Immunofluorescent Localization of Myosin, Alpha-actinin and Tropomyosin in Odontoblast Microfilament Bundles of the Rat Incisor

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ABSTRACT. The localization of alpha-actinin, tropomyosin, myosin and actin in odontoblasts was examined by fluorescence microscopy using well characterized antibodies and rhodamine-phalloidin. All the reagents labeled the distal end of the cell body in the form of an oval ring with a preferential axis along the tooth axis. This ring was often interrupted. In conventional electron microscopy, microfilament bundles with periodical dense spots were running along the tooth axis at the level of the distal end of the cell body. The periodicity was about 0.6-1.0 µm. It may be possible that this dynamic structure functions to keep odontoblasts in a layer by contracting in an isometric form.
MATERIALS AND METHODS

Fluorescence microscopy. Male adult Wistar rats (150–300 g, Clea, Japan) were sacrificed by decapitation under sodium pentobarbital anesthesia. The mandibular incisors were immediately removed from the alveolar bone. The incisors were cut with a razor blade 3 to 4 mm from the apical end of the incisors. The apical tips were frozen by dipping them into liquid nitrogen, and cryosections (4 or 6 µm in thickness) were cut using a cryotome (Tissue Tek II, Miles, IN, USA). Most of the sections were immunolabeled without fixation. However, some were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 10 min on glass microscope slides. The antibody staining patterns seen in the fixed sections were basically the same as those of unfixed cells although the fixed sections exhibited relatively high background staining.

The following rabbit antisera were used: anti-chicken gizzard tropomyosin (7), anti-chicken gizzard alpha-actinin (8), anti-human platelet myosin (6), and anti-chicken gizzard vinculin (18). These antisera were kind gifts from Dr. K. Fujiwara, of the National Cardiovascular Center Research Institute, Osaka, Japan. The monoclonal antibody against desmoplakin I/II was a kind gift from Drs. J. Kartenbeck and W.W. Franke, of the Institute of Cell and Tumor Biology, German Cancer Research Center, Heidelberg, FRG. The fluorescein-conjugated donkey anti-rabbit Ig and anti-mouse Ig were purchased from Amersham Corp. Amersham, Bucks, UK. For staining the tissue sections, the rabbit antisera were diluted 50 to 100 times with PBS containing 1% bovine serum albumin (BSA/PBS). The mouse monoclonal antibody was used at a concentration of 15 µg/ml in BSA/PBS. The fluorescent secondary antibodies were diluted 20 times with BSA/PBS before use.

The cryosections on glass microscope slides were incubated with one of the appropriately diluted primary antibodies at room temperature for 30 min and washed three times, 5 min each, with PBS. Actin filament localization was studied by staining the sections with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) diluted 1:20 (165 nM) in PBS for 30 min, followed by three washings with PBS. The sections were mounted in Perma Fluor (Lipshaw Immunon, Detroit, MI, USA) and examined with an Olympus microscope equipped with epifluorescence optics (Olympus, Tokyo, Japan). Photomicrographs were recorded using 35 mm Kodak Try-X pan film (Eastman Kodak, Rochester, NY, USA).

Some of the sections labeled with one of the antibodies were further labeled with rhodamine-phalloidin. Double-labeled sections were examined with the Olympus epifluorescence microscope using a G520 filter (Olympus) at B excitation to block rhodamine fluorescence.

Some of the sections were incubated with normal rabbit serum (Cappel, Malvern, PA, USA) diluted 1:50 or 1:100 in BSA/PBS, with normal mouse IgG (Sigma, ST. Louis, MO, USA) at 15 µg/ml, or with rhodamine-phalloidin (165 nM) in the presence of an excess of unlabeled phalloidin (Sigma, 82.5 µM). The sections incubated with normal rabbit serum or normal mouse IgG were further incubated with fluorescein isothiocyanate-conjugated anti-rabbit Ig or anti-mouse Ig, respectively.

