ARTICLE VI

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MICROFILAMENTS IN CELLS ASSOCIATED WITH INDUCED HETEROTOPIC BONE FORMATION IN GUINEA PIGS

An Immunofluorescence and Ultrastructural Study

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Sera from patients with active chronic hepatitis were used to demonstrate actin microfilaments in cells of a bone-induced model. Demineralized and freeze-dried allogenic dentine was implanted in the abdominal muscles of Guinea pigs. After 21 and 28 days the tissue was excised and showed: a) resorption areas of the dentine contained dentinoclasts and macrophages. b) osteoid and bone and c) areas of fibroblastic proliferation. Osteoblasts and young osteocytes showed the strongest fluorescent staining. Electron microscopy of these cells demonstrated abundant 5–7 nm microfilaments interpreted as actin in the outer parts and cell projections, in which some microtubules also were observed. Fewer microfilaments were present in mature osteocytes although the cell projections in mineralized bone also showed microfilaments. Microfilaments were also found in some dentinoclasts where the actin was located in projections from the ruffled border protruding into the dentine. The localization in the ruffled border corresponded to the distribution of the fluorescent staining of actin. The high amount of actin in osteoblasts and osteocytes may be correlated to the synthesis and transport of the new matrix components. The microfilaments in the clear sealing zone of dentinoclasts were considered to have a function in the sealing of the ruffled border.

Key words: Actin; bone; immunofluorescence; transmission electron microscopy.

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Microfilaments with a diameter of 5–7 nm are found beneath the plasma membrane of many non-muscle cells, and it has been shown ultrastructurally that these microfilaments interact with heavy meromyosin (*Ishikawa et al.* 1969, *Wessells et al.* 1971, *Pollard & Weihing* 1974). The actin-containing microfilaments are suggested to be involved in cell mobility (*Pollard & Weihing* 1974), cytoplasmic streaming (*Palewitz et al.* 1974) and cytokinesis (*Schroeder* 1970). *King & Holtrop* (1975) have demonstrated binding of heavy meromyosin to microfilaments in osteoblasts, osteocytes and osteoclasts.

Smooth muscle antibodies (SMA) appear in serum from patients with active chronic hepatitis (*Johnson et al.* 1965, *Gabbiani et al.* 1972, *Andersen et al.* 1976). *Gabbiani et al* (1976, 1973), *Gabbiani & Ryan* (1974) and *Lidman et al.* (1976) have shown that this SMA is directed against actin. This feature has been used to demonstrate actin by indirect immunofluorescence in smooth muscle (*Johnson et al.* 1965) and other cell types (*Gröschel-Stewart & Gröschel* 1974, *Lazarides* 1975).

Induction of heterotopic bone formation has been shown to occur after implantation of demineralized allogenic dentine in the abdominal muscles of rats and guinea pigs (*Urist et al* 1967, *Bang* 1973). Previous electron microscopic investigations using this experimental model demonstrated microfilaments peripherally in osteoblasts (*Nilsen* 1977).

In this study the presence and distribution of microfilaments have been studied ultrastructurally and correlated with the actin distribution, as demonstrated by indirect immunofluorescence.

MATERIALS AND METHODS

Ten young male guinea pigs, randomly bred, were used. Each animal received 4 implants of allogenic demineralized and freeze-dried dentine in the pouches of the abdominal muscles (*Bang* 1973). The animals were fed a standard diet (Norwegian standard for guinea pigs and rabbits, Statens Institutt for Folkehelse, Oslo, Norway) supplemented with swedes and hay.

After 21 and 28 days, respectively, the animals, 5 in each group, were anesthetized with Nembutal^R and the implants were excised with some surrounding tissue.

Electron Microscopy

One part of each implant was divided into small pieces (1 mm^3) (altogether 80 specimens) and subsequently immersed in 2% glutaraldehyde in 0,1 M cacodylate buffer (pH 7.2) containing 7.5% sucrose (some at room temperature and some at + 4 °C) and then postfixed in 2% OsO_4 in the same buffer. The specimens were then dehydrated and embedded in Epon 812. One-µm thick sections were cut and stained with toluidine blue, and the actual cellular zones were chosen, prepared and cut at 50–60 nm with a diamond knife on a Reichert Om U3 ultramicrotome. The sections were collected on uncoated grids and double-stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope.

