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Immunohistochemical localisation of amelogenin-like proteins and type I collagen and histochemical demonstration of sulphated glycoconjugates in developing enameloid and enamel matrices of the larval urodele (*Triturus pyrrhogaster*) teeth

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ABSTRACT

The presence of collagen in enameloid distinguishes it clearly from true enamel, but little is known about the phylogenetic relationship between these 2 tissues. It has previously been reported that amelogenins are the principal proteins of the enamel matrix, that type I collagen and chondroitin sulphates are the predominant matrices in dentine, and that amphibian and reptilian aprismatic enamels contain no sulphated glycoconjugates, although certain sulphated substances are secreted into mammalian prismatic enamel during matrix formation. The larval urodele (*Triturus pyrrhogaster*) teeth are known to be composed of enameloid, dentine, and enamel-like tissue. To characterise the tooth matrices, the localisation of amelogenin-like proteins, type I collagen, and sulphated glycoconjugates was investigated. Chondroitin sulphates and fine fibrils immunoreactive for type I collagen were elaborated as the enameloid matrix inside the dental basement membrane. After the matrix had been deposited in full thickness, coarse collagen fibrils also immunoreactive for type I collagen and chondroitin sulphates were deposited below as the first dentine matrix. Further, enamel-like matrix with no collagen fibrils or sulphated glycoconjugates but strongly immunoreactive for amelogenins was deposited on the dentine. Although no immunolabelling for amelogenins was found over the enameloid matrix, at least at the formation stage, the zone of coarse collagen fibrils of dentine was partially immunoreactive as observed in mammalian mantle dentine. From the ontogeny and matrix constituents of larval urodele teeth, it is suggested that enameloid is originally a dentine-like tissue.

Key words: Amphibian tooth matrix; dentine.

INTRODUCTION

The involvement of amelogenins in enameloid formation in lower vertebrates has been controversial (Herold et al. 1980, 1989; Slavkin et al. 1983*b*; Samuel et al. 1987; Ishiyama et al. 1993). A mammalian type of enamel protein, amelogenins are widely distributed not only in mammalian prismatic enamel (Nanci et al. 1985; Inage et al. 1989) but also in amphibian and reptilian aprismatic enamels (Herold et al. 1989; Ishiyama et al. 1993). It has been reported that a fish (garpike) enamel contains amelogenin-like proteins but that its enameloid does not (Ishiyama et al. 1993). On the other hand, Slavkin et al. (1983*b*) have demonstrated immunohistochemically the presence of amelogenin polypeptides in shark tooth enameloid and inner dental epithelial (IDE) cells. It has been also shown that mouse amelogenin probes were hybridised with genomic cDNA from seawolf (*Anarrhichas*) but not with Atlantic salmon (*Salmo salar*), suggesting that the DNA for amelogenins could be deleted or modified in some fishes but in others it has been preserved during their evolution (Lyngstaas et al. 1990). Metatherian enamel proteins are also revealed to be highly homologous with those of mammals (Hu et al. 1996).

Although it has been shown that amelogenin antigens are localised immunohistochemically in the

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pokal cells of hagfish horny teeth (Slavkin et al. 1983*a*), most recent immunohistochemical and ultrastructural studies have failed to detect the antigens (Yokoyama & Ishiyama, 1998). It is suggested that amelogenins of extant vertebrates originate from a common ancestral gene (Slavkin & Diekwisch, 1996). Most recently, amelogenin-like proteins have been demonstrated in the genoine layer of the scales of primitive osteichtyan fishes (Kogaya, 1997; Zylberberg et al. 1997). This suggests that the ganoine-forming cells have also preserved a gene for amelogenin-like proteins and that when considering the origin of teeth from the dermal armour the gene might have already been expressed, associated with the formation of exoskeletal elements of 500 myr-old ancestors (Girondot & Sire, 1998).

The highly mineralised tissues covering the tips of the larval monocuspid and adult bicuspid teeth of the urodeles (*Ambystoma mexicanum*, *Triturus vulgaris*, and *Triturus pyrrhogaster*) are themselves different: that is, the former is enameloid and the latter true enamel (Kerr, 1960; Smith & Miles, 1971; Kogaya, 1994, 1995). Although it has been suggested that enameloid could have evolved into true enamel via a prolongation of secretory activity of IDE (Poole, 1971) and that evolution of enamel may result from heterochrony that represents changes in the relative time of appearance and rate of various characters, including gene expression, associated with tooth development (Slavkin & Diekwisch, 1996), it is still controversial whether 1 of these 2 tissues precedes the other. Smith (1992) has maintained that enamel is the primitive tissue. Kogaya (1994, 1995) has reported that enameloid should be recognised as quite a different mineralised tissue from true enamel, i.e. a modified dentine, since the mineralisation pattern and matrix components of enameloid which is elaborated both by odontoblasts and IDE, deposited before true dentine is formed, usually includes collagen fibrils and chondroitin sulphates, but contains no minerals at least during the matrix formation stage. These features are quite different from those of true enamel, which is produced by ameloblasts after true dentine formation, contains no mesodermal-derived collagen or chondroitin sulphates but ectodermal proteins, amelogenins and enamelins, and already includes some minerals even at the matrix formation stage. On the other hand, it is well known that both enameloid and dentine matrices are composed of collagen fibrils (Sasagawa, 1988, 1995; Prostak et al. 1991; Wakita, 1993) and that type I collagen is a major component of the mammalian dentine matrix (Veis, 1984). However, little is known about the type of enameloid collagen, although Sasagawa (1996) and Prostak et al. (1992) have suggested that from its ultrastructural features and biochemical analysis it may be type I collagen.

