Immunolocalization of corticosteroid hormone receptors in the mechanoreceptors in rat oral tissues

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ABSTRACT

Previous studies have confirmed the presence of corticosteroid receptors including mineralocorticoid (MCR) and glucocorticoid receptors (GCR) in the glia cells of both central and peripheral nervous systems. However, no report has been offered as yet on the detailed localization of these receptors in the specialized Schwann cells associated with mechanoreceptors. Thus, the present study examined the immunolocalization of MCR and GCR in the specialized Schwann cells (terminal and lamellar Schwann cells) associated with the mechanoreceptors in rat periodontal ligament and palatal mucosa by the use of immunohistochemical techniques and confocal laser scanning microscopy. It further attempted to detect the localization of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD II), a key enzyme for signal transduction via MCR. Immunostaining with antisera against GCR and MCR demonstrated intense immunoreactions in the terminal or lamellar Schwann cells but not nerve fibers associated with the periodontal and palatal mechanoreceptors. A double staining with propidium iodide and either GCR- or MCR-antiserum revealed the intranuclear and cytoplasmic localization of these receptors in the terminal Schwann cells. 11β-HSD II-immunoreactivity was found in the cytoplasm and on the nuclear envelope of the terminal Schwann cells, indicating the co-localization of MCR and 11β-HSD II in them. These findings suggest that the terminal or lamellar Schwann cells are target tissues for corticosteroid hormones. Taken together with previous reports on the functional significance of the corticosteroids, the results indicate that glucocorticoids and mineralocorticoids might be respectively involved in the proliferation and/or differentiation and Na⁺/K⁺-homeostasis in the terminal or lamellar Schwann cells associated with the mechanoreceptors.

Adrenal corticoids are divided into two types: mineralocorticoids and glucocorticoids. Mineralocorticoids play a major role in regulating sodium and potassium homeostasis, while glucocorticoids mediate a variety of developmental, stress-induced, and circadian responses. The biological effects of these steroids are initiated by the binding of two kinds of intracellular receptors: mineralocorticoid receptors (MCR) or the type I receptor, and glucocorticoid receptors (GCR) or the type II receptor (14, 39). These two types of corticosteroid receptors also have been identified in the brain (11, 29, 38). The physiological mineralocorticoid (aldosterone) and glucocorticoids (cortisol and corticosterone) equivalently bind MCR, while glucocorticoids show an affinity only to GCR (40). The steroid-receptor
complexes translocate to the nucleus, bind to the specific DNA recognition elements, and thereby stimulate the transcription of target genes. In addition, selectivity of the mineralocorticoid action within a target organ is mediated by the action of the cytosolic enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD II) (13, 15), which converts the functional form of glucocorticoids (cortisol and corticosterone) to the inactive metabolites, cortisone, and 11-dehydrocorticosterone. Because glucocorticoids circulate in serum at levels 100–1000 times greater than aldosterone, the co-localization of 11β-HSD II and MCR facilitates the selective action of aldosterone on its target organs.

Previous studies and clinical experiences have indicated a large number of mechanoreceptors in the periodontal ligament (for reviews, 8, 25). In particular, the periodontal Ruffini ending, categorized as low threshold slowly adapting type II mechanoreceptors (4, 9), is essential for periodontal mechanoreception. Morphologically, this nerve ending is characterized by extensive arborizations of expanded axon terminals and an association of special Schwann cells termed terminal or lamellar Schwann cells (6, 23, 24). These special Schwann cells are regarded as an analogue of lamellar or laminar cells in cutaneous mechanoreceptors (for reviews, 25, 30). Although the involvement of steroid hormones in glial cells has been shown in both central and peripheral nervous systems (cf. 20, 26, 27, 42), no report has discussed the expression of GCR and MCR in the specialized Schwann cells associated with the mechanoreceptors. The present study therefore was undertaken to examine the expression of corticosteroid hormone receptors and a key enzyme of MCR, 11β-HSD II, in the periodontal ligament of rat incisor using immunohistochemical techniques. It further made a comparison of their immunoperoxidase procedure. The sections were reacted with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1 : 1,000; Vector Lab.) and avidin-peroxidase complex (ABC Kit, Vector Lab.). A solution of 0.01 M PBS was used to dilute the antisera and to rinse the sections throughout the procedure. Final visualization used 3, 3′-diaminobenzidine (0.04%) and hydrogen peroxidase (0.03%) in a 0.05 M Tris-HCl buffer (pH 7.6). The sections were counterstained with 0.05% methylene blue.

