Distribution of ABH Blood Group Epitopes on Inner Surface of Dental Hard Tissue: Serological, Immunohistochemical and Ultrastructural Study on Odontoblasts

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Nakayama, Y. and Aoki, Y. Distribution of ABH Blood Group Epitopes on Inner Surface of Dental Hard Tissue: Serological, Immunohistochemical and Ultrastructural Study on Odontoblasts. Tohoku J. Exp. Med., 1998, 184 (4), 267-276 —— This study was designed to investigate localization of ABH antigens on the inner surface of human tooth hard tissues. The tooth samples, extracted therapeutically, were cut and dried at room temperature for a few weeks. Scanning electron microscopic observation disclosed that the inner surface was exclusively covered with the odontoblastic zone and virtually no blood vessels remained in the samples. Blood group activities of the tooth fragments, which were detected with absorption-elution test, were markedly decreased when the odontoblastic zone was scraped off. To visualize the activity, the avidin-biotin-peroxidase complex immunostaining was performed on the odontoblasts of fresh teeth followed by embedding. Decalcification process was omitted to preserve the antigenicity. Group specific colorization was successfully developed on the cell surface of the odontoblasts. Immunoreactivity of the cell membrane of the odontoblast including the odontoblastic process was also confirmed by immunotransmission microscopic observation. On the other hand, localization of epitopes on intrinsic dentine without cell components could not be visualized. The results of these experiments indicate that the odontoblasts are one of potent sources of blood group antigenicity for blood grouping of the human teeth. ——— forensic dentistry; ABO blood group; avidin-biotin-peroxidase complex immunostaining; immuno-electron microscopic study; odontoblasts © 1998 Tohoku University Medical Press

ABH blood group activity is detectable in human teeth using, for example, absorption-elution (AE) technique (Neiders and Standish 1977). In the dental pulp, vascular endothelium and red blood cells are regarded as a source of ABH antigenicity. It is considered that the dentine would be also available for blood grouping (Shimizu 1971; Takata 1973; Mukai et al. 1975), localization of the

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blood group antigens in it. However, it is still a subject of controversy. Smeets et al. (1991) presumed that the blood group substance was located in the dentinal tubule, however, complete removal of the pulp components from dentine samples was not confirmed. The odontoblasts, which form a single layer lining the periphery of the pulp and have processes extending into the dentinal tubule, would also be a cell component with the epitopes of ABH blood groups (Shimizu 1971). However, antigenic activities on the cells have not been histologically demonstrated.

In this study, we investigated the localization of ABH blood group antigens on the inner surface of the dentine as a carrier using serological, immunohistochemical and ultrastructural analyses.

Materials and Methods

Specimens. Permanent teeth without caries, which were therapeutically extracted from patients ranging in age from 14 to 75 years at the Dental Hospital of Iwate Medical University, were used for the experiments. Cotton gauzes applied to the extraction wound were also obtained as reference samples for blood grouping. The teeth were scrubbed in tap water, and split vertically at the center using a diamond disc within one day after extraction to remove dental pulp. Subsequently, the teeth and pulps were dried at room temperature for 1-3 weeks. For immunohistochemical study, the tooth samples were split and fixed immediately after extraction as described later on.

Antibodies and reagents. Polyclonal anti-A and anti-B sera were purchased from Kokusaishiyaku (Tokyo), and Ulex europaeus anti-H lectin was prepared by ethanol precipitation method (Tsutsubuchi et al. 1984). Mouse monoclonal anti-A and anti-B antibodies were obtained from Biotest (Dreieich, Germany), and Anti-H were from Kokusaishiyaku. An avidin-biotin-peroxidase complex (ABC) immunostaining kit (Vectastain PK-4010; Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine tetrahydrochloride (DAB, Dojindo, Kumamoto) were used for immunohistochemical staining.

ABO blood grouping by AE technique. For ABO blood grouping by AE technique, two types of 2×2×2 mm dentine fragments, namely fragments with and without the odontoblastic zone were obtained (referred to as D+OZ and D-OZ, respectively) from crown side of the teeth (19 molars, 6 premolars and 5 incisors). The latter was prepared by scraping inner surface of the dentine with a round steel bur. Specimens of the pulp, D+OZ and D-OZ were sensitized with polyclonal anti-A and anti-B sera, and anti-H lectin. The sensitized fragments were immersed in two drops of saline and heated at 56°C to obtain the eluates. Threads of the cotton gauzes were also typed by this method.

Scanning electron microscopic (SEM) examination. Five split and dried teeth and their pulps were treated for SEM study to observe the inner surface of the dentine and the periphery of the pulp. The samples were fixed in 2.5% glutaral-
dehydrole for 1 hour at room temperature. After dehydration with graded ethanol, the specimens were dried again by t-butyl alcohol freeze-drying method (Inoue and Osatake 1988). The dried samples were then mounted on an aluminum stubs, coated with platinum and observed with SEM (S-2300, Hitachi, Tokyo) at 15 kV. A few D-OZ specimens used for AE test were also subjected to SEM examination.

