Alkaline phosphatase in developing teeth and bone of man and macaque monkey

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The activity of nonspecific alkaline phosphatase (E.C. 3.1.3.1) in developing teeth and bone of human fetuses and young macaque monkeys has been studied by means of histochemistry. The incubations for alkaline phosphatase were performed at pH 8.2 using naphthol-AS-MX-phosphate as substrate and Fast Blue RR salt or Fast Red Violet LB salt as couplers. By means of pretreatment with heat (56 °C), or addition of sodium metavanadate, ortho- or pyrophosphate, two alkaline phosphatases were demonstrated in the developing teeth.
Prior to hard tissue formation all alkaline phosphatase activity was inhibited by the addition of vanadate, phosphate, or by pretreatment with heat. Pretreatment with heat or addition of vanadate or phosphate also inhibited alkaline phosphatase activity in the odontoblasts and in the pulpal connective tissue, whereas the activity in the subodontoblastic cell layer, stratum intermedium, outer enamel eptithelium, and the outer cells of the reduced enamel epithelium were much less affected. A week resistant activity was also noted in odontoblasts and pulpal connective tissue.

Key-words: Enzyme; histochemistry; odontogenesis

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Alkaline phosphatase activity in developing teeth has been studied by means of histochemical methods in several investigations (15, 16). Most of these studies have been performed in rodents and there are few investigations carried out on human or monkey teeth. Previously, it has been found that there are species differences in enzyme activity between developing rodent and primate teeth. Thus, differences have been noted concerning alkaline phosphatase (14) and acid phosphatase (7).

Recently, two alkaline phosphatases have been demonstrated histochemically in developing rat teeth by means of specific inhibitors or pretreatment with heat (8). It has also been possible to separate two alkaline phosphatase isoenzymes electrophoretically from homogenates of rat dental pulps (4).

Therefore, the aim of this investigation was to study the alkaline phosphatase activity in developing teeth and bone in man and monkey by histochemical means and to see if different isoenzymes could be distinguished by means of specific inhibition.

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MATERIAL AND METHODS

Tooth germs of primary teeth from four human fetuses and developing permanent teeth from two macaque monkeys (Macaca irus) were used.

The human fetuses were removed by Caesarian section in connection with legally approved abortions at an estimated fetal age of 12 to 24 weeks. The fetuses were kept on ice while the jaws were removed. The jaws were then placed in an aqueous solution of carboxymethylcellulose on a microtome stage and frozen by immersion in hexane cooled with solid CO_2 (–75 °C).

The monkeys that were about one year old were killed by an overdose of mebumal sodium, decapitated, and their heads frozen in CO_2-cooled hexane. After radiography, the parts of the jaws containing developing teeth were cut out with a saw and mounted in an aqueous solution of carboxymethylcellulose on a microtome stage and immersed in CO_2-cooled hexane. Frozen sections 10–20 μm thick were taken according to the method devised by Ullberg (20). After freezedrying the sections were incubated for histochemical demonstration of alkaline phosphatase (E.C. 3.1.3.1) at pH 8.2 using naphthol-AS-MX-phosphate as substrate and Fast Blue RR salt or Fast Red Violet LB salt as couplers (1). The incubations were performed at room temperature for 30 to 60 min. To study the effect of heat, the sections were kept in isotonic saline at 56 °C for 60 min prior to incubation. Sodium orthophosphate (Na_2HPO_4, to a final concentration of 100 mM in the incubation medium) or sodium pyrophosphate ($Na_4P_2O_7$, 100 mM) or sodium metavanadate ($NaVO_3$, 10 mM) were added to the incubation medium as possible enzyme inhibitors (10 mM of $NaVO_3$ did not totally dissolve in the incubation medium).

To test a possible effect of an increased ion strength 100 mM sodium chloride was added to the incubation medium. Control incubations without substrate were also performed and showed no staining.

After incubation the sections were washed in distilled water and gas bubbles were removed in vacuo before the sections were embedded in glycerin jelly. Some sections were counter stained with methyl green. (All chemicals were obtained from the Sigma Chemical Co., St. Louis, U.S.A.)

RESULTS

Alkaline phosphatase activity was found in most cells of developing teeth and alveolar bone. There was a weak alkaline phosphatase activity in all cells of the dental papilla prior to dentin formation, except in the differentiating odontoblasts where a higher activity was seen. A rather high activity was noted in blood vessel walls (Fig. 1). At the onset of predentin formation the odontoblastic enzyme activity increased and at the beginning of the mineralization of the dentin there was a marked increase in the alkaline phosphatase activity in the subodontoblastic cell layer (Figs. 2, 3). Staining for enzyme activity was seen in the odontoblastic processes of the predentin whereas no activity could be demonstrated in the tubules of the mineralized dentin (Fig. 2).

