Immunoexpression of aquaporin-1 in the rat periodontal ligament during experimental tooth movement

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ABSTRACT
This study examined the immunoexpression pattern of aquaporin-1 (AQP1), first identified as a water channel protein, in the periodontal ligament of rat molars during experimental tooth movement to clarify its role in periodontal responses in an overloaded model by the insertion of a piece of elastic band. In the control group without any treatment, the cementoblasts and osteogenic cells as well as the vascular endothelial cells showed AQP1 immunoreaction. In the experimental group, hyalinized tissue and intensely AQP1 positive amorphous structures which were identified as degenerated endothelial cells by immunoelectron microscopy, occurred at the compression side on Days 1 and 3. AQP1 immunoreaction came to be stronger in the intact endothelial cells around the hyalinized tissue. The hyalinized tissue had almost disappeared by Day 5 when many macrophages reactive to acid phosphatase activity appeared. The periodontal width on Day 7 became almost the same as that in the control group. These findings indicate that the hyalinized tissue and damaged AQP1 positive endothelial cells are phagocytized by macrophages which have temporarily migrated, and suggest that the surviving endothelial cells with intense AQP1 reaction are involved in periodontal regeneration by capillary sprouting.

Tooth movement by appropriate mechanical forces depends on the biological tissue reaction—including bone formation/resorption—to maintain homeostasis of the periodontal ligament which receives a rich vascular network with delicate three-dimensional extensions. A well-coordinated tissue remodeling is able to induce orthodontic tooth movement with minor, reversible injury to the supporting tissues (for review, 39). During tooth movement, undermining bone resorption occurs on the compressed side while bone formation takes place on the tension side. However, an application of inadequate or excess orthodontic forces easily results in cell death on the compressed side of the periodontal ligament known as hyalinized tissue; this often delays smooth tooth movement (for review, 36).

The blood vessels in the periodontal ligament are involved in the regulation of tissue remodeling during orthodontic treatment. These blood vessels have been reported to respond differently depending on the orthodontic force magnitude (10, 13), suggesting different microvasculature reactions to mechanical forces. A heavy force apparently enables the compressed periodontal ligament to induce severe damage, resulting in a vascular deformation of degeneration. A previous ultrastructural study reported rapid vascular reactions on the compressed side of the rat periodontal ligament: the packing of erythrocytes in blood vessels occurred within 30 min, their fragmentation after 2–3 h, and the disintegration of blood vessel walls after 1 day (27). Such a breakdown of blood vessels due to pressure force has been considered one of the factors resulting in the

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formation of hyalized tissue (23, 24, 27, 28). Indeed, hyalinization has been reported to occur in not only the initial but also in the later stages of orthodontic tooth movement (37). Although numerous studies over the past 100 years have produced hyalinization on the locally pressured side of the periodontal ligament, the relationship between force level, timing onset, and extent of hyalinization remains unclear (for review, 36).

Aquaporins (AQPs) are a family of transmembrane channel-forming glycoproteins that provide a major pathway for osmotically driven water transport through cell membranes (2, 5, 7, 31, 35). AQPs have at least 13 subtypes and display a widespread tissue distribution ranging from mammals to plants and microorganism (17, 33). In the AQP family, AQP1 is strongly expressed throughout the microvascular endothelia in various organs including the kidney, lung, skin, secretary glands, skeletal muscle, and peritoneum (11, 19). In addition to its involvement in osmotically-driven transendothelial water movement, AQP1 has been reported to facilitate endothelial cell migration (12) and tumor angiogenesis (29). Recently, an experimental study using a rabbit chronic myocardial infarction model clearly demonstrated a temporal up-regulation of AQP1 mRNA and protein levels in response to ischemia, which were down-regulated by an administration of acetazolamide, a carbonic inhibitor, suggesting that AQP1 plays a role in postnatal angiogenesis (22). Furthermore, a high expression of AQP1 in endothelial cells of the choroid plexus has been reported in the case of vasogenic edema after experimentally induced ischemia (16). These experimental data lead us to hypothesize that changes in AQP1 immunexpression in the endothelial cells reflect the degeneration/regeneration of the periodontal ligament during orthodontic tooth movement because the compressed area induced by orthodontic forces can be regarded as one ischemia/reperfusion site (13).

The present study was therefore undertaken to examine the expression pattern of AQP1 in the rat periodontal ligament during experimental tooth movement by an immunohistochemical method. The double immunostaining with rat endothelial cell antigen (RECA-1) (9) and AQP1 was also employed to demonstrate the relationship between AQP1-positive cells and blood vessels. Enzymatic histochemistry for acid phosphatase (ACPase) and tartrate-resistant acid phosphatase (TRAPase) activities was also employed to demonstrate the behavior of the macrophage lineage and osteoclasts incident to tooth movement in this experimental model. As a first step in this study, we applied a heavier force by Waldo’s method (38) to produce hyalinized tissue on the compressed side.