The present study was designed to characterise the tooth matrices of the larval urodele (*Triturus pyrrhogaster*) which have both enameloid and enamel-like tissue.

MATERIALS AND METHODS

Mandibles and maxillae with tooth germs were dissected from 20 larval newts (*Triturus pyrrhogaster*) anaesthetised with cold water and fixed in a mixture of 1% glutaraldehyde/4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3–6 h. They were divided into 3 groups to assess the different preparation procedures: (1) the immunolabelling group (7 larval newts) in which the specimens were decalcified with 5% EDTA for 1–3 weeks, dehydrated and embedded in LR White; (2) the high iron diamine (HID) staining group (6 larval newts); and (3) the group for ultrastructural observation (7 larval newts) in which the specimens without decalcification were embedded in Taab 812 resin.

HID-TCH-SP staining

This staining method has been found to localise sulphated glycoconjugates specifically (Sannes et al. 1979). Further, the specificity was established on the basis of staining most sites known from biochemical analysis or radioautography with $^{35}SO_4$ to contain sulphated complex carbohydrates. The precise histochemical properties of the sulphated materials can be identified by use of the enzymatic digestion method (Kogaya & Furuhashi, 1988).

Specimens were incubated for 18 h at room temperature in high iron diamine (HID) solution, as previously described (Sannes, 1984; Kogaya & Furuhashi, 1987). This solution was prepared by addition of 1.6 ml 40% $FeCl_3$ (Nakarai Chemical Co, Kyoto) to a freshly prepared diamine solution containing 120 mg of *N*,*N*-dimethyl-*m*-phenylene diamine (HCl) (Sigma, St Louis) and 20 mg *N*,*N*-dimethyl-*p*-phenylene diamine (HCl) (Sigma) in 50 ml of distilled water. Control specimens were similarly incubated in a solution prepared by addition of 1.6 ml 40% $MgCl_2$ to a freshly prepared diamine solution as mentioned above, the pH of which was adjusted to 1.6 with 0.1 M HCl. These specimens were rinsed several times in distilled water, postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4),

dehydrated and embedded in Taab 812 Resin. Ultrathin sections, cut with a diamond knife and floated onto stainless steel grids, were reacted with 2% thiocarbohydrazide (Merck, Darmstadt), in 10% acetic acid for 15 min, rinsed in distilled water, and treated with 1% aqueous silver proteinate (Merck) for 20 min in the dark. The silver proteinate background staining was eliminated by filtering (Whatman filter no. 2) the silver proteinate solution twice before use. Sections were then washed in 2–3 changes of distilled water. The ultrathin sections were observed without uranyl acetate or lead citrate staining.

Enzymatic degradation

To identify the histochemical properties of HID-TCH-SP stain deposits, some specimens were exposed for 6 h at 37 °C to a 0.05% testicular hyaluronidase (Sigma, type 1S) solution in 0.1 M sodium acetate buffer (pH 5.8) containing 0.15 M sodium chloride before HID staining.

Immunostaining for amelogenins and type I collagen

The protein A-gold immunocytochemical technique (Nanci et al. 1985) for the detection of antigenic sites of amelogenins and type I collagen was used. The thin sections of LR White embedded specimens, mounted on formvar coated nickel grids, were floated for 5 min on a drop 0.01 M phosphate buffered saline (PBS) containing 1% ovalbumin and then placed onto a drop of antiserum (rabbit antiserum against bovine amelogenins diluted 1:200 in PBS ovalbumin; gift from Dr H. Shimokawa, Tokyo Medical and Dental University, rabbit antisera against chicken type I collagen diluted 1:100 in PBS ovalbumin; Chemicon, Temecula) for 1–3 h. The specificity of these antibodies has been determined by ELISA and immunoblots (Shimokawa et al. 1984) and radioimmunoassays (Mauger et al. 1982). The sections were rinsed with PBS to remove excess unbound antibodies, transferred onto a drop of PBS containing 1% ovalbumin for 5 min, and incubated for 30 min with the protein A-gold complex (EY Laboratories, Tokyo). The sections were then thoroughly washed with PBS and followed by distilled water. Controls consisted of sections incubated with (1) antibody previously exposed to an excess of amelogenins (gift from Dr Doi, Asahi University), (2) ovalbumin, (3) protein A-gold alone. The sections were conventionally stained with uranyl acetate and lead citrate and examined with a JEM 1200 EX electron microscopy.