Immuno-light microscopic and immuno-transmission electron micro-sco-pic (TEM) studies. Immunohistochemical examination was carried out on several intact premolars and upper wisdom teeth extracted from patients aged 17-25 years. The teeth were split, the pulps were removed from the inner surface of the dentine and the remaining tooth samples were immediately treated with a mixture of 4% paraformaldehyde and 8% sucrose in 0.1 M cacodylate buffer (pH 7.4) for 5 minutes. The samples were sensitized with mouse monoclonal anti-A, anti-B and anti-H diluted 1:50 with 13 mM phosphate-buffered saline (pH 7.4) for overnight at 4°C. ABC immunostaining (Hsu et al. 1981) was performed according to the manufacture's directions except for the prolonged incubation time (1 hour) with the biotinylated antibody. Subsequently, the samples were fixed with 2.5% glutaraldehyde for 1 hour and postfixed with 1% osmic acid solution for 1 hour. After dehydration with graded ethanol and substitution with n-butyl glycicyl ether (QY-1; Nisshin EM, Tokyo), the odontoblastic zone was scraped off from the inner surface of the dentine with dental scaler at the final stage of epon mixture. The odontoblasts in suspension were then embedded in Quetol 812 (Nisshin EM). Semithin (0.5 μm) and ultrathin sections were prepared using an ultramicrotome and examined with a light microscope and TEM (H-7100S, Hitachi) at 100 kV without further staining, respectively.

RESULT

Blood grouping on teeth by AE test. Table 1 lists the results of blood grouping obtained from the 30 teeth by AE test. The blood types determined using the gauze specimens and the results obtained from the pulps matched in 27 cases. Amongst the remaining 3 samples of which the blood group could not be confirmed, the two were from old patients aged 66 and 71 years, and the one was

<table>
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<th>Type of Specimen</th>
<th>Blood grouping results</th>
<th>Percentage of consistency (%)</th>
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<td>Consistent</td>
<td>Inconsistent</td>
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<tr>
<td>Pulp</td>
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<tr>
<td>D+OZa</td>
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<td>0</td>
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<td>D-OZc</td>
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*a* Blood group could not be determined.

*b* Dentine fragments with odontoblastic zone

*c* Dentine fragments scraped odontoblastic zone
markedly worn out. All D+OZ specimens were typed correctly but for 2 samples with inconclusiveness. On the other hand, the blood group antigen could be detected from only one out of 30 D-OZ samples.

SEM findings. The inner surface of the dentine is covered with the odontoblastic zone (arrow) and scattered clusters of the odontoblasts (Fig. 1a). Observation with higher magnification disclosed the clusters of shrunken cells and dentinal tubules (Fig. 1b). Capillary vessels could not be found on the odontoblastic zone. However, blood vessels run sporadically on the peripheral region of the pulp, passing through fibers and probably nerve bundles (Figs. 1c and d).

Fig. 1. SEM findings of inner surface of the dentine (a and b) and periphery of the dental pulp (c and d). a: At the slightly brighter part (arrow), the dentine is covered with the odontoblastic zone. On the upper side, clusters of the odontoblasts are scattered over the dentine (scale bar: 1 mm). b: Higher magnification of odontoblast clusters and the dentinal tubules. No capillary vessels are found in this area. c: Periphery of the pulp is covered with fibers. d: Higher magnification of periphery of the pulp. Loops of capillary vessel exist at a rate of approximately one per square millimeter.
Aside from the absence of cell components, SEM view of the inner surface of D-OZ disclosed that the dentinal tubules were choked by shaving (Fig. 2).

*Immunohistochemical findings with light microscopic examination.* Figs. 3 and 4 show the immunohistochemistry of a type A sample sensitized with anti-A, anti-B and anti-H. Group specific color development was observed at intercellular spaces. The predentine which is an unmineralized dentine matrix lining the innermost layer of the dentine was not stained by this method (Fig. 4). Essentially identical group specific reaction was demonstrated on the specimens of other blood types.

*Staining reactivity on cell membrane of odontoblasts.* In TEM observation, intercellular spaces were exclusively stained with the chromogen. In some areas, the group specific deposition widened where the cell membrane would have been cut obliquely, and gave bead-like appearances presumably because of deformity of the membrane (Fig. 5, arrowheads). Few nonspecific staining was noted, and the group specific reaction could be discriminated from the high electron density areas including desmosomes and gap junctions (Fig. 5b). Intensity of the reactivity would somewhat vary at different cutting levels of odontoblasts. On the cells cut through the level of nuclei, the membranes showed relatively faint marks. At the level of the predentine, staining reactivity was occasionally observed on the outlines of the odontoblastic processes (Fig. 6a). No group specific reaction was found in intrinsic dentine, although electron-dense structure produced by osmium black was in dentine matrix and tubular lumen (Fig. 6c).