MATERIALS AND METHODS

All animal experiments were reviewed and approved by the Niigata University Faculty of Dentistry Intra-mural Animal Use and Care Committee prior to the study (approval number #42). The animals were housed in a temperature-controlled room under normal 12 h light/12 h dark laboratory conditions with free access to chow and water.

Experimental procedures and tissue preparation.

Twenty-five male Wistar rats, aged 8 weeks (body weight 250–300 g), were used in this experimental study. The animals were divided into experimental and control groups. While the experimental animals were under anesthesia with a gas mixture of halothane and oxygen, experimental tooth movement was initiated by the unilateral insertion of a piece of elastic band (#6L; TOMY International, Fukuoka, Japan) between the maxillary first and second molars (Fig. 1), according to Waldo’s method (38). Animals in the experimental group were allowed to survive for 1, 3, 5, and 7 days (n = 5 at each stage). The untreated upper molars of 5 rats served as controls.

After appropriate survival periods, the rats were deeply anesthetized by an intraperitoneal injection of 8% chloral hydrate (400 mg/kg), and perfused transcardially with a cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The removed upper jaws were decalcified with 10% EDTA-2Na (Dojindo Laboratories, Kumamoto, Japan) solution for 3–4 weeks at 4°C. Tissue blocks were either embedded...
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and AQP1, AQP1-immunostained cryostat sections with DAB development were subjected to ACPase activity reaction by an azo-dye method (3). For double immunostaining with AQP1 and RECA1, the cryostat sections were primarily incubated with a mouse monoclonal antibody to RECA1 (1:100; Serotec, Oxford, UK), followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:100; Vector Lab. Inc.). They were then reacted with a polyclonal antiserum against AQP1 (1:1000; Millipore Corporation) and subsequently by Texas Red™-conjugated anti-rabbit IgG (1:100; Vector Lab. Inc.).

The specificities of antiserum and antibody have been reported in previous literature (7, 9).

RESULTS

The rats undergoing artificial tooth movement decreased in body weight by Day 1, but thereafter gained weight throughout this experimental period as did in the control rats (data not shown; cf. 18). No remarkable changes were recognizable in their drinking or grooming behavior.

Enzymatic histochemistry for TRAPase reactivity.

For the histochemical demonstration of TRAPase activity, a marker for osteoclasts, the azo-dye method (3) was utilized with slight modifications as reported previously (18). The sections were faintly counter-stained with 0.03% methylene blue.

Double staining. In the double staining of ACPase and AQP1, the immunoreacted sections were counter-stained with 0.03% methylene blue.
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Although the distal periodontal space remained narrow with remaining hyalinized tissue, intense AQP1 reactive structures with a granular appearance lay between the hyalinized tissue and the alveolar bone on Day 3 (Fig. 3c). Other areas including the mesial ligament show no changes in immuno-intensity in spite of higher immunoreaction for AQP1 in the compressed area. The observation of plastic sections demonstrated the localization of these immunoreactive materials in the cytoplasm with a granular appearance (Fig. 3d), as confirmed by immunoelectron microscopic examinations (Fig. 3g). Under the electron microscope, the AQP1 positive cells contained an accumulation of membranous structures—including fragmented cell organelles and amorphous substrates—and that the luminal structure was filled with cell debris (Fig. 3g). Double immunofluorescent staining demonstrated a localization of RECA1, a rat endothelial cell marker, in the AQP1 positive structures at the periphery of the hyalinized tissue (Fig. 3h–j). Large and granular structures with AQP1 immunoreaction decreased in number after Day 5 (Fig. 3e). The area at the periphery of the remaining hyalinized tissue contained more intense AQP1 positive endothelial cells, presumably regenerating/regenerated endothelial cells (Fig. 3e). The AQP1 positive structures surrounding the hyalinized tissue completely disappeared from

Reactions of AQP1-immunopositive cells in the periodontal ligament during tooth movement

In the control group (Fig. 3a), immunostaining with AQP1 depicted vascular endothelial cells (arrows in Fig. 3a) as well as in the cementoblasts and osteogenic cells as Kawano et al. (15) have reported. Furthermore, the fibroblasts and endothelial cells in the dental pulp were also positive for AQP1 immunoreaction (Fig. 3a).