RESULTS

Development of the larval urodele teeth

In the present study 3 stages of tooth development as shown in Figure 1 were surveyed: (a) enameloid matrix formation stage at which fine fibrils with no obvious cross bands are deposited as the first tooth substances, (b) dentine matrix formation and mineralisation stage at which coarse collagen fibrils oriented parallel to the tooth surface are elaborated and initial mineralisation associated with the dentine collagen fibrils subsequently occurs, (c) enameloid maturation and enamel-like matrix formation stage at which the distal cell membrane of the IDE cells covering cap enameloid becomes deeply folded to form a ruffled border while enamel-like matrix begins to form on the mineralised shaft dentine.

The enameloid matrix was composed of fine fibrils with no obvious cross bands and amorphous material (Fig. 2*a*). The fine fibrils in the surface layer of the enameloid appeared parallel to the dental basement membrane, and the density of the fibrils was much higher in the surface layer than in the central portion (Fig. 2*a*). After the fine fibril matrix had been deposited in full thickness, coarse collagen fibrils, oriented parallel to the tooth surface, and matrix vesicles appeared below and spread downwards along the distal surface of the IDE cells (Fig. 2*b*). Initial mineralisation occurred associated with matrix vesicles and coarse collagen fibrils of the dentine matrix (Fig. 2*c*) and then rapidly spread into the enameloid matrix (Fig. 2*d*). At this stage the outermost part of enameloid was ultrastructurally different from the central part, that is, the former still contained

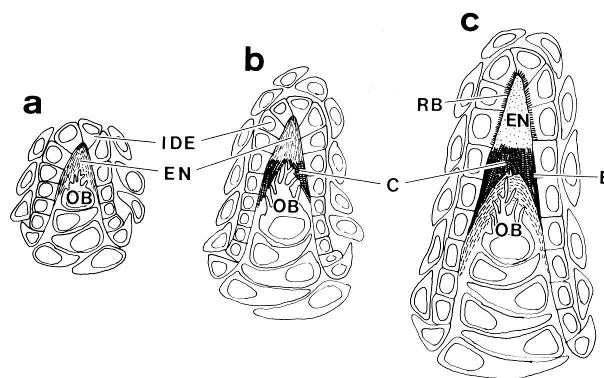

Fig. 1. Schematic representation of the development in the larval urodele (*Triturus pyrrhogaster*). (*a*) Enameloid matrix formation stage, (*b*) dentine matrix formation and mineralisation stage, (*c*) enameloid maturation and enamel-like matrix formation stage. IDE, inner dental epithelium; OB, odontoblasts; EN, enameloid matrix composed of fine collagen fibrils; C, coarse collagen fibrils of dentine; E, enamel-like matrix; RB, ruffled border.