A rather high alkaline phosphatase activity was observed in the outer enamel epithelium, stellate reticulum and stratum intermedium (Fig. 1). In some areas the inner enamel epithelium as well as the secretory and post-secretory ameloblasts showed a very weak enzyme activity which seemed to be situated at the cell limits. Also, a weak alkaline phosphatase activity was seen in the proximal part of secretory ameloblasts.

In bone the highest alkaline phosphatase activity was found in osteoblasts. Osteocytes showed a moderate activity. A weak enzyme activity was noted in osteoclasts.

Pretreatment with heat as well as addition

of sodium metavanadate, orthophosphate or pyrophosphate to the incubation medium inhibited the activity of the pulpal cells prior to the onset of hard tissue formation (Figs. 4, 5). When hard tissue formation had started there was marked inhibition of enzyme activity in the dentin producing odontoblasts and the pulpal connective tissue, whereas the enzyme activity in the subodontoblastic cell layer appeared to be unaffected (Fig. 6).

In the enamel organ prior enamel matrix formation, alkaline phosphatase activity in stratum intermedium, stellate reticulum, and outer enamel epithelium was inhibited by pretreatment with heat or by vanadate or phosphate (Figs. 4, 5). During the stage of enamel matrix formation the enzyme activity in stratum intermedium, stellate reticulum, outer enamel epithelium, and outer cells of the reduced enamel epithelium was unaffected by pretreatment with heat, or the addition of vanadate or phosphate to the incubation medium (Figs. 4, 6).

Pretreatment with heat as well as the addition of phosphate or vanadate to the medium reduced the osteoblastic alkaline phosphatase activity. The osteocytic and osteoclastic enzyme activity was found to be very low after these procedures.

Addition of sodium chloride (100 mM) to the incubation medium had no effect on the alkaline phosphatase activity.

DISCUSSION

In the present study alkaline phosphatase activity was demonstrated in most cells of the developing teeth and bone. The finding of an increased alkaline phosphatase activity in differentiating odontoblasts of primate teeth corroborates the results of earlier studies (13, 18). The increase in alkaline phosphatase activity which was noted in the subodontoblastic cell layer at the onset of dentin mineralization has recently been observed in rats (8).

The finding of alkaline phosphatase activity in the odontoblastic processes of the predentin is in agreement with the results of earlier studies (e.g. 3, 13). In the mineralized parts of the dentin no alkaline phosphatase activity was noted and it may seem that the odontoblastic processes withdraw as the dentin formation proceeds (19).

In agreement with earlier studies (13, 17) enzyme activity was observed in the stratum intermedium, the stellate reticulum, the outer enamel epithelium, and in the outer cells of the reduced enamel epithelium. However, the authors (13, 17) did not report alkaline phosphatase activity at any stage of the ameloblastic layer. In the present study a weak enzyme activity which seemed to be localized to cell limits was observed in some areas of presecretory, secretory and post-secretory ameloblasts although most of these cells showed no enzyme activity. Thus, the finding of alkaline phosphatase activity in some parts of the ameloblastic layer whereas other parts showed no enzyme activity, may suggest that these cells have rythmic metabolic changes in addition to their main functional stages. In the inner enamel epithelium of developing human teeth alkaline phosphatase has previously been demonstrated in an electron microscopic study (11).

Previously, a difference in cellular localization of alkaline phosphatase between human and rodent ameloblasts has been demonstrated (14). Formalin fixed specimens were used and staining for enzyme activity was found in the ameloblastic layer in mice, whereas no staining was noted in human ameloblasts. Generally, this was confirmed in the present study although a slight activity was occasionally noted in the human and monkey ameloblasts. This finding was in striking contrast to the observations in rats where a high enzyme activity has been found in the short post-secretory ameloblasts (5, 8, 15).

The osteoblastic and osteocytic alkaline

phosphatase activity which was demonstrated in the present study corroborates the results of several previous studies (e.g. 2, 6, 12, 22). The reports on osteoclastic alkaline phosphatase activity differ in their findings (for review, see (9). It is known that osteoclasts are rich in acid phosphatase (23) and it may well be that the enzyme activity demonstrated in the present study is due to this acid phosphatase (9).