**Discussion**

The failure of detection of blood group antigens from D-OZ by AE test would
Fig. 3. Staining reactivity of anti-A (a) and anti-B (b) with a specimen from a type A individual. a: Group specific staining is developed on the intercellular spaces (arrows). b: Immunonegative cells tend to be shrunken and scattered, making a contrast with the positive cells whose integrity was well preserved. OZ, odontoblastic zone; PD, predentine (magnification ×1000).

be partly due to relative insensitivity of the methods employed. Use of dentine powder, not fragment, as specimens would give improved results (Shimizu 1971; Takata 1973; Mukai et al. 1975). Nonetheless, the contrast between the results of blood grouping obtained from D+ OZ and D-OZ specimens strongly suggested that the portion scraped off to prepare D-OZ samples should contain considerable amount of blood group antigens. SEM observation disclosed that the inner surface of D+ OZ was only covered with the odontoblasts, and virtually no blood vessels remained in the specimens. This confirmed that the detected ABH antigens would have their origin not on the vascular endothelium or red blood
cells but on the odontoblasts or innermost part of the dentine.

The localization of ABH blood group epitopes on the odontoblasts had not been satisfactorily visualized. In their immunohistological study, Ogata et al. (1992) reported that the odontoblasts were not immunoreactive against monoclonal anti-ABH antibodies. Several reasons including physical fragility of the cell membrane and loss of antigenicity by decalcification has been alleged to be responsible for the difficulty of detecting blood group epitopes on the odontoblasts.

In the present study, decalcification process was omitted and sections were prepared by pre-embedding method (Knox 1982; Horisberger 1985) to preserve the activity of epitopes. PBS of increased concentration was used for immunostaining to avoid rupture of insufficiently fixed cells. The pre-embedding method is suitable for detection of cell surface antigens, whereas it would hardly visualize those in cytoplasm, especially with the use of antibodies of large molecular size (Ohtani 1991). In this study, some bead-like components appeared to be stained (Fig. 5, arrowheads). They would, therefore, most likely represent the portions of deformed cell membrane partly because of PBS of high osmotic pressure.

Odontoblasts are columnar in shape with nucleus displaced from its dentinal extremity. Relatively weak immunoreactivity of sections cut through the nuclei may be due to poor preservation of cell membrane at the boundary between the pulp and the odontoblasts. Addition of 0.25% glutaraldehyde to the mixture of 4% paraformaldehyde and 8% sucrose increased the preservability of the cell membrane but resulted in the marked loss of antigen activity (data not shown). It is of interest that the immunonegative odontoblasts tended to be shrunken and
Fig. 5. TEM findings of immunoreactivity of anti-H (a) and anti-A (b) with a specimen from a type O individual. a: Band-like group-specific staining is observed on the cell membranes. The reactivity gives a linear appearance where membrane has been cut at exactly right angle (circle). Arrowheads indicate bead-like appearances which probably represent artificially deformed cell membrane. b: Desmosomes could be discriminated from group-specific reaction (arrows). (scale bar: 5 μm)

scattered making a contrast with the positive cells whose integrity was well preserved. This would suggest that some products of immunostaining might have contributed to maintenance of intercellular connectivity.

The results of our experiments indicate that the odontoblast including the dentinal process was one of the positive sources of blood group antigenicity. On the other hand, the immunoreactivity of the predentine could not be confirmed in
Fig. 6. TEM findings at level of the predentine. a: Group-specific reactivity of anti-B with a specimen from a type B individual is on membrane of the odontoblastic processes (arrow) (scale bar: 3 μm). b, c: Findings of type B sample sensitized with anti-A. b: Arrowheads indicate membrane of the processes unstained (scale bar: 3 μm). c: Electron-dense structure surrounds the tubular lumen. In this tubule, no odontoblastic process is visible presumably because of shrinkage artifact.

This study and few significant results were obtained immunohistochemically on the intrinsic dentine. Thus, the localization of the blood group antigens in intrinsic dentine without cell components still remained unclear. Marked decrease in the surface area due to obstruction of the dentinal tubules by shaving might be another cause of the negative results of AE test with D-OZ fragments. It is well recognized that there is an electron-dense structure surrounding the tubular lumen whose components is still unknown (Torneck 1989) (Fig. 6c). This structure might conceal immunopositive epitopes. Immuno-electromicroscopic
investigation on the vicinity of this structure should be performed as a prospective study.

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References