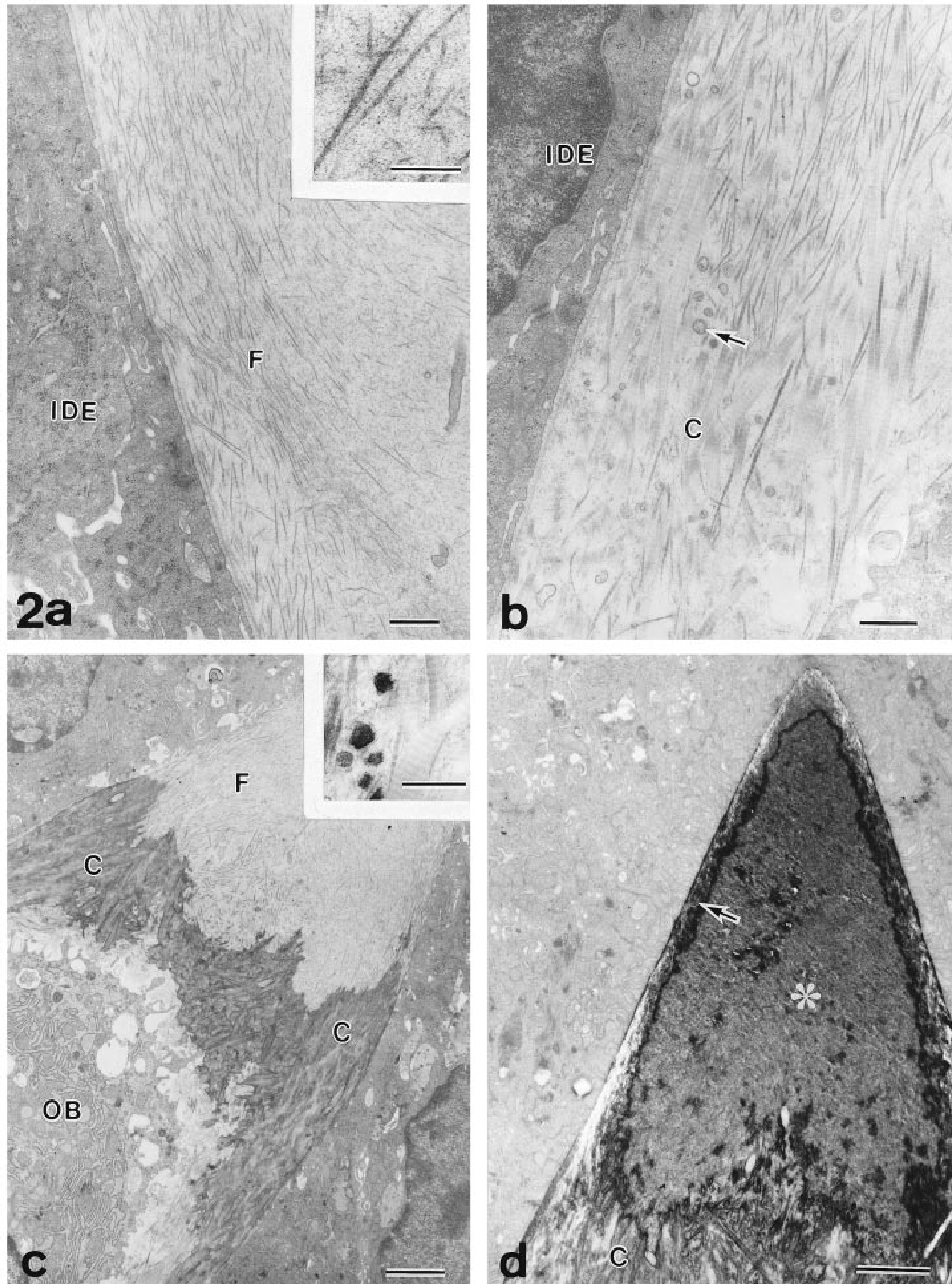

Fig. 2. (*a*) Enameloid matrix composed of fine fibrils (F) which have no obvious cross striation Bar, 0.5 μm (inset bar, 0.2 μm). (*b*) After the full thickness enameloid matrix has been formed, coarse collagen fibrils (C) begin to be deposited below. Arrow indicates matrix vesicle. Bar, 0.5 μm. (*c*) The first mineralisation occurs associated with matrix vesicles (inset bar, 0.5 μm) and then coarse collagen fibrils (C). F, enameloid matrix composed of fine fibrils; OB, odontoblast. Bar, 2 μm. (*d*) At the maturation stage of enameloid, the distal cell membrane of IDE develops a ruffled border. Note differences in structure between outermost part (arrow) and inner part (asterisk). C, mineralised coarse collagen fibrils of dentine. Bar, 2 μm.

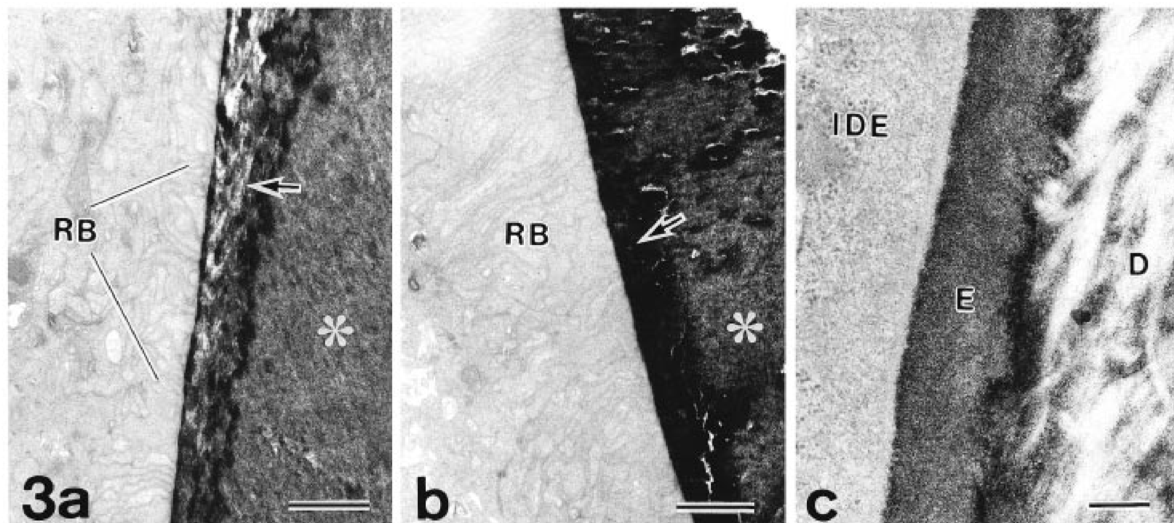

Fig. 3. Enameloid maturation and enamel-like matrix formation. (*a*) Higher magnification of enameloid matrix at the maturation stage. Note that fibrous structures (arrow) parallel to the tooth surface are still observed and that the inner part (asterisk) of the enameloid appears homogenous. RB, ruffled border. Bar, 1 μm. (*b*) At the late maturation stage the outermost part (arrow) is observed as an electron-dense heavily mineralised layer compared with the inner part (asterisk). RB, ruffled border. Bar, 1 μm. (*c*) Enamel-like matrix (E) is formed on the mineralised shaft dentine (D). Note that the distal cell membrane of IDE has no ruffled border. Bar, 0.2 μm.

