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Heterogeneous features of selachian enameloids

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Abstract

Chemical properties of the enameloid of sharks (*Lamna ditropis*, *Carcharodon carcharias* and *Prionace glauca*) were analyzed using EPMA for inorganic components, and using histochemical methods for organic components, in order to discuss evolution of enameloids. The distribution patterns and concentrations of Na, Ca and Mg in the shark enameloid were similar to those of the mammalian enamel. This fact suggests that ion transport is performed by epidermal cells in enameloid calcification as in mammals. Histochemical and immunohistochemical observations showed compositional heterogeneity in the developing enameloid matrix of *C. carcharias*, that corresponds to the structural heterogeneity of mature teeth acquired by advanced selachians for more effective feeding. Since the heterogeneity of matrix reflects plural stages of matrix secretion by odontoblasts, the acquisition of the structural heterogeneity may have attained by the emergence of new secretory stages of the odontoblasts.

Introduction

Morphogenesis of teeth is one of the most well-studied organogenesis in vertebrates because teeth are formed by the epithelio-mesenchymal interaction, the important process in development (Davidson, 1991). And teeth are suited for researches on the relationships between development and morphological evolution. In this study chemical properties of shark teeth were investigated focussing on the following problem.

On the basis of morphological observations (Goto, 1978; Kemp, 1985; Kemp and Park, 1974; Prostak and Skobe, 1988) there have been a conflict as to whether the selachian enameloid is homologous with the mammalian enamel, which epidermal cells form, or not. And biochemical and immunohistochemical analyses by Herold *et al.* (1980, 1989) and Slavkin *et al.* (1983a, b) demonstrated the presence of the mammal-type enamel proteins secreted by epidermal cells in the shark enameloid. But Ishiyama *et al.* (1994) stressed the mammal-type enamel proteins are absent in the shark tooth germs. From these researches arguments on the homology of enameloid with enamel became confusing. The confusion is probably due to the difference of developmental aspects focussed in each study. Sasagawa (1993) proposed an interesting scheme that the enameloid formation includes three aspects, namely calcification, matrix formation, and cellular activity. In other words, the studies of developing enameloid should be performed based on these three aspects.

This paper focuses upon this problem. EPMA analysis was performed to investigate the physiological condition in the enameloid calcification. And in order to reveal the developmental origin of the enameloid matrix, the matrix formation was studied by SEM and histochemical observations.

Materials and methods

Materials

In the present study three shark speces were investigated.

Teeth and tooth germs of *Lamna ditropis* (1.5m in total body length and 1.5cm in tooth crown height) were obtained in a commercial landing at Kesennuma port, northeastern Japan. They were fixed with 10% neutralized formalin for EPMA and histochemical investigations. Tooth germs were detached from the frozen specimens, and only enameloid matrix was homogenized in distilled water.

Teeth and tooth germs of *Carcharodon carcharias* (2cm in tooth crown height) was provided by Dr. Goto of Tsurumi University and were preserved in 10% neutralized formalin.

Teeth of *Prionace glauca* (1.5cm in tooth crown height) was provided by Dr. Taniuchi of the University of Tokyo. Some of them were fixed with 10% neutralized formalin for EPMA analysis.

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Methods

EPMA analysis

Polished thin sections of hard tissues were coated with carbon and then set in an electron probe micro analyzer (JEOL model JCMA-733mkII) to qualify the contents of five elements (Ca, F, Mg, P and Na) in the tissues. The diameter of electron beam was 10 μm and the acceleration voltage was 1.5kV. Bleaching of teeth was performed in 10% (v/v) sodium hypochlolite solution overnight before embedding in resin. The element contents were measured straight from the surface of enameloid to the dentin at the interval of 20 μm.

SEM observation of soft tissues

Soft tissues fixed with 10% formalin were dehydrated. They were embedded in paraffin at 50℃ and cut longitudinally by a microtome to obtain flat surface for observation as in the case of thin sections. Paraffin was eliminated from the specimens and then immersed in *t*-buthyl alcohol, and freeze-dried (Hitachi Freeze drier). The freeze-dried specimens were coated with Pt-Pd alloy prior to observation by a scanning electron microscope (S-2400S; Hitachi).

