Immunohistochemical Localization of Amelogenins in Enameloid of Lower Vertebrate Teeth

Abstract. *The indirect method of immunofluoresence was used to demonstrate the presence of amelogenins in the enameloid of teeth and dermal denticles of Chondrichthyes; in the enameloid of Teleostei and Amphibia; and in the enamel of Reptilia. Nonmammalian amelogenins are formed in the ectodermal cells of tooth organs and chemically are so similar to mammalian amelogenins that they interact with antiserum prepared from bovine enamel matrix.*

Vertebrate teeth are covered by highly mineralized tissues called enamel in mammals and reptiles and enameloid in fish and amphibians (*1*). Although these tissues are similar in function and location, their true relation is controversial, especially with respect to the embryonic cells that form them and the chemical composition of their organic matrix proteins (*2*). It is well known that enamel matrix proteins (amelogenins) are formed by ectodermal cells of the tooth organ (*3*) and constitute a unique class of glycoproteins with a high content of proline, histidine, glutamic acid, and leucine (*4*). Enameloid matrix proteins are less well known; most reports claim that they are of mesodermal origin and include collagen as a major component (*1, 2*). Indirect evidence suggests that some of the enameloid matrix proteins are derived from the ectodermal cells of the tooth organ (*5, 6*).

In the present study, we tried to determine whether enameloid matrix proteins in nonmammalian vertebrates include ectodermally derived amelogenins that chemically are closely related to mammalian amelogenins. We found that amelogenins are present in the enameloid of teeth and dermal denticles of Chondrichthyes, in the enameloid of teeth of Teleostei and Amphibia, and in the enamel of Reptilia. The amelogenins in these nonmammalian vertebrates are chemically so similar to mammalian amelogenins that they interact with antiserum prepared from bovine enamel matrix. Nonmammalian amelogenins are produced in the ectodermal cells of the tooth organs.

Heterologous bovine antiserum to amelogenin (*3, 7*) was prepared as follows. Soft, immature enamel matrix was scraped from the teeth of a 6-month-old fetal calf and dialyzed against 0.5*M* and 0.01*M* EDTA to solubilize the amelogenins. Two milligrams of solubilized amelogenin in 1 ml of 50 percent complete Freund's adjuvant was injected subcutaneously at several sites along the back of 2.5-kg rabbits once every 2 weeks until each rabbit had received eight injections. The rabbits were bled 1 week after the final injection and the serums precipitated by 70 percent ammonium sulfate. Redissolved serum was purified by affinity column chromatography (*3, 7*). The specificity of this antiserum to amelogenin was demonstrated previously (*3*) by radioimmunoassay and by immunohistochemical studies of tooth odontoblasts, dentine, pulp and stellate reticulum, tongue, salivary glands, liver, kidney, and bone in which there was a complete absence of fluorescence when the antiserum was used.

Blocks of jaw tissue containing developing and mature teeth or of skin tissue containing denticles were obtained from a cow, mouse, pig, tokay gecko, salamander, cod, and spiny dogfish shark and were fixed in 95 percent alcohol, decalcified in 10 percent trichloroacetic acid, and embedded in paraffin (*8*). Histological sections were made and treated by the indirect method of immunofluorescence (*9*) in which purified antiserum (1:40) to bovine amelogenin was followed by goat antiserum (1:10) to rabbit immunoglobulin conjugated to fluorescein isothiocyanate. After washing, the sections were counterstained with Evans blue (0.1 percent) to suppress nonspecific fluorescence (*9*) and then examined with a Leitz Ortholux fluorescence microscope.

The enamel and enameloid matrices and adherent inner dental epithelium (IDE) of developing tooth organs of all the vertebrate teeth examined showed some degree of fluorescence (Fig. 1). Immature enamel matrices from all mammalian species tested exhibited strong fluorescence. In the developing bovine tooth, newly deposited enamel matrix and attached ameloblasts showed intense fluorescence (Fig. 1A). Newly deposited enamel matrix in the cusp of a developing mouse molar (Fig. 1B) and the cervical region of a developing pig molar (Fig. 1C) was brightly fluorescent, but less so than that of the bovine enamel matrix.