Confocal laser scanning microscopy. Following incubation with the same primary antisera for the immunoperoxidase procedure, the sections were reacted with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1 : 100; Vector Lab.) for 1 h at room temperature. After thorough washes in PBS, the immunoreacted sections were counterstained with propidium iodide (1 : 3,000; Molecular Probes, Inc., Eugene, OR) for demonstration of nuclei, and were examined under a confocal laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

MATERIALS AND METHODS

All experiments were conducted under the Animal Experimentation of the Institutional Animal Use and Care Committee of the Niigata University School of Dentistry.

Animals and Tissue Preparation. Five Wistar rats (8 weeks old, weighing 250–300 g) were used in this study. The rats were deeply anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg), and transcardially perfused with a fixative containing 4% paraformaldehyde and 0.025% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4). The upper jaws were removed en bloc and immersed in the same fixative overnight. After demineralization with 10% EDTA-2Na for 3–4 weeks at 4°C, the tissue blocks were dehydrated through ascending ethanol and embedded in paraffin. The paraffin-embedded specimens were sagittally cut with a microtome at a thickness of 5 μm.

Immunoperoxidase procedure. Paraffin sections were processed for the avidin-biotin complex (ABC) method according to Hsu et al. (18). After inhibition of endogenous peroxidase activity with absolute methanol containing 0.3% hydrogen peroxide for 30 min at room temperature, sections were soaked in 2.5% normal goat serum (NGS; Vector Lab., Burlingame, CA) for 60 min. They were then incubated overnight with the primary antisera as shown in Table 1. Following washes in 0.01 M phosphate buffered saline (PBS, pH 7.4), the sections were reacted with two consecutive incubations of biotinylated goat anti-rabbit IgG (1 : 1,000; Vector Lab.) and avidin-peroxidase complex (ABC Kit, Vector Lab.). A solution of 0.01 M PBS was used to dilute the antisera and to rinse the sections throughout the procedure. Final visualization used 3, 3′-diaminobenzidine (0.04%) and hydrogen peroxidase (0.03%) in a 0.05 M Tris-HCl buffer (pH 7.6). The sections were counterstained with 0.05% methylene blue.

PGP 9.5 and S-100 protein immunohistochemistry. Additional two rats were perfused with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4). The removed upper jaws were decalcified in the same manner described above, and immersed in 30% sucrose solution for cryoprotection. Frozen sections, including incisors, at a thickness of 30 μm
were processed for immunohistochemistry for PGP 9.5, a general neuronal marker (12, 41), and S-100 protein using a commercially available ABC kit (Vector Lab.). The details of the immunohistochemical protocol have been described in previous reports (17, 31). The immunostained sections were counterstained with 0.05% methylene blue.

**Immunocytochemical controls.** Immunocytochemical controls were performed by: (1) replacing the primary antiserum with normal non-immune rabbit serum or with PBS; and, (2) omitting biotinylated anti-rabbit IgG or avidin-peroxidase complex. These sections did not show any immunoreactivity in the periodontal ligament or associated tissues.

**Observation area.** The mid-region of the lingual periodontal ligament of the upper incisors was selected as the area to be examined because the distribution and terminal formation of periodontal Ruffini endings had already been demonstrated in this region (cf. 25). The palatal antemolar rugae were also chosen as an observation area as they have been reported to contain many mechanoreceptors (5, 28, 46).

**RESULTS**

**Distribution and terminal morphology of mechanoreceptors in the periodontal ligament and palatal mucosa.**

The periodontal ligament of the rat lower incisor contained many PGP 9.5-immunoreactive nerve fibers. These nerves were restricted to the alveolar half of the ligament, and never appeared in the tooth half (Fig. 1a). They were referred to as the alveolus-related part (ARP) and tooth-related part (TRP), respectively (2). In the ARP, thick nerve fibers branched repeatedly to form Ruffini endings. These Ruffini endings displayed extensive ramifications with expanded terminal portions (Fig. 1a). In addition, thin nerve fibers, frequently beaded in appearance, terminated as free nerve endings in the ARP.

A dense distribution of S-100 immunopositive Schwann cells was found in the ARP but not in the TRP. The S-100-positive structures also branched in a dendritic fashion and formed Ruffini endings which were accompanied by some round cells called terminal Schwann cells (Fig. 1b). These positive cells had an indented or ovoid-shaped nucleus in their respective rich cytoplasm. Ordinary Schwann cells, slender in shape, were also distributed throughout the ARP.

The connective tissue papillae of the rat antemolar rugae received both comparatively thick and thin nerve fibers with PGP 9.5-immunoreaction (data not shown). These fibers terminated at the top of the connective tissue papillae. Some thin PGP 9.5-positive nerve fibers entered the epithelial layer (data not shown). The connective tissue papillae also contained round cells with S-100 immunoreactivity (Fig. 1c). These positive cells possessed rich cytoplasm and indented nuclei, indicating that they were lamellar Schwann cells.

