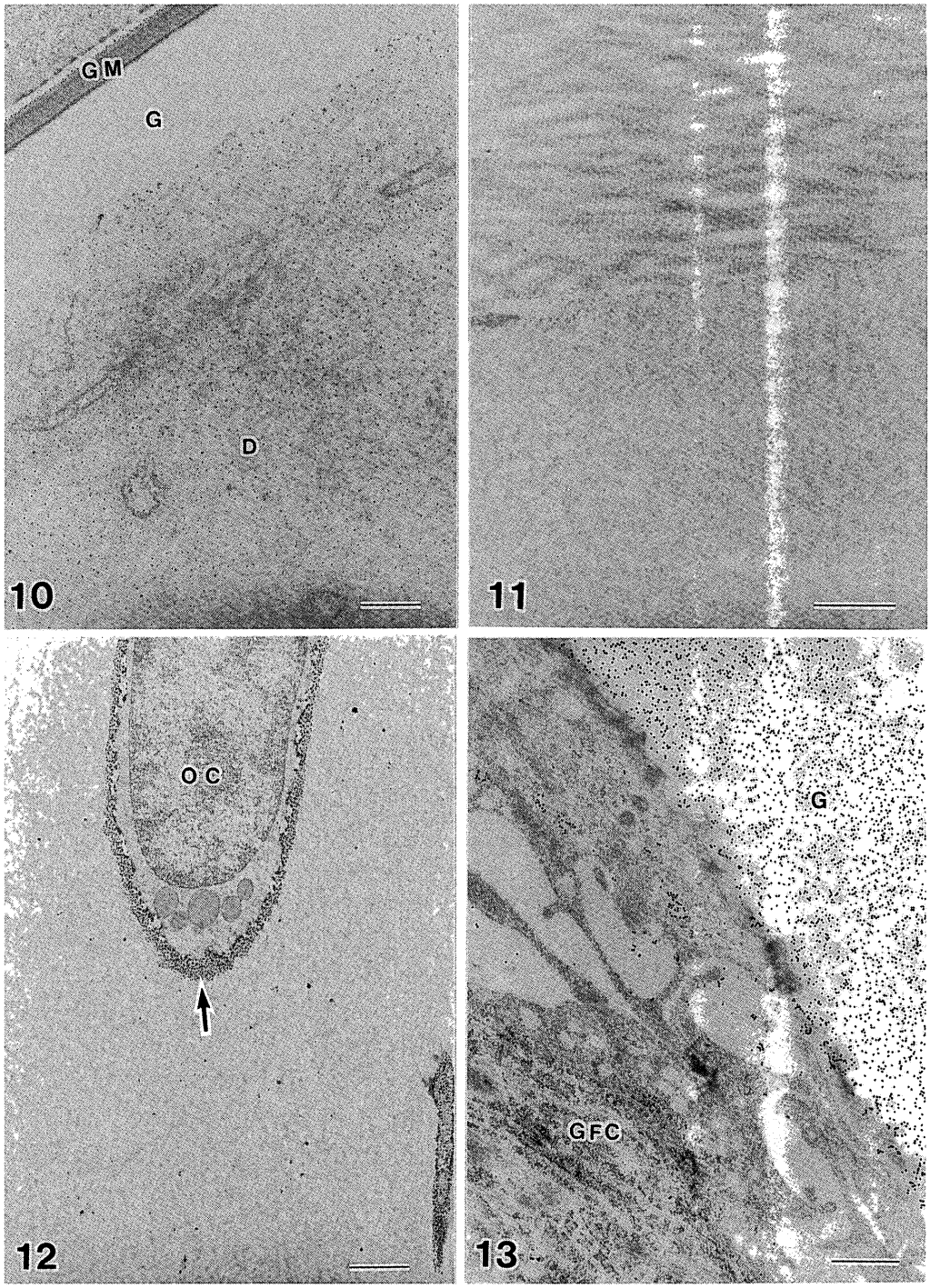

Fig. 10. Sulphated glycoconjugates are detected over the dentine matrix (D). GM : ganoine membrane, G : ganoine layer dissolved by decalcification Bar=500nm

Fig. 11. There are few sulphated glycoconjugates associated with the parallel-fibered bone matrix. Bar=1μm

Fig. 12. Note that there are large amounts of sulphated glycoconjugates are found in the periphery matrix (arrow) of the osteocyte-like cell (OC), but few over the bone matrix. Bar=1μm

Fig. 13. Immunocytochemical localization of mammalian amelogenin-like proteins. The specific gold labeling is present over the ganoine matrix (G). GFC : ganoine forming cell Bar=1μm

esses of teeth. The scales of the living primitive fishes, identified as a tissue homologous with the dermal armor that appeared in agnathus 500 million years ago, are composed of enamel, dentine, and bone and it is conceivable that they have retained more primitive cellular processes involved in hard tissue formation. From this viewpoint, the present study examines with development and structure of the scales of *Polypterus senegalus* and describes a partial characterization of extracellular matrices of

ganoine, dentine, and bone.