Electron microscopy. Three male Wistar rats (170–200 g, Clea) were anesthetized and perfused through the left ventricle with 5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, at room temperature. After 15–20 min the alveolar bone covering the enamel organs of the incisors of the lower jaw was carefully removed. The enamel organs of the incisors were removed and sliced into 1 mm thick segments, and placed in the same fixative for 2 hr. After washing overnight in a 0.05 M phosphate buffer solution, they were transferred to a phosphate-buffered 1% osmium tetroxide solution at 4°C for 2 hr. They were dehydrated in a graded series of ethanol and embedded in Epon 812. Sections made with a LKB Ultrotome V.
Fig. 1. Electron micrographs of odontoblasts at the level of distal end of the cell body in a tangential section. In a, abundant filament bundles are found predominantly along the long axis of the incisor (the long axis is indicated by a large arrow). The filament bundles are composed of dense portions (small arrows) and lucent portions (arrowheads). Typical alignment of the filament bundles exhibiting periodicity is shown in b. The filament bundle terminates at the intermediate junction area (arrows in b). PD: predentin, OP: odontoblast process, OB: odontoblast body. a: bar = 5 µm, b: bar = 0.5 µm.
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ultramicrotome (LKB, Stockholm, Sweden) were placed on naked grids, stained with uranyl acetate in 50% ethanol and lead citrate, and examined with a Jeol 100B or 200CX electron microscope.

RESULTS

Ultrastructure of odontoblast microfilament bundles. A longitudinal section of the incisor shows polarized columnar odontoblasts, which are packed in a layer. Odontoblast nuclei are located at the proximal end which is far from dentin and predentin. At the opposite end, an odontoblast process extends to the predentin and dentin. At the base of the odontoblast processes, a dense line stained with toluidine blue is seen (Fig. 2a, arrows). Ultrastructurally it is microfilament bundles composed of actin (17). In cross sections of odontoblasts at the level of the distal microfilament bundles, the cell profile designated an elongated polygon even though adjacent cell profiles were accurate polygons or circles. Dense microfilament bundles had intermittent electron dense spots within the elongated polygon (Fig. 1). The periodicity was about 0.6–1.0 μm between the centers of a dense spot and an adjacent spot. The long axis of the elongated polygons of the cell profile was oriented roughly along the axis of the incisor. The microfilament bundles of the odontoblast also ran along the long axis of the elongated polygon, and, therefore, along the incisor axis. The microfilament bundles terminated at both ends to plasma membrane which formed an intermediate junction with adjacent cells. The plasma membrane in the junction area was highly interdigitated (Fig. 1).

Odontoblasts stained with rhodamine-phalloidin, antimyosin, anti-alpha-actinin, antitropomyosin, antivinculin and anti-desmoplakin I/II. In longitudinal sections, odontoblasts were strongly stained with antibodies against alpha-actinin, tropomyosin, and myosin, as well as rhodamine-phalloidin for actin at the distal end of the cell body in the form of linear fluorescence. Antibodies against alpha-actinin, tropomyosin and myosin rarely or only faintly labeled other portions of the odontoblast body and process. However, fine fibrous actin fluorescence by rhodamine-phalloidin was found to be numerous in the predentin (Fig. 2e). In cross sections of the odontoblasts at the level of the distal end of the cell body, where microfilament bundles were located ultrastructurally, myosin lines were seen to run roughly parallel to the long axis of the incisor (Fig. 2f). Likewise, the location of actin lines was exactly the same as myosin lines (Fig. 2e). The overall distribution of myosin and actin formed fluorescent ovals at the distal end of the odontoblast body, although the oval was often interrupted (Fig. 2e, f). Alpha-actinin was also located at the distal end of the cell body, where the fluorescent lines generally ran along the tooth axis. Although the oval pattern was discernible, it had considerable

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Fig. 2. a, A light micrograph of odontoblasts sectioned along their long axis. A 1 μm Epon section stained with toluidine blue. Arrows show the position of microfilament bundles of odontoblasts. D: dentin, PD: predentin, O: odontoblasts. Bar = 10 μm. b–f, Fluorescence micrographs of odontoblasts sectioned tangentially (b, c, e, f) and a phase-contrast micrograph (d) corresponding to e and f. They were stained with anti-alpha-actinin (b), antitropomyosin (c), rhodamine-phalloidin (e) and antimyosin (f). In e and f, micrographs were taken from a double-labeled section. Long axis of the incisors is indicated by arrows. Alpha-actinin, tropomyosin, actin and myosin are colocalized at the distal end of the cell body basically in the form of the oval along the tooth axis, although the oval pattern has considerable irregularity. Bars = 10 μm.
irregularity and the contour of the fluorescent lines was intermittent (Fig. 2b). Anti-tropomyosin stained the same portion as those of rhodamine-phalloidin and the other two antibodies described above. The fluorescence intensity of anti-tropomyosin was stronger than the others, and the fluorescent oval pattern had some irregularity (Fig. 2c). Consequentially actin, myosin, alpha-actinin and tropomyosin were colocalized at the distal end of the cell body basically in the form of the oval along the tooth axis. From our previous reports using fluorescent phallacidin (16) and heavy meromyosin (17), the stained structures in this immunofluorescence study are closely related to microfilament bundles at the distal end of cell body on an ultrastructural basis.