Histochemistry

Carbohydrates were detected using the PAS method. Sections were incubated in 1% (w/v) periodic acid solution for 10 minutes to oxidize polysaccharide components and then washed with distilled water. The washed sections were immersed in Schiff's reagent for 10 minutes at room temperature and then washed three times with 0.5% (w/v) sodium pyrosulfate solution.

Antisera preparation

Antiserum, denoted as LDE-1, was prepared against whole teeth extracts of *Lamna* using a rabbit by Sawady Technology Corporation (Tokyo). Preserum was collected prior to immunization for a control.

Immunohistochemistry

The paraffin-embedded sections were deparaffinized, hydrated, and washed in phosphate-buffered saline (PBS: 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, 2.7mM KCl, 0.14M NaCl). The sections were incubated with primary antiserum or preserum appropriately diluted in PBS for 60 minutes at room temperature, washed three times with PBS, and then incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma BioScience) for 30 minutes at room temperature. The sections were washed three times with PBS and then incubated with reaction solution containing nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for several minutes (NBT and BCIP solutions were purchased from Gibco-BRL). Reaction with alkaline phosphatase was stopped by PBS containing 20mM EDTA, and then the stained sections were dehydrated. After completing dehydration the histological sections were enclosed in resin and then observed under an optical microscope.

Results

EPMA analysis (Fig. 1)

In the three shark speces examined, the calcium content was about 50-55 wt%, throughout the enameloid layer regardless of whether the preparation was bleached or not. At the enameloid-dentin junction a discrete decrease of CaO was observed and dentin contained less calcium (ca. 40 wt%) than enameloid.

In the outer enameloid layer of the unbleached tooth of *L. ditropis* the content of Na_2O was lower (<1 wt%) than that of the inner layer (1.1-1.3 wt%). Teeth of other samples including bleached samples of *L. ditropis* and bleached and unbleached samples of the other two species showed a similar distribution pattern. In the teeth of the three species examined, dentin contained a smaller amount of sodium than enameloid.

In the outer enameloid layer of the teeth of *L. ditropis*, the content of magnesium was almost equal to that of the inner enameloid layer, being about 0.1 wt%. But the innermost enameloid layer adjacent to the dentin contained two times more magnesium than the other layers. The same result was obtained for the teeth of *C. carcharias* and *P. glauca*.

Histochemistry (Fig. 2)

The sections of the tooth germ of *C. carcharias* were stained using the PAS method. At the stage of matrix completion, in the enameloid matrix, fibers being arranged in parallel to the enameloid surface were red-stained. Their density was higher in the inner layer than in the other layers.

SEM observation (Fig. 3)

The enameloid matrix of tooth germ of *C. carcharias* was observed using SEM. At the early stage of matrix