Although in this study the growth series of scale ontogeny was not successively investigated, observation of the growth sites (margins of the scales), confirmed that (1) Dentine formation precedes ganoine formation. (2) When the deposition of the initial dentine matrix begins, ganoine-forming cells faced the outer surface of the dentine and secrete ganoine matrix onto it. (3) Subsequently, when a certain thickness and width of dentine and ganoine is formed, ganoine-forming cells further cover the lateral growing surface of the dentine and also secrete ganoine matrix there. This secretion leads to the hook-shape configuration of the ganoine layer. (4) After the ganoine matrix maturation, the ganoine-forming cells move away, and newly-differentiated dentine-forming cells arranged to faced the hook-shaped ganoine layer and begin to secrete dentine matrix. These processes are repeated, such that the scales gradually grow. Before differentiation into dentine forming cells, there is a stage when undifferentiated mesenchymal cells are localized just under future ganoine forming cells (Fig. 9a). Because morphogenesis and cell differentiation in the developing tooth are controlled by a series of reciprocal interactions between the epithelial and mesenchymal tissue 16), this might represent the epithelial-mesenchymal interaction stage at which dentine-forming cells are induced. Although epithelial elements might be involve in the differentiation of bone forming cells17), there is no direct morphologic evidence at the growing sites of the scales. Studies are now in progress to examine whether various elements (TGF-beta, FGFs 1, 2, etc.) related to cell differentiation are expressed.

During dentinogenesis, mantle dentine is formed first and subsequently circumpulpal dentine is formrd. There are distinct differences in the compositions of the extracellular matrices, mineralization mechanism, and ultrastructure between the two. For example, the mantle dentine includes large amounts of sulphated glycoconjugates, but not phosphophoryn. Matrix vesicles involved in initial mineralization are observed only in the mantle dentine. Further, the calcium distribution of odontoblasts at the mantle dentine formation stage is rather similar to that of osteoblasts. These differences in organic matrix composition and in mineralization processes between the two seem very important, because the mantle dentine is thought to be phylogenetically primitive and morphologically resembles woven bone. It might be that circumpulpal dentine evolved as a further specialization of primitive mantle dentine-like tissue, which might have barely been distinguishable from bone at a very early evolutionary stage. This study demonstrated that dentine matrix of the scales contains large amounts of sulphated glycoconjugates, even after mineralization. Our previous studies[12] revealed a diverse distribution of sulphated glycoconjugates in the osteoid of mammalian membrane-bone. In some regions, there are large amounts, while in other regions, there are few. Further work is needed to confirm calcium distribution pattern of dentine or of bone-forming cells of the scales and/or involvement of matrix vesicles in the mineralization.

Because teeth of extant vertebrates originate from the dermal armor found in the earliest jawless vertebrates, some of which retained ectodermally-derived enamel, it is conceivable that a gene for enamel proteins is expressed that is associated with the enamel formation. One type of mammalian enamel proteins, amelogenins, is widely distributed in aprismatic and prismatic enamels[10,11]. The detection of a mammalian type of enamel proteins in the ganoine layer suggests that the gene is preserved in teeth as well as in scales.

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Polypterus senegalus の鱗の成長側における発生様式と組織構造

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キーワード：ガノイン鱗，エナメル質，象牙質，骨，系統進化

抄録　ポリプテルス（*Polypterus senegalus*）の鱗は，ガノイン（エナメル質），象牙質および骨から構成され歯と相同器官である．歯の発生や構造をより深く理解するためには，その進化的起原や進化過程を認識することが重要である．現生の原始的魚類のガノイン鱗の研究は，歯の系統進化解明の手掛かりを提供しうる．本研究は，その鱗の細胞分化や構造を含む歯性ならびに骨性組織の発生様式について述べる．