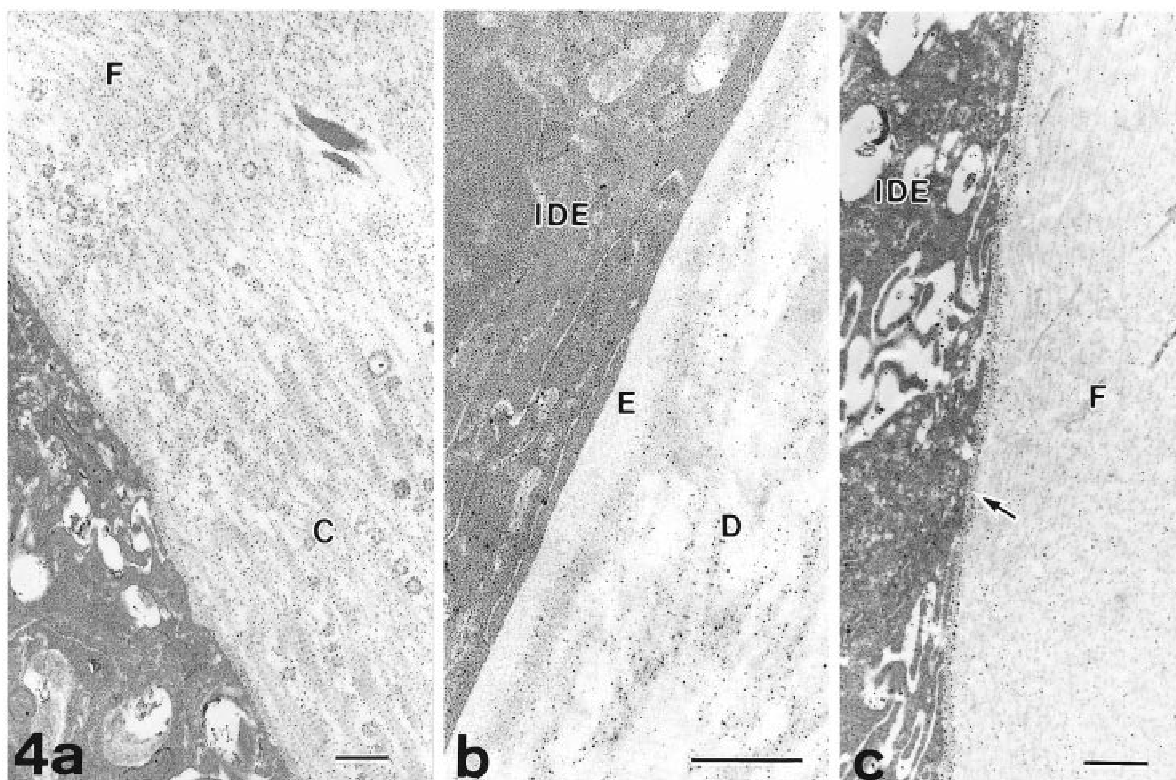

Fig. 4. Detection of sulphated glycoconjugates in enameloid, dentine and enamel-like matrices by use of the HID-TCH-SP staining method. (*a*) HID-TCH-SP stain deposits are observed over both enameloid fine fibrils (F) and dentine coarse collagen fibrils (C). (*b*) No HID-TCH-SP staining is found over enamel-like matrix (E) on the shaft dentine (D). (*c*) Most HID-TCH-SP stain deposits associated with the enameloid matrix (F) are removed by the enzymatic degradation of testicular hyaluronidase, indicating that the sulphated glycoconjugates are chondroitin sulphates. Note that the stain deposits associated with the dental basement membrane (arrow) survive. Bars, 0.5 μm.

fibrous structures oriented parallel to the tooth surface but the latter appeared almost homogenous (Figs 2*d*, 3*a*, *b*). In addition, as the mineralisation proceeded, the outermost part appeared as a thin electron-dense layer (Fig. 3*b*), suggesting that it was a much more heavily mineralised area. Subsequently, a thin layer of