Immunofluorescence

The other part of each implant was directly frozen in isopentane precooled to −140 °C in liqued nitrogen and stored at −80 °C. Serum was taken from a patient with chronic active hepatitis, containing specific antibodies against muscles (SMA serum). The titre was 1/128. The serum was also tested for antimitochondrial and anti-DNA-antibodies and showed negative reactions. In order to test the reproducibility of the results, serum with smooth muscle antibodies from three other patients with chronic active hepatits was used on all specimens in the same manner. Cryostat sections (7 µm) were obtained and washed twice with phosphate buffered saline (PBS) before immersion in cold acetone at + 4 °C for 5 minutes. The sections were incubated with SMA serum diluted 1:8 to 1:20 in PBS and incubated in a moist chamber for 60 minutes. The preparations were then washed 3 times in PBS for 30 minutes and incubated for another hour in a 1:20 dilution of rabbit antihuman IgG coupled to fluorescein isothiocyanate (FITC) (DAKO-immunoglobulin, Copenhagen, Denmark) (F/P molar ratio 2,3 antibody titre: 200 µg/ml). The sections were then washed 3 times for 30 minutes and examined immediately by incident light fluorescence microscopy. (Leitz; Ploem-opaque illuminator, filters: 3,5 mm BG 12 and K 510).

Control sections were treated with: 1. PBS or normal human serum, 2. SMA-serum neutralized by absorption with homogenates of smooth muscle from rat stomach.

RESULTS

Transmission Electron Microscopy

The light and electron microscopical study demonstrated three main types of cellular reactions appearing consistently in all animals: a) resorption zones with dentinoclasts (multinucleated dentine resorbing giant cells morphologically identical to osteoclasts) and macrophages; b) matrix production zones with osteoblasts, osteocytes, and a few chondroblasts and chondrocytes; c) areas consisting of fibroblasts. All three zones wer found after 21 days as well as 28 days. At 28 days, however, more mineralized bone was observed. For a detailed description of these cells, see *Nilsen* (1977). Large amounts of new matrix were found to be produced in all animals.

A young connective tissue which surrounded the three zones exhibited only scattered granulocytes and lymphocytes. Skeletal muscle fibres occupied the outer parts of the specimens, representing the abdominal muscles.

Osteoblasts and osteoid osteocytes regularly showed a distinct accumulation of microfilaments along and parallell to the cell membrane facing the newly formed osteoid (Fig. 1). These microfilaments had a diameter of 5–7 nm and seemed to be attached to the cell membrane and extended also into cell projections (Fig. 2). Quite a few of the cell projections of adjacent cells showed tight junctions. Scattered microtubules were observed in both cell projections and the cortical part of the osteoblasts and osteocytes when the specimens were fixed at room temperature. Tissue fixed at + 4 °C showed fewer microtubules.

Both osteoid and mineralized bone showed abundant microfilaments in the cell projections. These microfilaments were longitudinally orientated. The mature osteocytes, however, lacked the distinct accumulation of microfilaments just beneath the cell membrane which was evident in osteoblasts and young osteocytes. Deeper parts of the cytoplasm of young matrix-producing cells showed smaller bundles of microfilaments. often