On Day 1, when the formation of hyalinized tissue (an asterisk in Fig. 3b) had begun, the distal periodontal ligament with hyalinized tissue contained some large structures with intense AQP1 immunoreactions (Fig. 3b). These positive structures lost their lumen appearing as knob-like structures (arrows in Fig. 3b). Although the distal periodontal space remained narrow with remaining hyalinized tissue, intense AQP1 reactive structures with a granular appearance lay between the hyalinized tissue and the alveolar bone on Day 3 (Fig. 3c). Other areas including the mesial ligament show no changes in immuno-intensity in spite of higher immunoreaction for AQP1 in the compressed area. The observation of plastic sections demonstrated the localization of these immunoreactive materials in the cytoplasm with a granular appearance (Fig. 3d), as confirmed by immunoelectron microscopic examinations (Fig. 3g). Under the electron microscope, the AQP1 positive cells contained an accumulation of membranous structures—including fragmented cell organelles and amorphous substrates—and that the luminal structure was filled with cell debris (Fig. 3g). Double immunofluorescent staining demonstrated a localization of RECA1, a rat endothelial cell marker, in the AQP1 positive structures at the periphery of the hyalinized tissue (Fig. 3h–j). Large and granular structures with AQP1 immunoreaction decreased in number after Day 5 (Fig. 3e). The area at the periphery of the remaining hyalinized tissue contained more intense AQP1 positive endothelial cells, presumably regenerating/regenerated endothelial cells (Fig. 3e). The AQP1 positive structures surrounding the hyalinized tissue completely disappeared from
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the distal periodontal ligament by Day 7, when the width of the ligament became almost the same as that of the control group (Fig. 3f).

**Double staining of AQP1 immunoreaction and ACPase reactivity**

A combination of immunohistochemistry and enzy-

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**Fig. 3** AQP1 immunoreaction in the periodontal ligament (PDL) during experimental tooth movement. AQP1 immunostaining with DAB development (a-c, e, f), toluidine blue-stained plastic section by post-embedding (d), immunoelectron micrograph (g), and double immunofluorescent staining (h–j). In the untreated control (a), endothelial cells (arrows) as well as cementoblasts and osteogenic cells are positive for AQP1 immunoreaction. On Day 1 (b), when the hyalinized tissue (-) has been formed, intense AQP1 immunoreactive structures appear around this degenerated area (arrows). (c) On Day 3, when the hyalinized tissue (-) remains, knob-like structures with intense AQP1 immunoreaction exist around this remaining hyalinized area. A higher magnified view shows large structures having AQP1 immunoreactions with granular appearances (arrows in inset (c)). These immunoreactive deposits with dot-like appearances are seen to be localized in the cytoplasm (d). A part of the hyalinized tissue (-) remains on Day 5 (e), but completely disappears by Day 7 (f). Note that regenerating endothelial cells (arrows) around the hyalinized tissue have intense AQP1 immunoreaction on Day 5 (e). In immunoelectron microscopy (g), these large structures with AQP1 reactivity on Day 3 are identified as degenerated endothelial cells. (h–j) Double staining with RECA1 (green) and AQP1 (red) on Day 3. Figure j is a merged image showing a co-localization of RECA1 and AQP1. AB: alveolar bone, B: buccal, D: distal, M: mesial, P: palatal sides. A direction in all figures is the same as shown in Fig. 1a. Scale bars: a–c, e, f: 100 μm, c inset: 50 μm, d: 50 μm, g: 5 μm, h–j: 25 μm.
Orthodontic tooth movement causes active periodontal tissue remodeling—bone deposition and resorption on tension and pressure sides, respectively, resulting in maintenance of the periodontal ligament structure. Unlike human teeth, rat molars display continuous distal movement under physiological conditions, confirmed by the restricted distribution of TRAPase reactive osteoclasts on the tooth surface and osteoclasts on the bone surface shown in this study (Fig. 2d). In this experiment model, histochemistry for TRAPase activity demonstrated the temporally up-regulated occurrence of these cells on the distal side of alveolar socket (Fig. 2e–g), indicating that this can be an accelerated compressive model at the distal ligament. Indeed, we could observe the formation of hyalinized tissue in addition to the occurrence of many osteoclasts at that site. Waldo’s method (38), which has been widely utilized for the analysis of tissue remodeling during artificial tooth movement has a disadvantage of an unavoidable inflammatory