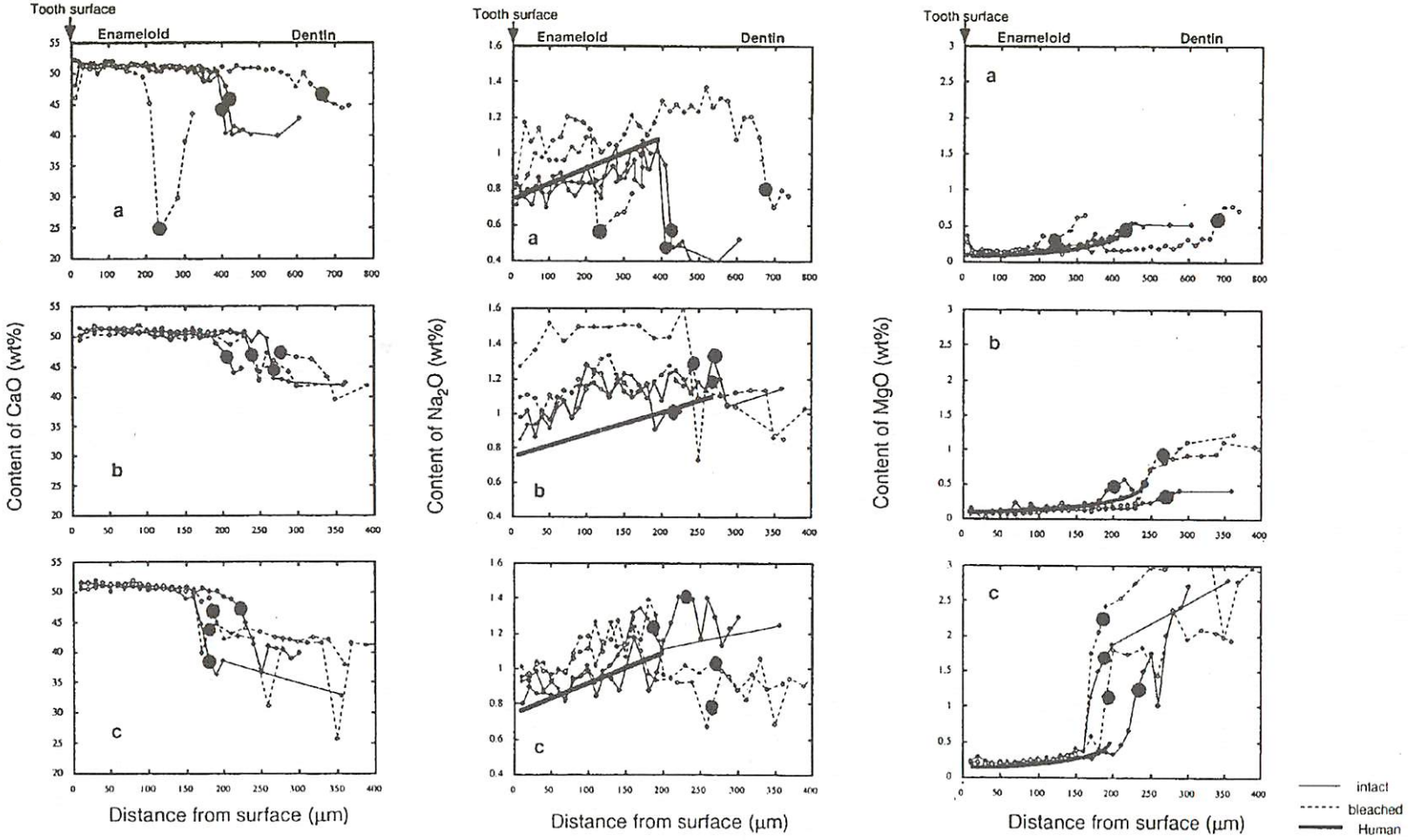

Figure 1. Distribution patterns of CaO (left column), Na_2O (middle column), and MgO (right column) contents in enameloids of *Carcharodon carcharias* (a), *Lamna ditropis* (b), and *Prionace glauca* (c). Solid circles show the contents at the enameloid-dentin junction. Human data are calculated from data of Weatherell and Robinson (1973).

formation, in the outer layer, a thick basement membrane was observed in a reticulate figure. Beneath the membrane, sheet-like fiber bundles were observed. In the middle layer three components were identified; they are, 1) sheet-like fiber bundles (collagen fibers, from histochemical data not shown) that are arranged in vertical to the enameloid surface, 2) thin fibers that are arranged in parallel to the enameloid surface, and 3) vacuoles, a few μm in diameter. In the inner layer, the sheet-like fiber bundles were absent, and the thin fibers were arranged more irregularly than those in the middle layer of the matrix. The vacuoles in the inner layer were smaller than those in the middle layer.

After the matrix formation stage in the outermost layer, a thick reticular basement membrane was absent and the fibers were densely arranged in parallel to the enameloid surface. The middle layer contained two types of fibers, arranged in vertical and parallel to the enameloid surface. The vertical fibers occurred in a sheet-like figure, and the directions of the parallel fibers were independent of one another. In the inner layer irregularly arranged fibers were observed. The fibers comparable to the sheet-like fibers were not observed in it.

Immunohistochemistry (Fig. 4)

The enameloid matrix of the tooth germ in *C. carcharias* was investigated with immunohistochemical technique. The vacuoles and the microfibers were well-stained not with preserum but with the anti-*Lamna* teeth extract (antiserum LDE-1). At the matrix completion stage the LDE-1 reacted more strongly with the inner layer of the enameloid matrix than with the outer layer at the dilution of 10^{-4}. Preserum also reacted the inner layer very weakly. The tooth germs in *L. ditropis* indicated similar results.