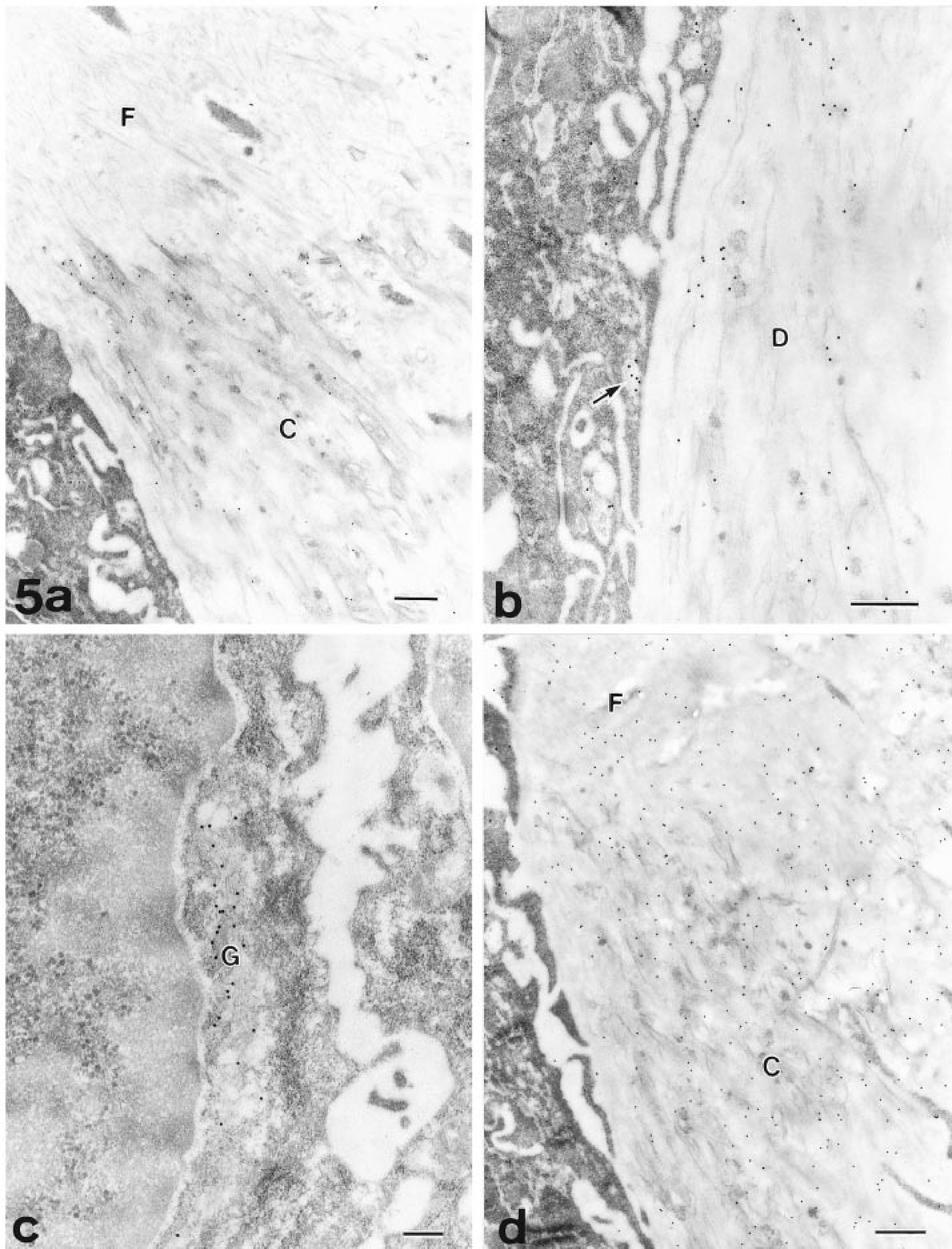

Fig. 5. Immunohistochemical localisation of amelogenin-like proteins and type I collagen. (*a*) Amelogenin immunoreactivity is observed over coarse collagen fibrils (C) of dentine but not over fine collagen fibrils of enameloid matrix (F). Bar, 0.5 µm. (*b*) A secretory granule-like structure (arrow) in the IDE cells is also immunoreactive for amelogenins. D, dentine. Bar, 0.5 µm. (*c*) Amelogenin immunoreactivity is found over Golgi apparatus (G) of IDE covering the cap enameloid matrix. Bar, 0.2 µm. (*d*) Both enameloid (F) and dentine (C) collagen matrices are immunoreactive for type I collagen. Bar, 0.5 µm.

enamel-like structure, which contained no fibrous elements and was histologically quite different from the enameloid and dentine matrices, was deposited on the shaft dentine (Fig. 3*c*). The distal portion of the IDE cells covering the cap enameloid showed a well developed ruffled border but those facing the enamel-like structure on the shaft dentine had no cytoplasmic infoldings (Figs 2*d*, 3*a*–*c*).