**DISCUSSION**

Orthodontic tooth movement causes active periodontal tissue remodeling—bone deposition and resorption on tension and pressure sides, respectively, resulting in maintenance of the periodontal ligament structure. Unlike human teeth, rat molars display continuous distal movement under physiological conditions, confirmed by the restricted distribution of TRAPase reactive osteoclasts on the alveolar bone surface of the distal periodontal ligament as shown in this study (Fig. 2d). In this experiment model, histochemistry for TRAPase activity demonstrated the temporally up-regulated occurrence of these cells on the distal side of alveolar socket (Fig. 2e–g), indicating that this can be an accelerated compressive model at the distal ligament. Indeed, we could observe the formation of hyalinized tissue in the distal side in addition to the occurrence of many osteoclasts at that site. Waldo’s method (38), which has been widely utilized for the analysis of tissue remodeling during artificial tooth movement has a disadvantage of an unavoidable inflammatory
reaction just beneath the inserted elastic band (cf. 18). Thus, we eliminated the distal roots of first molars and the mesial roots of second molars from the observation area. Furthermore, our preliminary observation showed no formation of hyalinized tissue in the mesial roots of first molars whose direction of movement was reversed by the insertion of an elastic band (40). Therefore, we selected the disto-palatal root of a second molar as an area to observe the changes in AQP1 immunoreaction in this study.

One highly interesting finding in this study is the occurrence of structures with intense AQP1 immunoreaction near the hyalinized tissue. These structures co-localized with immunoreaction for RECA1, an endothelial cell marker (9), and had a lumen-like structure, indicating they are possibly endothelial cells. In general, a highly compressive force induces the formation of hyalinized tissue due to damage to blood vessels (for a review, 36). Since blood vessels in the periodontal ligament provide gases and nutrients and actively participate in tissue remodeling associated with orthodontic treatment, there is no doubt that an inadequate orthodontic force causes pathological damage in the periodontal ligament, often resulting in its degeneration or necrosis when a heavier force is applied (32). A series of electron microscopic studies has shown the degeneration of the endothelial cells and edema on the compression side during the early stages of orthodontic tooth movement (23, 26, 28). The present study demonstrated a rapid formation of hyalinized tissue (Days 1 and 3), suggesting that blood vessels were damaged at a comparatively early stage. Anastasi et al. (1) reported a reduction in the number of blood vessels after 3 days of treatment in humans. On the other hand, Noda et al. (20) demonstrated that a heavy continuous force of over 4 g (16.22 g/cm²) caused a disappearance of vascular structures on the compression side in rats in spite of the absence of any degeneration by the application of a light continuous force of 0.8–2.6 g (3.21–6.24 g/cm²). The current immunoelectron microscopy exhibited that AQP1 positive structures found on Day 3 contained fragmented membranous materials and/or amorphous substances (see, Fig. 3g); these ultrastructural configurations resembled cell necrosis due to hypoxia. It is plausible to consider that the excess orthodontic force easily damaged the endothelial cells on the distal side to attain the status of hypoxia there.

The AQP1 in the endothelial cells is likely involved in osmotically driven water transport through cell membranes in the periodontal ligament of control animals because active tissue remodeling constantly takes place in the ligament (30). However, little information is available regarding the functional significance of AQP1 in the periodontal ligament; to our knowledge, this is the first report to describe AQP1 immunoreaction during orthodontic tooth movement. Recent experimental studies have revealed the temporal up-regulation of AQP1 mRNA and protein levels in response to ischemia (16, 22). It is thus reasonable to posit that AQP1 immunoreaction reflects the status of the ischemia/perfusion process during the formation/disappearance of hyalinized tissue at the compressive area due to excess orthodontic force since the damage to blood capillaries is believed to result in the formation of hyalinized tissue (for a review, 36).

Another possibility for the temporal up-regulation of AQP1 is the involvement of AQP1 in endothelial cell migration as suggested by an analysis of mice lacking AQP1 (29). Interestingly, AQP-dependent cell migration may be a general phenomenon not restricted to vascular endothelial cells (34). The current immunoelectron microscopy showed degeneration of the endothelial cells with AQP1 immunoreaction near the hyalinized tissue, but AQP1 immunoreactivity in the endothelial cells around the hyalinized area appeared to become more intense (see, arrows in Fig. 3e). Probably, non-damaged endothelial cells with stronger AQP1 reaction migrate into the damaged tissue after phagocytosis of degenerated endothelial cells by ACPase reactive macrophages which temporally migrated in and around the damaged area. Indeed, it has been reported that AQP1 promotes microvascular generation by accelerating cell migration in tumor models (29), and that a lack of AQP1 inhibits angiogenesis in vivo and in vitro (4, 14). However, the revascularization process incident to tissue remodeling has not yet been elucidated. Extracellular matrices surrounding blood vessels have been thought to play crucial roles in neovascularization (6). Although the key angiogenic molecules including vascular endothelial growth factor (VEGF) can induce the sprouting of blood vessels (21), neovascularization occurs without VEGF in certain conditions, including hind limb ischemia (8) and retinopathy (25). The mechanism of revascularization incident to tissue remodeling in the periodontal ligament constitutes one subject for a future study.

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