Discussion

Heterogeneous distribution of inorganic components

The distribution patterns of sodium and magnesium in the enameloid of the three shark species displayed a gradual increase toward the enameloid-dentin junction like that demonstrated in mammalian enamels (Fig. 1). The concentrations of these two elements in the shark enameloid showed values of the same order as those reported for mammal enamel. The Mg content of the shark enameloid was much lower than that of dentin as in mammalian teeth. The fact that bleaching of teeth did not change the distribution patterns and the contents of Na, Mg, and Ca shows that they are independent of organic components . Therefore it is expected that the mechanisms of calcification and ion transport in the enameloid are intrinsic for sharks, and that the shark enameloid is more related to the mammalian enamel than to the shark dentin.

The above results and enameloid and the fact that mammalian enamel is calcified with inorganic ions transported by ameloblast (Nagai and Frank, 1975; Ozawa *et al.*, 1979) both imply the participation of epidermal cells in enameloid calcification. The fact that in the calcification stage of the enameloid epidermal cells display active shapes (Goto, 1978; Kakizawa, 1984) strongly support this interpretation.

Origin of enameloid matrix

In the case of shark enameloid matrix in *C. carcharias*, comparison of SEM observations with histochemical ones revealed that the sheet-like material comprised collagen fibers (data not shown). The perpendicular fibers observed by Kakizawa (1984) in the tooth germ of *L. ditropis* may represent to these sheet-like collagen fibers. Vacuoles, each about 2 μm in diameter, and fine fibers (referred to as "microfiber" in this paper) being arranged in parallel to the enameloid surface were green-stained with the collagen stain method (data not shown), indicating that they contained non-collagenous proteins. SEM observation revealed that they existed around the odontoblasts (Fig. 3), indicating the mesenchymal origin of them. The microfibers in the elasmobranch enameloid matrix may correspond to the giant fiber (Kemp and Park, 1974; Prostak and Skobe, 1988) or the odontoblastic process (Goto, 1978; Kemp, 1985; Prostak and Skobe, 1988).

Kiso (1997) indicated that the enameloid was related to the dentin as far as soluble proteins are concerned. Protein shared by the enameloid and dentin matrices is abundant in the dentin. It is well known that dentin matrix is secreted only by odontoblasts. Strong reactivity of the vacuoles and the microfibers with LDE-1 in *C. carcharias* (Fig. 4) indicates involvement of matrix proteins having the same epitopes as those of *L. ditropis*. This indication supports the mesenchymal origination of vacuoles and microfibers. Therefore the shark enameloid is not homologous with the mammalian enamel in matrix formation. The results of this study are consistent with the interpretations by Sasagawa (1994) and Ishiyama *et al.* (1994).

Maturation of enameloid matrix

This study revealed the structural change of the enameloid matrix in the teeth of *C. carcharias*. Under the

SEM the vacuoles are eliminated and microfibers changed into thick fibers that were arranged in parallel to the enameloid surface in the middle and the inner layers (Fig. 3). The dence enameloid matrix changed into sparse fibrous matrix. This observation proposes that non-collagenous proteins decrease during the late stage. These results and the previous biochemical work (Kiso, 1997) indicate that the proteolytic activities in shark enameloid are evidently present during enameloid development in *C. carcharias* as in bony fish.

Biochemical analysis clarified that the amount of amelogenin decreases and the enamel matrix becomes enamelin-rich during the enamel maturation in mammals (Termine *et al.*, 1980). In mammalian enamel proteases work for the maturation, and histochemical reactivities of the enamel matrix change during the matrix maturation (Suga, 1987). Kawasaki *et al.* (1987) made clear with histochemical and biochemical techniques that bony fish protease activities are responsible for elimination of matrix proteins during matrix maturation as in mammals. The proteolytic activity in mammalian enamel is derived from the proteases secreted by ameloblasts (Ozawa *et al.*, 1983). Judging from these lines of evidence, in the morphogenesis of the shark enameloid, the epidermal cells are expected to secrete not initial matrix but the enzymes responsible for matrix maturation.