When tested with the bovine antiserum to amelogenin, enamel and enameloid matrices from the nonmammalian species were less fluorescent than mammalian enamel. The surface enamel layer in a functional tooth from the gecko (Fig. 1D) exhibited moderate fluorescence as did the enameloid layer in a functional tooth from the salamander (Fig. 1E). There was also moderate fluorescence in the enameloid layer of functional teeth from the cod (Fig. 1F) and shark (Fig. 1G). Both the newly deposited enameloid matrix and the adherent IDE cells in the developing shark tooth bud exhibited moderate fluorescence (Fig. 1H). The enameloid layer of functional dermal denticles (Fig. 1I) in the shark's skin showed more intense fluorescence than the enameloid layer of its developing or functional teeth. Sections of control teeth, prepared with normal rabbit serum or phosphate-buffered saline, showed no fluorescence.

The fluorescence observed in the teeth of these representative vertebrates demonstrates that amelogenin-like proteins are present in all enamel and enameloid tissues of vertebrate teeth and toothlike dermal denticles and that they are immunologically cross-reactive with bovine antiserum to amelogenin. Cross-reaction implies a chemical and structural similarity between specific antigenic proteins. Immunological cross-reaction between two proteins occurs when the respective amino acid sequences of their molecules differ by less than 40 percent (*10*). Organ-specific antigenic proteins have been demonstrated in kidney, gastrointestinal tract, skin, heart, and genital tissue in a wide range of species (*11*). Both enamel and enameloid matrices showed fluorescence when treated with bovine antiserum to amelogenin, indicating that amelogenins are the organ-specific proteins in both tissues and that chemically they are very similar. This conclusion is supported by studies in which it was shown that amino acid composition (*12*) and histochemical reactions (*13*) in enameloid organic matrix resemble those of enamel.

Bovine ameloblasts synthesize and secrete amelogenins into enamel matrix (*3*). In the present study, the IDE cells adherent to developing enameloid matrices showed moderate fluorescence, indicating that they contain amelogenins. This result (i) suggests that the IDE cells of fish and amphibian tooth organs synthesize and secrete amelogenins into enameloid matrix and (ii) confirms and extends the finding that an ectodermal protein, amelogenin, contributes to enameloid formation (*5, 6*). Therefore enameloid matrix contains ectodermally derived amelogenins as well as mesodermally derived proteins (*6*).

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Mature enameloid matrices showed substantial fluorescence, indicating the presence of amelogenins. Mature mammalian enamel matrix showed no fluorescence, indicating that amelogenins are removed during maturation (*3*). Mature enameloid is 5 to 10 percent organic and thus is not as highly calcified as mammalian enamel (2 percent organic). Collagen fibrils present in enameloid during the early stages of its development disappear during maturation (*6*). Thus the organic matrix of mature enameloid probably consists of amelogenins.

The presence of amelogenins in both enamel and enameloid tissues suggests that they play a part in the development of these extremely hard tissues. The growth of large calcium hydroxyapatite crystals in enamel (*14*) and enameloid (*5*) appears to be related to the tissue's hardness and durability and could be favored by certain physicochemical characteristics of amelogenins.

We have shown that dermal denticles contain amelogenins in their enameloid coverings. This demonstrates that ectoderm contributes protein to the enameloid of the placoid scale (dermal denticle) and initiates mesodermal appositional activity.