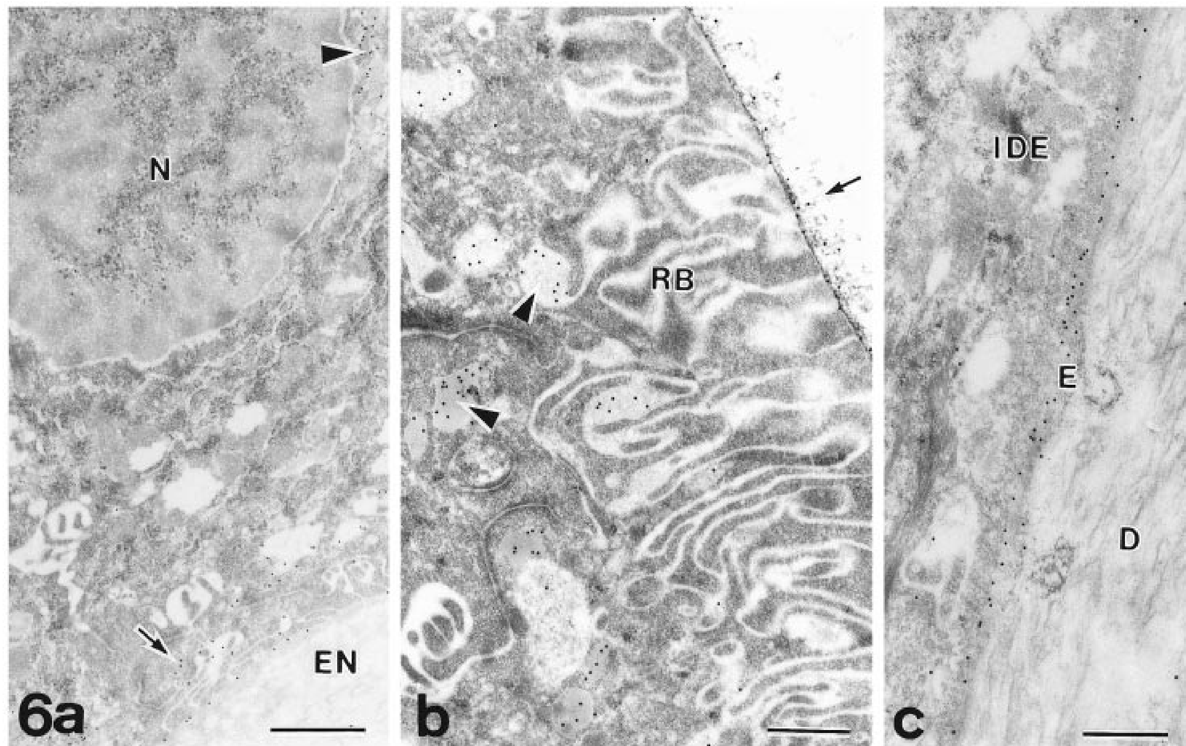

Fig. 6. Immunohistochemical localisation of amelogenin-like proteins in the enameloid matrix and IDE cells at the early (*a*) and late (*b*) maturation stage and in the enamel-like matrix (*c*). (*a*) Immunolabelling for amelogenins is observed in the poorly developed ruffled border spaces (arrow) and on the outermost surface of maturing enameloid (EN). Note the immunoreactivity over Golgi apparatus (arrowhead). N, nucleus. Bar, 1 μm. (*b*) Amelogenin immunoreactivity is found associated with the remnants (arrow) of enameloid matrix being degraded and inside phagosome-like structures (arrowheads) adjacent to the ruffled border (RB). Bar, 0.5 μm. (*c*) Intense immunoreactivity for amelogenins is observed over enamel-like matrix (E) on the shaft dentine (D). Bar, 0.5 μm.

Sulphated glycoconjugates

Sulphated glycoconjugates were detected by use of the HID-TCH-SP staining technique. HID-TCH-SP stain deposits were observed in the enameloid and dentine matrices but not in the enamel-like structure on the shaft dentine (Fig. 4*a*, *b*). Testicular hyaluronidase digestion resulted in the removal of most HID-TCH-SP stainable materials in the enameloid matrix (Fig. 4*c*), indicating that the sulphated glycoconjugates are chondroitin sulphates.

Immunohistochemical localisation of amelogenin-like proteins and type I collagen

No amelogenin immunoreactivity was observed over the enameloid matrix composed of fine collagen fibrils throughout the formation stage (Fig. 5*a*). Amelogenins were first detected immunohistochemically over the coarse collagen fibrils of the dentine matrix (Fig. 5*a*) and over Golgi apparatus and secretory granule-like structures of IDE cells covering the cap enameloid (Figs 5*b*, *c*, 6*a*). On the other hand, both the fine fibrils of enameloid and coarse collagen fibrils of dentine were immunoreactive for type I collagen (Fig. 5*d*). At the beginning of the enameloid maturation stage, amelogenin-like proteins were often found in the poorly developed ruffled border spaces of the distal portions of IDE cells and sometimes in the outermost surface layer of enameloid (Fig. 6*a*). At the late maturation stage when the IDE cells developed a ruffled border, amelogenin immunoreactivity was observed over phagosome-like structures adjacent to the ruffled border and over the remnants of the enameloid matrix being degraded (Fig. 6*b*). An intense immunolabelling for amelogenins was usually seen over enamel-like structure on the shaft dentine (Fig. 6*c*). In control specimens no significant immunolabelling was observed.

DISCUSSION

Our attention has been focused on the distribution pattern and histochemical properties of sulphated glycoconjugates in various types of ectoderm-derived hard tissues (ganoine, fish enameloid, amphibian and reptilian aprismatic enamel, and mammalian prismatic enamel), and it has been shown that the developing enameloid matrix usually contains chondroitin sulphates (Kogaya, 1989; Kogaya &

Akrisaka, 1989), that no sulphated glycoconjugates are detected in the ganoine layer and aprismatic enamel (Kogaya et al. 1992; Kogaya, 1997) and that certain sulphated glycoconjugates whose histochemical properties remain undefined are localised preferentially in the surface layer of developing prismatic enamel (Kogaya & Furuhashi, 1988). In the present study, it was revealed that chondroitin sulphates are detected in the enameloid matrix of the larval urodele but no sulphated glycoconjugates are observed in the enamel-like tissue on the mineralised shaft dentine. Thus the present study provides evidence to support the conclusion that the former is enameloid and that the latter is a tissue belonging to the aprismatic enamels.