Heterogeneity of organic matrix in enameloid

This study revealed that vacuoles and microfibers were heterogeneously distributed in the enameloid matrix of *C. carcharias*. SEM observations showed in *C. carcharias* the presence of large amounts of vacuoles and microfibers in the outer layer of the enameloid, and of randomly arranged fibers in the inner layer adjacent to the enameloid-dentin junction at the matrix formation stage (Fig. 3). The heterogeneity of the distribution of the vacuoles and fibers was also confirmed in this species by immunohistochemical observations of thin sections. At the late stage (matrix completion stage) the heterogeneity was recognized as difference of orientations of fibers.

At the stage of matrix completion polysaccharides in a fiberous form occurred abundantly in the inner enameloid layer of *C. carcharias* (Fig. 2). The fact that the polysaccharides were detected on the odontoblast side in the enameloid matrix implies that the polysaccharides are secreted by odontoblasts. This implication is inconsistent with the epidermal origin of polysaccharides suggeted by Kakizawa (1984) and Goto (1978). Immunohistochemical observations revealed the difference between materials of the outer and inner enameloid layers in this species. The inner layer reacted both with the preserum and the antiserum, while the outer layer reacted only with the antiserum.

The histochemical heterogeneity observed in the shark enameloid matrix may be related to the layered structure of mature teeth. Reif (1973) stressed that the three layered structure evolved to enable more effective feeding. According to his observation, the three layered shark enameloid consists of "shiny layer", "parallel fibered layer", and "haphazardly fibered layer" from the outer side to the inner side (Fig. 5). At the matrix completion stage the structural heterogeneity resembled Reif's three layered structure from the view of morphology (Fig. 3). Especially, Reif's "haphazardly fibered layer" appears to correspond to the region enriched with polysaccharides and preserum-positive materials. In this study the structural heterogeneity within the shark enameloid was revealed to accompany with the histochemical heterogeneity. Because of odontoblastic origin of enameloid matrix the enameloid structures may have arisen by evolutionary emergence of switching of mechanisms for odontoblastic activity. According to Reif (1973) primitive elasmobranchs, including hybodontids and ptychodontids, do not have the haphazardly fibered layer, but more advanced elasmobranchs have it (Fig. 5). Therefore, if we accept the recent opinion of elasmobranch phylogeny (Shirai, 1996), the emergence of switching of odontoblastic activity would have been achieved when Neoselachii appeared. Sasagawa (1993) considered that the shiny layer is formed by epidermal cells. Therefore the increase of epidermal activity also may be important in the evolution of the shiny layer.

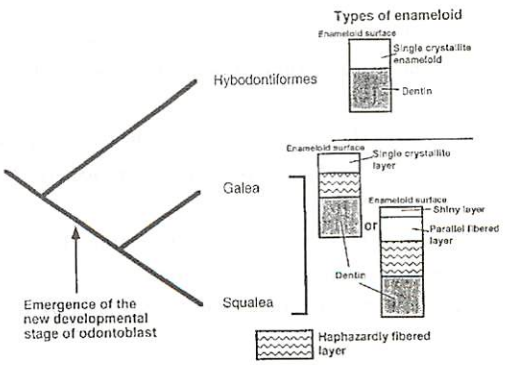

Figure 5. Distribution of enameloid types in elasmobranch phylogeny. The phylogenetic tree is after Shirai (1996). The enameloid types and the terminology are from Reif (1973). *C. carsharias*, *Lamna ditropis*, and *Prionace glauca* are included in Galea.

Conclusion

As a results of this study, it was supported that in the sharks the mesenchymal cells are responsible for matrix formation but the epidermal cells probably take part in calcification during enameloid development. And proteolytic process during enameloid development was clarified in elasmobranch as seen in other vertebrates. Therefore shark enameloid is similar to the mammalian type enamel in part and to the dentin in part.

Moreover, these histogenic activities construct a layered structure in shark enameloid. The emergence of the haphazardly fibered layer appears to have developed by switching of odontoblastic activity at the appearance of the Neoselachii. Histogenetic activities differ among the species with the same structure of enameloid, especially between the lamniform sharks and other sharks.

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References

Davidson E. H. (1991) Spatial mechanisms of gene regulation in metazoan embryos. *Development* 113: 1-26.

Goto M. (1978) Histogenetic studies on the teeth of leopard shark (*Triakis scyllia*). *J. Stomatological Society, Japan* 45: 527-584.