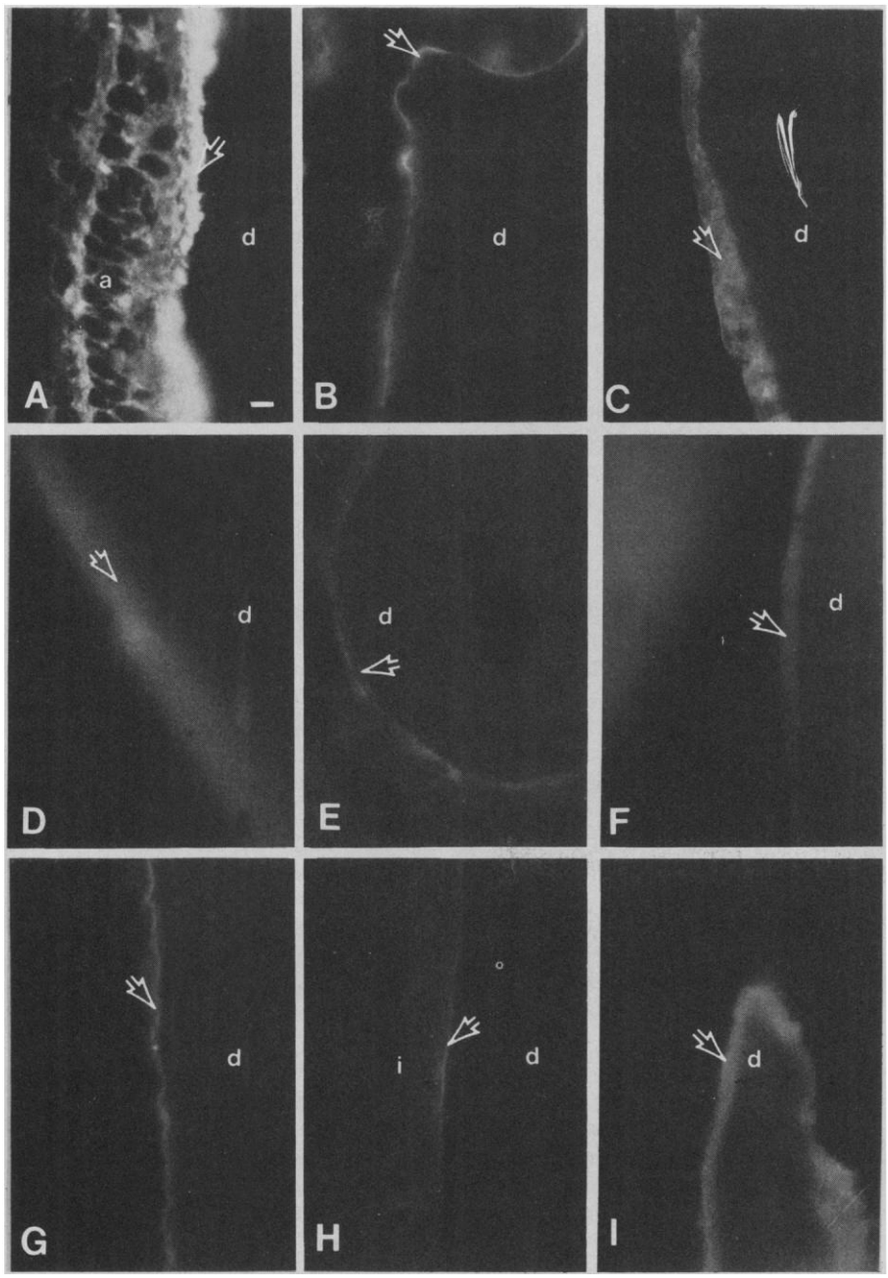

Fig. 1. Sections of vertebrate teeth showing immunofluorescence after treatment with antiserum to bovine amelogenin and fluorescein-conjugated antiserum to rabbit. All sections are longitudinal except the cross section shown in (E). (A) Section of a developing tooth from the bovine *Bos taurus* showing very bright fluorescence in the newly deposited enamel matrix (arrow) and in the IDE cells of the ameloblast layer (*a*); the dark area to the right is dentin (*d*). Scale bar, 10 μm. (B) Section of a cusp, stripped of cells, of a molar from the mouse *Mus musculus* showing bright fluorescence in newly deposited enamel matrix (arrow). (C) Section of the cervical region, stripped of cells, of a molar from the pig *Sus scrofa* showing bright fluorescence in the newly deposited enamel matrix (arrow). (D) Section of a functional tooth from the gecko *Gekko gecko* showing moderate fluorescence in the surface enamel layer (arrow). (E) Section of the base of a functional tooth from the salamander *Notophthalmus viridescens* showing moderate fluorescence in the surface enameloid layer (arrow). (F) Section of a functional tooth from the cod *Gadus callarias* showing moderate fluorescence in the surface enameloid layer (arrow). (G) Section of a functional tooth from the shark *Squalus acanthias* showing moderate fluorescence in the surface enameloid layer (arrow). (H) Section of a developing tooth from the shark showing moderate fluorescence in the newly deposited enameloid layer (arrow) and in the IDE cells (*i*). (I) Section of a functional denticle from the shark showing more intense fluorescence in the surface enameloid layer (arrow).

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