**Immunohistochemical localization of GCR.**

Immunohistochemistry for GCR was able to demonstrate an intense immunoreaction in the periodontal ligament of rat incisors (Fig. 2a). These immunoreactive structures displayed dendritic profiles in the ARP of the periodontal ligament. However, the TRP of the ligament did not contain any specific immunoreaction. From their morphology and location, the dendritic ramifications with GCR-immunoreactivity appeared to be identical to the periodontal Ruffini endings (Fig. 2a, b) though they were comparatively thicker than these periodontal Ruffini endings immunostained with antisera against PGP 9.5. At higher magnifications, these immunopositive structures were frequently observed as two parallel lines with a space (Fig. 2b). Furthermore, the round cells, probably terminal Schwann cells associated with the periodontal Ruffini endings, also showed GCR-immunoreactivity. It was, however, difficult to identify the detailed immunoreactive sites in the terminal Schwann cells by immunoperoxidase techniques. In contrast, the nerve fibers and their associated ordinal Schwann cells in the nerve
bundles which penetrated into the periodontal ligament through the bony slits lacked GCR-immunoreactivity (Fig. 2a), while the osteoblasts on the surface of the alveolar bone also showed intense GCR-immunoreactivity.

Confocal laser scanning microscopic observation of double stained sections for GCR and nuclei clearly demonstrated the outlines of the periodontal Ruffini endings as dendritic ramifications (Fig. 4a). The nucleus of the terminal Schwann cells displayed a merged color (yellow), while the Schwann sheaths around the axon terminals were observed as green (Fig. 4a). The nuclei of the periodontal fibroblasts were colored red by propidium iodide, indicating that the periodontal fibroblasts were devoid of GCR-immunoreactivity.

Observation of the rat palatal mucosa showed that the lamellar cells in the dermal papillae immunoreacted with GCR-antiserum (Fig. 2c). The immunopositive reaction was distributed throughout their rich cytoplasm as well as their nuclear envelope. In addition, the epithelial cells of rat palatal mucosa exhibited intense GCR-immunoreaction except for the thick keratinized layer.

**Immunohistochemical localization of MCR and 11β-HSD II**

Immunoreactions for MCR (Fig. 3a) and 11β-HSD II (Fig. 3e) were found in the ARP of the rat incisors. The intensity was higher in MCR-immunostaining than in 11β-HSD II-immunostaining. These immunoreactive structures arborized repeatedly to form the periodontal Ruffini endings, as observed in GCR-immunostaining (Fig. 2a). However, neither an MCR- nor 11β-HSD II-immunoreaction was recognizable in the nerve bundles (Fig. 3b, e). The osteoblasts in the alveolar bone also exhibited MCR- (Fig. 3a, b) and 11β-HSD II-immunoactivities (not shown). The terminal Schwann cells contained intense immunoreactive products for MCR in their nuclei and cytoplasm (Fig. 3c). However, no 11β-HSD II-immunoreactions were recognizable in the nuclei of the terminal Schwann cells; they appeared only in their cytoplasm. These immu-
nolocalizations were more clearly demonstrated with confocal laser scanning microscopy; the co-localization of MCR and propidium iodide, colored yellow, was discernable in the nuclei of the terminal Schwann cells (Fig. 4b), while 11β-HSD II-immunoreactions were detectable in their nuclear envelope as well as cytoplasm (Fig. 4c). The Schwann sheaths around the axon terminals, frequently observed as parallel lines with an expanded space, were also positive in MCR and 11β-HSD II-immunoreactions.

In confocal microscopy, a double staining with each antiserum and propidium iodide demonstrated an immunonegative reaction in the periodontal fibroblasts.

In rat palatal mucosa, the expression patterns of MCR- and 11β-HSD II-immunoreactions were identical to those in the periodontal ligament; the cytoplasm of the lamellar cells displayed individual immunoreactions, but their nuclei immunoreacted only with MCR-antiserum.

DISCUSSION

Previous investigations have pointed out the possibility that the periodontal ligament is a target tissue for steroid hormones because cultured periodontal fibroblasts have been reported to possess specific binding sites for glucocorticoids (22). The present immunohistochemical study was able to demonstrate clearly the immunoreactivity for GCR in the terminal Schwann cells associated with the periodontal Ruffini endings in the rat periodontal ligament. Furthermore, the lamellar Schwann cells in the palatal sensory apparatus exhibited GCR-immunoreactions, which were not detectable in the ordinary Schwann cells. Since the present immunostaining could detect intense and specific immunoreactions for GCR in alveolar bone osteoblasts consistent with previous studies (10, 36), we can regard GCR-immunoreactions in the terminal Schwann cells as specific immunoreactions. As far as we know, this is the first report to reveal the presence of GCR in the specialized Schwann cells associated with mechanoreceptors.

