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Original Article

Organic anion transport during rat enamel formation

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ABSTRACT

Objective: The C-terminal end of nascent amelogenin is dissociated immediately after secretion and rapidly re-absorbed by ameloblasts, presumably by endocytosis. The purpose of this study was to test whether organic anion transporters (OATs) are also involved in the re-absorption process of enamel matrix proteins via non-endocytotic pathways.
Materials and methods: Localization of OAT1, OAT2, and OAT3 in rat tooth germs was examined by immunohistochemistry using specific antibodies. Actual translocation of organic anions through the ameloblast layer was further tested by systemic tracer experiments in rats in which Lucifer Yellow (LY), a fluorescent organic anion, was used as a tracer.
Results: In rat tooth germs, OAT2 was associated exclusively with the distal cell membranes of secretory ameloblasts where Tomes' processes were developed and disappeared when matrix formation was terminated. On the other hand, OAT1 was absent in secretory ameloblasts and was colocalized with the ruffled border of ruffle-ended ameloblasts in the maturation stage. OAT3 was undetectable in ameloblasts and located instead only in the stratum intermedium cells. Systemic administration of LY resulted in intense labeling of immature enamel and also a transient labeling of the cytosol of secretory ameloblasts immunopositive for OAT2. In the maturation stage, cytosolic labeling of LY was negligible in all cells of the enamel organs, including ameloblasts.
Conclusions: These data suggest the existence of OATs in rat tooth germs and their possible involvement in matrix re-absorption at least in the secretory stage of amelogenesis.

1. Introduction

The organic anion transporter family is known to play an important role in the elimination of a variety of endogenous and exogenous harmful substances from the body [1]. Almost 2 decades ago, several groups cloned and identified a transport protein that is responsible for the complete excretion of para-aminohippuric acid (PAH) by the kidney [2,3]. This PAH transporter was found to interact with and excrete a variety of other negatively charged endogenous and exogenous protein molecules [4] and hence was renamed as organic anion transporter1 (OAT1) [5,6]. Thus far, 6 isoforms of OAT, all belonging to the solute carrier SLC 22 gene family, have been identified; in addition to the kidney, they are located in various organs, including liver, brain, and placenta, where they are involved in the excretion of a wide range of xenobiotics and endobiotics [4].

Tooth enamel formation or amelogenesis is roughly divided in 2 consecutive stages, the secretory stage and the maturation stage. In the secretory stage, tall columnar ameloblasts synthesize and secrete enamel matrix proteins. Once the full thickness of the enamel is laid down, the ameloblasts become typical transporting cells and regulate calcium influx and matrix removal in and out of the enamel throughout the process of enamel maturation [7–9]. For the highly mineralized enamel to form, extensive degradation and re-absorption of the organic matrix are essential [7]. To date, the absorption of degraded enamel matrix proteins is proposed to be mediated by ameloblasts via fluid phase endocytosis, upon which the proteins are processed in the lysosomal system [10–12]. Alternatively, the majority of the partially broken down matrix is removed by osmotic pressure created at stages of enamel maturation that involve smooth-ended ameloblasts [13].

In independent *in vivo* tracer experiments, we intravascularly injected rats with a fluorescent anionic dye as a tracer in order to

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monitor its excretion from the kidneys. We noted intense fluorescence in the cytoplasm of secretory ameloblasts comparable to that in the transport epithelia of kidney tubules (unpublished data). We, therefore, hypothesized an involvement of the kidney-type non-endocytotic transcellular pathways in the elimination of degraded enamel matrix proteins by the enamel-forming cells. We envisaged this process might operate in addition to the classical endocytotic re-absorption pathways. To test this hypothesis, we performed an immunohistochemical study of the location of some organic anion transporters in the rat enamel organs. Moreover, we performed a systemic tracer experiment using a fluorescent tracer, Lucifer Yellow, a water-soluble organic anion, in order to monitor the actual movements of organic anions through the enamel organ *in vivo*.

2. Materials and methods

All experimental protocols were approved by the Animal Welfare Committee of Tokyo Medical and Dental University (No. 0120176B) and carried out under the institutional guidelines for animal experimentation.

2.1. Animals and tissue preparation for immunohistochemistry

Two- to three-week-old normal male Wistar rats weighing 25–40 g ($n=10$) were anesthetized by isoflurane inhalation and perfused through the ascending aorta with saline for 2 min, followed by perfusion with 4% paraformaldehyde (PFA) in 0.1 M cacodylate buffer (pH 7.4) for 15 min. The upper and lower incisors and molar tooth germs with surrounding bones were dissected and further immersed in the same fixative overnight at 4 °C. The specimens were then decalcified for 2 weeks in neutral 10% ethylenediaminetetraacetic acid (EDTA) at 4 °C and routinely embedded in paraffin. The kidneys were also embedded in paraffin.