Herold R. C., Graver H. T. and Christner P. (1980) Immunohistochemical localization of amelogenins in enameloid of lower vertebrate teeth. *Science* 207: 1357-1358.

Herold R., Rosenbloom J. and Granovsky M. (1989) Phylogenetic distribution of enamel proteins: Immunohistochemical localization with monoclonal antibodies indicates the evolutionary appearance of enamelins prior to amelogenins. *Calci. Tis. Int.* 45: 88-94.

Ishiyama M., Inage T., Shimokawa H. and Yoshie S. (1994) Immunocytochemical detection of enamel proteins in dental matrix of certain fishes. *Bull. de l'institut oceanographique, Monaco, n special* 14: 175-182.

Kakizawa Y. (1984) On the teeth of salmon shark, *Lamna ditropis* Hubbs and Follett. *Nihon Univ. Dent. J.* 58: 59-69.

Kawasaki K., Shimoda S. and Fukae M. (1987) Histological and biochemical observations of developing enameloid of the sea bream. *Advances in Dent. Res.* 1(2): 191-195.

Kemp N. E. (1985) Ameloblastic secretion and calcification of the enamel layer in shark teeth. *J. Morphology* 184: 215-230.

Kemp N. E. and Park J. H. (1974) Ultrastructure of the enamel layer in developing teeth of the shark *Carcharhinus menisorrah*. *Arch. Oral Biol.* 19: 633-644.

Kiso T. M. (1997) Fluoride-binding property and its relation to dentin of organic matrix in lamnid shark enameloid. *Arch. Comp. Biol. Tooth Enamel* 5: 1-11.

Nagai N. and Frank R. M. (1975) Transfert du ^{45}Ca par autoradiographie en microscopie electronique aucours de l'amelogenese. *Calcif. Tis. Res.* 19: 211-221.

Ozawa H., Yamamoto T. Yamada M. and Uchida T. (1979) Frozen ultrathin-sections for X-ray microanalysis of rat tooth germs. *J. Dent. Res.* 58(B): 1016-1018.

Ozawa H., Yamada M., Uchida T., Yamamoto T. and Takano Y. (1983) Fine structural and cytochemical studies of the golgi-SER system of ameloblasts with spacial reference to its resorptive function. In *Mechanisms of Tooth Enamel Formation* (Suga S. ed.) Quintessence, Tokyo: 17-48.

Prostak K. S. and Skobe Z. (1988) Ultrastructure of odontogenic cells during enameloid matrix synthesis in tooth buds from an elasmobranch, *Raja erinacae*. *Amer. J. Anat.* 182: 59-72.

Reif W-E. (1973) Morphologie und Ultrastruktur des Hai-"Schmelzes". *Zoologica Scripta* 2: 231-250.

Sasagawa I. (1993) Features of tooth enameloid in Elasmobranchs. *J. Fos. Res.* 26: 28-41.

Sasagawa I. (1994) The Fine structure of the dentin atrix and mineralization during dentinogenesis in sting rays, Elasmobranchs. *Bull. de l'institut oceanographique, Monaco, n special* 14: 167-174.

Shirai S. (1996) Phylogenetic interrelationships of neoselachians (Chondrichthyes: Euselachii) In *Interrelationships of Fishes, Revisited.* (Stiassny M. L. J., Parenti L. R. and

Johnson G. D. eds.) Academic Press, San Diego: 9-34.

Slavkin H. C., Graham E., Zeichner-David M. and Hildemann W. (1983a) Enamel-like antigens in hagfish: Possible evolutionary significance. *Evolution* 37(2): 404-412.

Slavkin H. C., Samuel N., Bringas Jr. P., Nanci A. and Santos V. (1983b) Selachian tooth development: II. Immunolocalization of amelogenin polypeptides in epithelium during secretory amelogenesis in *Squalus acanthias*. *J. Craniofacial Genet. Develop. Biol.* 3: 43-52.

Slavkin H. C., Bessem C., Bringas Jr. P., Zeichner-David M., Nanci A. and Snead M. L. (1988) Sequential expression and differential function of multiple enamel proteins during fetal, neonatal, and early postnatal stages of mouse molar organogenesis. *Differentiation* 37: 26-39.