The fact that pretreatment with heat, influence of sodium metavanadate, ortho- or pyrophosphate, selectively inhibited the alkaline phosphatase activity in the enamel organ at an early stage of development in the connective tissue of the pulp and in the odontoblasts, suggests that the enzyme in these cells is an isoenzyme which differs from the one found in the enamel organ at a late developmental stage and in the subodontoblastic cell layer after the onset of dentin mineralization. It is of interest to note that the resistant alkaline phosphatase seems to be associated with mineralization of dental tissues. An alkaline pyrophosphate phosphohydrolase has recently been found to have a cellular localization which is similar to the resistant isoenzyme noted in the present study (Larsson, Hasselgren and Hammarström, unpublished results).

Two alkaline phosphatase isoenzymes have been demonstrated in the developing teeth of rat (8). However, the isoenzyme which was resistant to the above mentioned inhibiting procedures was observed only in the subodontoblastic cell layer in the rat

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Fig. 1. Frozen section from a developing human deciduous incisor incubated for histochemical demonstration of alkaline phosphatase (E.C.3.1.3.1.) at pH 8.2 using naphthol-AS-MX-phosphate as substrate and Fast Red Violet LB salt as coupler. There is a high enzyme activity in odontoblasts (O) and a moderate activity in the pulpal connective tissue (P). Stratum intermedium (SI) and the outer enamel epithelium (OEE) show a high alkaline phosphatase activity whereas a moderate activity is seen in stellate reticulum (SR). D = dentin, E = enamel, SA = secretory ameloblasts, B = bone. X 35.

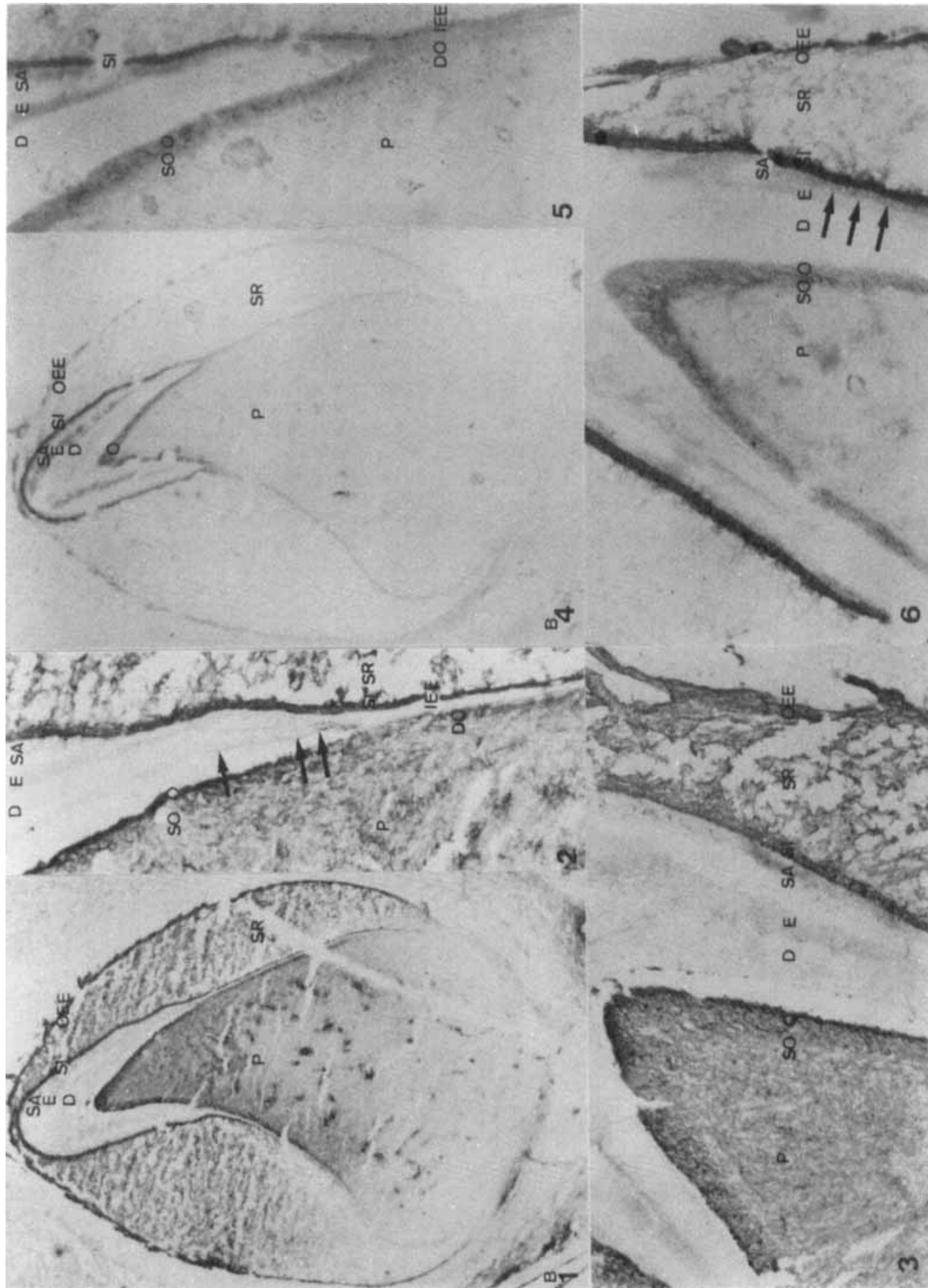
Fig. 2. Alkaline phosphatase in a developing human deciduous incisor. Detail of Fig. 1. Prior to dentin formation there is a higher enzyme activity in differentiating odontoblasts (DO) than in the pulpal connective tissue (P). After the onset of dentin formation a high activity is seen in odontoblasts (O). Note the alkaline phosphatase activity in the pulpal part of the dentinal tubules of the predentin (arrows). D = dentin, SO = subodontoblastic cell layer, E = enamel, SA = secretory ameloblasts, SI = stratum intermedium, SR = stellate reticulum. X 120.