Collagen fibrils and sulphated glycoconjugates are major components of the enameloid matrix of most fishes (Sasagawa, 1988, 1995; Kogaya, 1989, 1994; Wakita, 1993), which is quite similar to the mantle dentine matrix of tetrapods. Most enameloid collagen fibrils are generally thought to be derived from odontoblasts (Sasagawa, 1996), although Prostak et al. (1991) maintained that they can be synthesised by IDE cells. Unlike the dentine matrix in which collagen fibrils persist after mineralisation, enameloid matrix finally becomes a highly mineralised tissue with almost complete loss of organic matrices. Removal of the organic materials from the enameloid matrix has been suggested to be due to enzymatic degradation and absorption of the matrix proteins by IDE cells (Kawasaki et al. 1987). Shellis & Miles (1974) have suggested the interaction of epithelial proteins secreted by IDE with enameloid collagen fibrils, by which the collagen can be solubilised prior to or during mineralisation. Although it had been uncertain whether enameloid collagen fibrils are biochemically different from true dentine collagen, the present study showed that thin and nonbanded enameloid collagen fibrils of the larval urodele teeth are immunoreactive for type I collagen. It has been reported that the diameter of type I collagen in various tissues can vary from 0.02 to 0.2 μm and that type III collagen is involved in the regulation of fibril diameter (Romanic et al. 1991). Most recently, we have confirmed that both the enameloid and dentine collagen matrices of the teeth of *Polypterus senegalus* are also immunoreactive for type I collagen but that no amelogenin-like proteins are detectable in either matrix (unpublished data).

True enamels elaborated only by epithelial cells, including the ganoine layer of the scales of osteichtyan fishes (Kogaya, 1997; Zylberberg et al. 1997) and an enamel-like layer of garpike teeth (Ishiyama et al. 1993), are usually immunoreactive for a mammalian type of enamel protein, amelogenins. However, in fish enameloid matrix which is believed to be formed both by IDE and odontoblasts, some studies (Herold et al. 1980; Slavkin et al. 1983*b*) have revealed the presence of amelogenin-like proteins, but in others no amelogenin-like proteins have been detected immunohistochemically or biochemically (Graham, 1985; Samuel et al. 1987; Herold et al. 1989; Ishiyama et al. 1993). In the larval urodele teeth, amelogenin immunoreactivity was first observed associated with the coarse collagen matrix of dentine at the initial mineralisation stage. In mammalian tooth development, it has also been recognised that amelogenins are first observed in the mantle dentine before the onset of its mineralisation (Nanci & Smith, 1992). On the other hand, the cap enameloid matrix showed no immunoreactivity, at least during matrix formation stage, despite the presence of amelogenin-like proteins in the IDE cells covering the tooth tip. Amelogenin-like proteins were, however, found over the outermost surface layer of the maturation stage enameloid matrix and over the phagosome-like structures adjacent to the ruffled border of IDE cells. Two possibilities are proposed: first, amelogenin-like proteins may be involved in the maturation of the outermost enameloid layer; second, amelogenin-like proteins are easily transferred into the enameloid matrix because of the structural changes of dental basement membrane for resorption of enameloid matrices by IDE cells. Enameloid is usually found in most fish teeth but never in tetrapods except the larval urodele teeth, whereas enamel is distributed not only in the teeth of tetrapods but also in the teeth and scales of some fishes. It is, however, still uncertain which of enamel and enameloid first appeared, whether enamel evolved from enameloid, or whether they developed independently. Smith (1992) suggested that enamel is as primitive as enameloid and may first be found in the fossil record as a thin covering to the denticles of the dermal armour. Slavkin & Diekwisch (1996) pointed out that molecular and morphological features are common to the early ontogeny of all vertebrates. As far as the ontogeny of the larval urodele tooth hard tissues (enameloid, dentine and enamel) is concerned, it seems that enameloid is as primitive as mantle dentine and that true enamel evolved independently somewhat later.

Kogaya (1997) and Zylberberg et al. (1997) have revealed that the epidermis covering the ganoine scales of primitive osteichtyan fish, *Calamoichthys calabaricus* and *Polypterus senegalus*, synthesises a mammalian type of enamel protein, amelogenin-like

proteins, but that no amelogenin immunoreactivity is observed associated with the enameloid matrix of the teeth (unpublished data). Thus in *Polypterus senegalus*, amelogenin-like proteins are synthesised by epidermal cells of the trunk but not by oral epithelial cells directly associated with tooth development. The ganoine scales are phylogenetically derived from odontodes in the superficial part of the dermal skeleton which is thought to have been the most primitive type of mineralised tissue, appearing in extinct agnathans about 500 myr ago (Sire, 1989, 1990; Hall, 1992). The fact that the teeth of extant vertebrates are also derived from the dermal armour strongly suggests that a gene for amelogenin-like proteins had already been expressed, associated with the formation of exoskeletal elements in the ancestors. Although the prototype amelogenin proteins have probably undergone some modification during the long evolutionary history of vertebrates, it is conceivable that current common amelogenin antigens have been conserved in the living lower and higher vertebrates. Most recently Toyosawa et al. (1998) have reported that the 6 tyrosine residues of the N-terminal tyrosine-rich amelogenin protein region are conserved in all the amelogenins of monotremes, reptiles and amphibians.

In summary, from the ontogeny and matrix constituents of the larval urodele teeth it is suggested that the highly mineralised tissue enameloid is not a precursor of true enamel but originally a dentine-like tissue.

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