Suga S. (1987) Histochemistry of enamel matrix protein. In *Tooth enamel, its formation, structure, composition and evolution.* (Suga S. ed.) Quintessence, Tokyo: 163-173.

Termine J. D., Belcourt A. B., Christner P. J., Conn K. M. and Nylen M. U. (1980) Properties of dissociatively extracted fetal tooth matrix proteins. I Principal molecular species in developing bovine enamel. *J. Biol. Chem.* 225: 9760-9768.

Weatherell J. and Robinson J. A. (1973) The inorganic composition of teeth. In *Biological Mineralization* (Zipkin I. ed.), John Wiley and Sons, New York: 43-74.

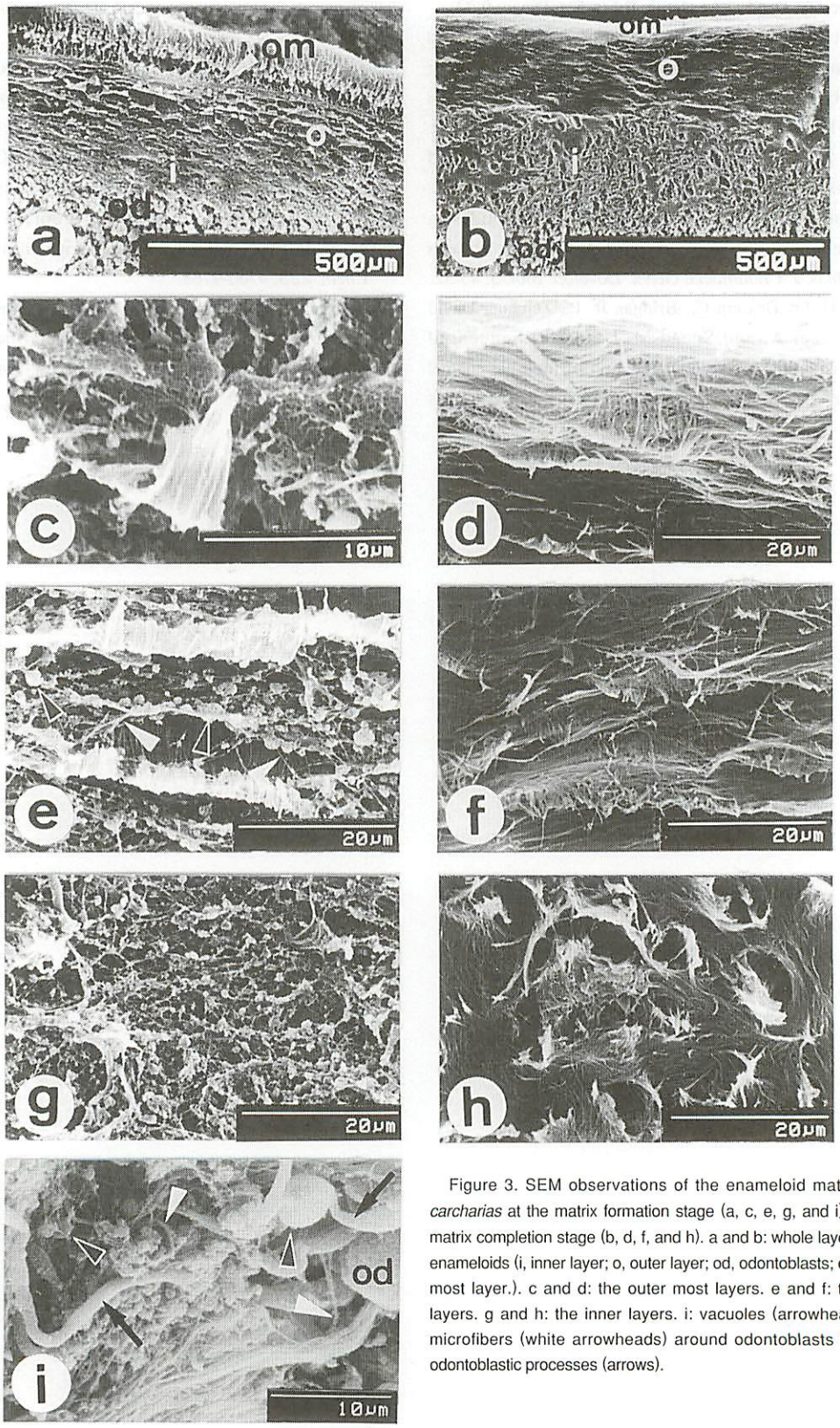

Figure 3. SEM observations of the enameloid matrix of *C. carcharias* at the matrix formation stage (a, c, e, g, and i) and the matrix completion stage (b, d, f, and h). a and b: whole layers of the enameloids (i, inner layer; o, outer layer; od, odontoblasts; om, outer most layer.). c and d: the outer most layers. e and f: the outer