On the other hand, antivinculin only faintly stained the terminal web and instead stained the proximal part of the odontoblast body. Antivinculin often stained blood capillaries within or under the odontoblast layer. Anti-desmoplakin I/II never labeled the odontoblasts.

Control sections incubated with rhodamine phalloidin in the presence of excess unlabeled phalloidin, normal rabbit serum or normal mouse IgG revealed no specific labeling of the odontoblasts.

DISCUSSION

The tooth is composed of three kinds of hard tissues which are enamel, dentin and cementum. Enamel is formed by characteristic protein secretion from epithelial ameloblasts and subsequent calcification. Dentin and cementum are elaborated by collagenous and other protein secretion from mesenchymal odontoblasts and cementoblasts and subsequent calcification. Dentinogenesis is initiated when odontoblasts differentiate to polarized columnar cells. Then these odontoblasts exhibit a structured cell layer beneath the inner enamel epithelia followed by the formation of the dentin matrix between the odontoblasts and the epithelia. The odontoblast inserts a long process into the predentin and dentin. Thin filament bundles which contain actin as by heavy meromyosin and NBD-phallacidin staining are present between the odontoblast process and the cell body (16, 17). The circumferential actin filament bundles of the intestinal and retinal epithelial terminal webs have been shown to be contractile (19, 20, 21). Because the basic ultrastructure and the composition of the cytoskeletal proteins of the odontoblast microfilament bundle are similar to those of the intestinal and retinal actin filament bundles, it may be reasonable to postulate that the odontoblast bundles are also contractile.

There is no circumstantial or substantial evidence in which odontoblasts actively slide past each other during dentin formation. Therefore, the microfilament bundles at the distal end of the cell body may function to keep odontoblasts in a layer by isometric contraction.

In this study, the profile of the odontoblast in a cross section at the level of microfilament bundles, designated the elongated polygon, is oriented along the axis of the tooth. Overall, the microfilament bundles run along the same axis. These results may be interpreted as follows. When dentin formation starts, its thickness increases. Since the whole incisor is cylindrical in shape and dentin is formed at the inner surface of the cylinder with continuing inward growth, the total surface area of the dentin-forming face must decrease during dentin formation. If this is the case, it could lead to the crowding of the underlying odontoblasts which are postmitotic.
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cells, and result in the shift of the configuration of odontoblasts from a simple single to a pseudostratified layer. Classical histological studies have shown that this shift occurs during dentin formation (2, 23). However, since odontoblasts are fixed with their neighbors at the junctional area where microfilament bundles are seen, the cell flattening at this level along the long axis of the cylinder could dissolve the crowding. It may be, therefore, postulated that the microfilament bundles play a role in resisting the pressure of crowding. Our previous study has shown that actin staining of odontoblasts, in which the intensity reflects the extent of microfilament development, is only weak at the stage of initial predentin formation, and then becomes stronger with increasing dentin thickness (16). Thus, the development of microfilament bundles is related to the dentin thickness formed rather than the establishment of the odontoblast as the secretory cell, which is consistent with the hypothesis described above.

The reason that antivinculin rarely labeled the odontoblast is unknown. Anti-desmoplakin I/II failed to label the odontoblast in this study. An earlier study has shown that antikeratin antibodies fail to stain the odontoblast (14). When these reports are considered together, it appears that the odontoblast lacks true desmosomes. Although "desmosomes" or "desmosome-like junctions" in the odontoblast have been described on the basis of electron microscopy (5, 12, 13, 22), it should be noted that their ultrastructure is different from that of the desmosomes in epithelial cells.

REFERENCES

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