Next, 4-μm thick longitudinal sections of the incisors and molar teeth were deparaffinized in xylene, rehydrated through a descending ethanol series, and rinsed in distilled water. In most cases, the sections were treated with TEG buffer (pH 9.0) for 15–20 min at 85 °C for antigen retrieval. After a brief wash in phosphate buffered saline (PBS), nonspecific binding sites were blocked by pre-incubating the sections in PBS containing 1% normal goat serum and 2% bovine serum albumin for 30 min.-Subsequently, the section were incubated overnight at 4 °C with affinity purified polyclonal rabbit antibodies raised against a synthetic C-terminal peptide of the rat renal organic anion transporter 1 (OAT1; Gene Accession ♯NP058920.1) and N-terminal peptide of rat renal OAT2 (Gene Accession ♯ 035913; Cat ♯ OAT11-A and -♯ OAT21-A; Alpha Diagnostics, San Antonio, TX, USA) and C-terminal peptide of rat renal OAT3 (Cat ♯ KE035; Transgenic Inc. Kobe, Japan). The specificity of the antibodies had already been validated by western blot and absorption tests [14–17]. After the sections were rinsed in PBS, they were incubated either with fluorescein isothiocyanate (FITC)- or streptavidin-conjugated goat-anti-rabbit IgG and processed for immunofluorescence and immunoperoxidase staining (ABC method) for the optical visualization of the immunoreactive sites. Negative controls were run by excluding primary antibodies from the reaction. Before microscopic examination, the sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole) or hematoxylin. Kidney sections were similarly immunostained and served as a positive control for OATs.

2.2. Tracer experiment with Lucifer Yellow

In order to trace the putative movement of organic anions in the growing tooth germs, we performed *in vivo* tracer experiments using Lucifer Yellow (dilithium salt, LYCH, MW, 457), a water soluble organic anion, as a fluorescent tracer. Under isoflurane anesthesia, 3-week-old male Wistar rats ($n=12$) were injected with 0.5% LY solution (1.5 mL/100 g body weight) into the external jugular vein within 30 s and euthanized by vascular perfusion as described elsewhere at 2–60 min after LY injection. Upper and lower jaws, including intact incisors and molars, were dissected and decalcified in 10% EDTA. Decalcified samples were dehydrated through an ascending ethanol series and embedded in Technovit 7100 (Heraeus, Wehrheim, Germany). All these processes were carried out in the dark to avoid loss of fluorescence in the tissue. Next, 3-μm Technovit sections were cut using a diamond knife and mounted on a glass slide with anti-fading mounting medium Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined under an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan).

3. Results

3.1. Localization of OATs in the enamel organ

3.1.1. OAT1

In rat incisors, no OAT1 immunoreaction was observed in the cells of the enamel organ throughout the presecretory, secretory, and transitional stages of amelogenesis (Figs. 1a and 2d). Immunoreaction for OAT1 first appeared along the distal membranes of ameloblasts at the beginning of the maturation stage and increased in intensity toward the incisal end (Fig. 2a). The OAT1 immunoreaction was associated with the ruffled distal membranes of ruffle-ended ameloblasts (RA; Fig. 2b) and fluctuated in intensity in accord with the cyclical modulation of ameloblast morphology from the ruffle-ended to the smooth-ended cell types (Fig. 2c and e). OAT1 expression was very weak or negative when the ameloblasts were in the smooth-ended mode (Fig. 2c and e). Cells of the papillary layer did not show detectable OAT1 immunoreactivity.

3.1.2. OAT2

In contrast, distinct immunoreactions for OAT2 were observed only during enamel matrix secretion (Fig. 1b and c) located along the distal membranes of the Tomes' processes of secretory ameloblasts (Fig. 1i). The OAT2 appeared at the onset of enamel matrix deposition (Fig. 1b) and persisted throughout the secretory stage terminating at the transition stage (Fig. 1c and d). In the molar tooth germs, an intense OAT2 immunoreaction was also located entirely to the distal end of secretory ameloblasts (Fig. 1k and l).

3.1.3. OAT3

In both incisors and molars, OAT3 immunoreactivity was confined exclusively to the cells of the stratum intermedium (Fig. 1e, f, g, j, and m), which showed a reaction corresponding to the peripheral part of the cells. In these cells, the OAT3 immunoreaction started in the presecretory stage (Fig. 1e) and extended into early maturation (Fig. 1g and m).

3.2. Localization of LY in the enamel organ

In rat incisors, 15 min after intravenous injection of 0.5% LY solution, intense fluorescence of LY appeared in the full thickness of the growing enamel matrix and in the cytoplasm of secretory