Fig. 3. Alkaline phosphatase in a developing human deciduous incisor. In the pulpal tissue (P) the highest enzyme activity is seen in odontoblasts (O) and in the subodontoblastic cell layer (SO). No enzyme activity is noted in the dentin (D). Staining for enzyme activity is seen in some areas (arrows) of the secretory ameloblasts (SA). Alkaline phosphatase activity is also noted in stratum intermedium (SI), stellate reticulum (SR), and in the outer enamel epithelium (OEE). X 100.

Fig. 4. Alkaline phosphatase in a developing human deciduous incisor. Sodium metavanadate ($NaVO_3$, 10 mM) has been added to the incubation medium. There is a marked inhibition of enzyme activity especially prior to hard tissue formation. Alkaline phosphatase activity is observed in the coronal part of the pulpal tissue (P), stratum intermedium (SI), stellate reticulum (SR), and in the outer enamel epithelium (OEE). O = odontoblasts, D = dentin, E = enamel, SA = secretory ameloblasts, B = bone. X 35.

Fig. 5. Alkaline phosphatase in a developing human deciduous incisor. Sodium metavanadate ($NaVO_3$, 10 mM) has been added to the incubation medium. Detail of Fig. 4. There is a marked inhibition of alkaline phosphatase activity, especially prior to hard tissue formation. After the onset of hard tissue formation the highest enzyme activity is noted in the subodontoblastic cell layer (SO), odontoblasts (O), and stratum intermedium (SI). P = pulp, D = dentin, E = enamel, SR = stellate reticulum. X 120.

Fig. 6. Alkaline phosphatase in a developing human deciduous incisor. Sodium metavanadate ($NaVO_3$ 10 mM) has been added to the incubation medium. There is a marked inhibition of enzyme activity. In the pulp (P) the highest activity is seen in the subodontoblastic cell layer (SO), odontoblasts (O) followed by the connective tissue. There is a high activity in stratum intermedium (SI) and alkaline phosphatase activity is seen in some areas of the secretory ameloblasts (SA) (arrows). X 100.



pulp. Also, in developing rat teeth the distribution pattern of this resistant alkaline phosphatase was found to be similar to that of an alkaline pyrophosphate phosphohydrolase (10).

Odontoblasts as well as osteoblasts showed a remaining alkaline phosphatase activity after pretreatment with heat or addition of inhibitors, and it is conceivable that there are different isoenzymes in these cells.

Vanadium forms a number of vanadates which are isomorphous with the corresponding phosphates (21). It therefore appears likely that the inhibitory effect of vanadate is due to its chemical similarity to phosphate. Vanadate and phosphate can form complex ions and it is possible that the inhibitors which were added to the incubation medium might have formed a complex compound with the naphthyl phosphate.

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