11β-HSD II has been thought to play a pivotal role in regulating systemic mineralocorticoid action. The target cells for glucocorticoids have been reported to co-express MCR and 11β-HSD II. 11β-HSD II is capable of converting cortisol to biologically inactive cortisone which can bind GCR with high affinity. This specific binding enables an increase in the binding affinity between MCR and mineralocorticoid (aldosterone), and induces the biological effects of aldosterone in the target organ (40). Thus, the co-localization of MCR and 11β-HSD II in the target cells is essential for the initia-
tion of the biological action of mineralocorticoids. In addition to GCR, this immunohistochemical study revealed that the terminal and lamellar Schwann cell elements in the mechanoreceptors exhibit the co-localization of MCR- and 11β-HSD II-immunoreactivities in the periodontal ligament and...
palatal mucosa, indicating a possible existence of a signaling pathway via MCR in the specialized Schwann cells. These findings suggest a systemic control exerted through the action of corticosteroid hormones in the mechanoreceptors.

There are certain morphological differences in terminal Schwann cells between periodontal ligament and palatal mucosa, but they have been categorized as the same cell type, i.e. telo-glia (cf. 30). The expression patterns of MCR and GCR in the specialized Schwann cells were identical between the periodontal ligament and palatal mucosa, indicating that they are equally target cells of corticosteroids via MCR and GCR. Although previous investigations have identified several marker substances for these cells, including non-specific cholinesterase and S-100 protein (cf. 24, 25, 30), MCR and GCR are also useful markers for the terminal and lamellar Schwann cells.

The intracellular localization of steroid receptors has been a controversial topic, ranging from an exclusively cytoplasmic to an exclusively nuclear localization. Current confocal microscopic observations were able to demonstrate both the nuclear and cytoplasmic localizations of GCR and MCR in the terminal Schwann cells of the periodontal Ruffini endings. However, 11β-HSD II-immunoreactions existed only in the cytoplasm, comparable with a previous finding that 11β-HSD II is exclusively localized on the cytoplasmic surface of the endoplasmic reticulum (32). In addition, current confocal microscopy detected 11β-HSD II-immunoreaction on the nuclear envelope, identical to a finding by Shimojo et al. (41). The traditional concept regarding the biological function of steroid hormones is based on the transcriptional activity regulated by target genes (genomic action). Indeed, various steroid hormones have been shown to induce gene expressions in glia cells (26, 27, 43). In contrast, recent investigators have proposed a new concept for the non-genomic action of the steroid hormones, which has been predicted under physiological conditions. Cytoplasmic complexes of the ligand/receptor appear to participate in signal transduction, resulting in the regulation of cell growth, survival, and migration (for review, 21). These receptors can be activated in a ligand-independent manner by a cell membrane receptor agonist, the neurotransmitter dopamine (37). It is reasonable to consider that the biological activity of the terminal Schwann cells is under the control of genomic and non-genomic actions of steroid hormones.

The present immunohistochemical study showed the terminal and lamellar Schwann cells associated with the periodontal and palatal mechanoreceptors to display intense GCR-immunoreactivity. However, little information is available on the action of glucocorticoids on the terminal Schwann cells. Glucocorticoid/GCR induces a decrease in the proliferation of glia cells in the central nervous system (3, 16, 44), but conversely stimulates Schwann cell proliferation (33). Glucocorticoids may serve as co-mitogens for Schwann cells under physiological conditions. In vitro studies have demonstrated that glucocorticoids modulate cellular proliferation and differentiation by regulating the expression of...
growth factor receptors in Schwann cells (33). The terminal Schwann cells associated with the periodontal ligament have been shown to express a low affinity neurotrophic factor (p75-NGFR) (7) and TrkB (1, 34). Thus, the terminal Schwann cells may have a potential for proliferation and differentiation via GCR. Indeed, a resection of the inferior alveolar nerve has shown to induce the migration and proliferation of these cells (for review, 45).

In contrast to GCR, the functional significance of the MCR system in glial cells is not well understood in both peripheral and central nervous systems. The immunohistochemical expression of MCR and 11β-HSD II in the terminal Schwann cells strongly suggests the physiological significance of mineralocorticoids including aldosterone in mechanoreceptors, especially in the regulation of ion concentration and transportation. The terminal Schwann cells associated with the periodontal Ruffini endings possess well-developed caveolae on the cell membrane of the terminal Schwann cells (6, 19, 23). Ochi et al. (35) succeeded in demonstrating the localization of Na’/K’ ATPase in their caveolae by immunoelectron microscopy. Since mineralocorticoids control trans-epithelial sodium and potassium vectorial transports, the MCR might participate in controlling the intracellular concentration of ions. Further investigations will have to clarify the complete functional significance of the MCR system in the terminal Schwann cells associated with mechanoreceptors.

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