layers. g and h: the inner layers. i: vacuoles (arrowheads) and microfibers (white arrowheads) around odontoblasts (od) and odontoblastic processes (arrows).

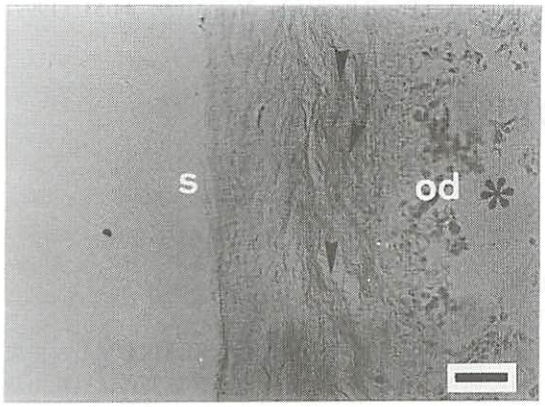

Figure 2. Thin section of *C. carcharias* tooth germ stained by PAS method at the matrix completion stage. The inner layer of enameloid matrix is well-stained (the arrowheads). s: surface of enameloid matrix. od: odontoblasts. Asterisk marks a region of artificial destruction. Bar = 50 μm.

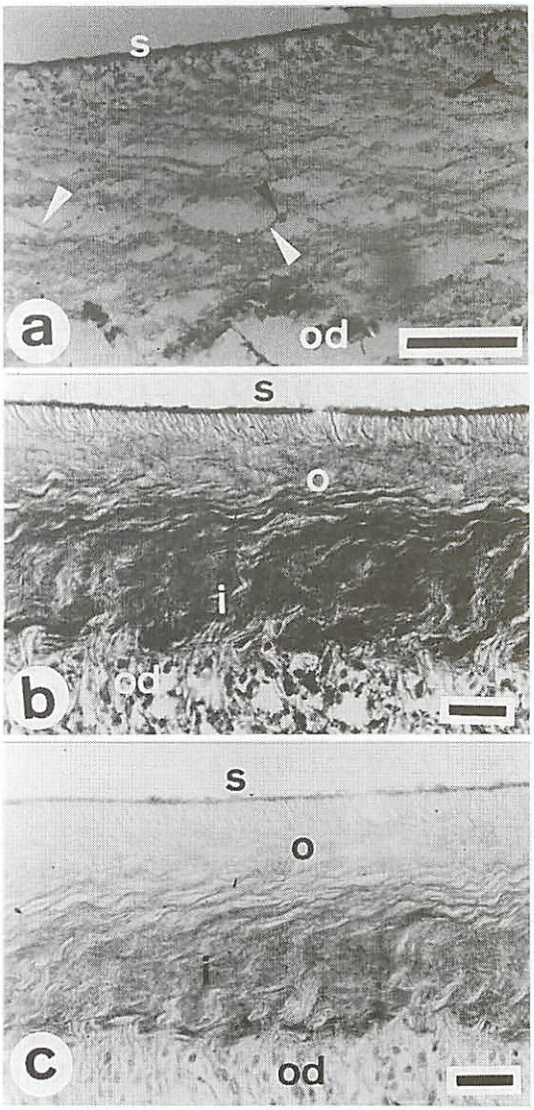

Figure 4. Immunohistochemistry of the enameloid matrix of *C. carcharias* at the matrix formation stage (a) and at the matrix completion stage (b and c; s, surfaces of enameloid matix; o, outer layers; i, inner layers; od, odontoblasts). a and b: reaction with antiserum LDE-1. c: with preserum. In the photograph of a, arrowheads point to well-stained vacuoles, and white arrowheads does stained microfibers. Bar = 50 μm.