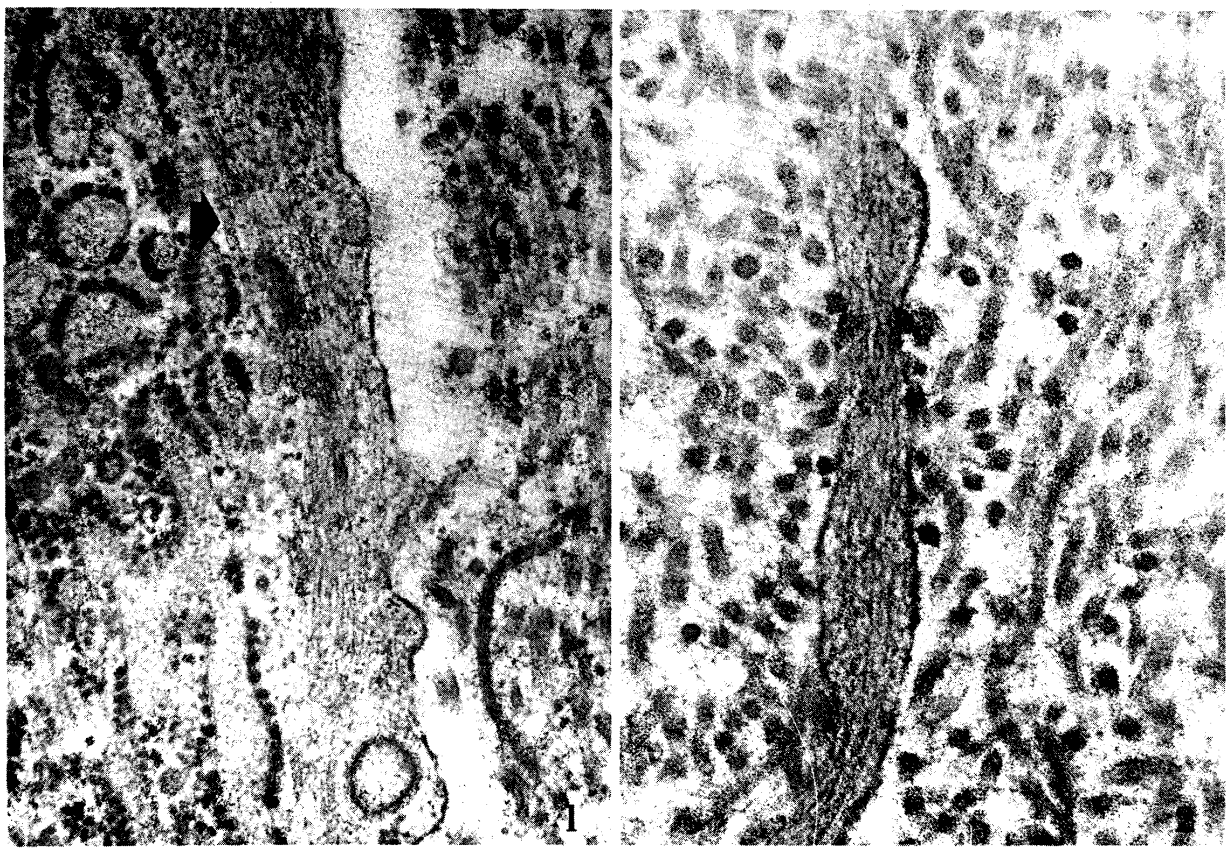

Fig. 1. Peripheral microfilaments in an osteoid osteocyte. Microtubule (Arrow) Collagen (C). × 50.000.

Fig. 2. Longitudinal section fo an extension from an osteoid osteocyte containing parallel microfilaments. × 80.000.

arranged parallel to the endoplasmatic reticulum.

The distribution of microfilaments was less evident in the resorbing cells than in the matrix-producing cells. Areas of the ruffled border in some of the dentinoclasts, however, contained bundles of microfilaments. These microfilaments were found especially in cellular extensions from the clear sealing zone of the ruffled border (Fig. 3). Microtubules, however, were not found in this area. Accumulation of microfilaments was also found in some macrophages. Fewer microfilaments were found in undifferentiated cells. In a few mature fibroblasts, however, a subcortical accumulation of microfilaments was demonstrated.

Immunofluorescence

In the young undifferentiated connective tissue surrounding the implants a bright green fluorescence was only found in vessels walls. A few fibroblasts, however, exhibited a moderate staining, and the skeletal muscles had a strong positive immunofluorescence in the I-bands.

All the osteoblasts and osteoid osteocytes showed strong cytoplasmic staining for actin (Fig. 4). The staining appeared manily in the periphery of the osteoid osteocytes. The osteoblast region exhibited also strong cytoplasmic fluorescence. This staining was more diffusely outlined than in osteoid osteocytes. Osteocytes in the mineralized bone had just a weak fluorescent staining.

In some dentinoclasts a strong intracellular fluorescence was observed in the ruffled border area (Fig. 4). Other dentinoclasts, however, had no evident fluorescence. Some macrophages in areas of resorption also showed cytoplasmic fluorescence. The cytoplasmic fluorescence staining in the matrix-producing cells as well as the positive staining in dentinoclasts was diffusely outlined.

All the control preparations were negative and gave only low background fluorescence.

DISCUSSION

The cellular reactions to the implanted allogenic dentine, including both osteoid and bone formation as well as resorption of dentine, confirmed the previous reports of a specific induction of bone. (*Urist et al.* 1967, *Bang & Urist* 1967, *Bang* 1973, *Nilsen* 1977). The close relationship between the

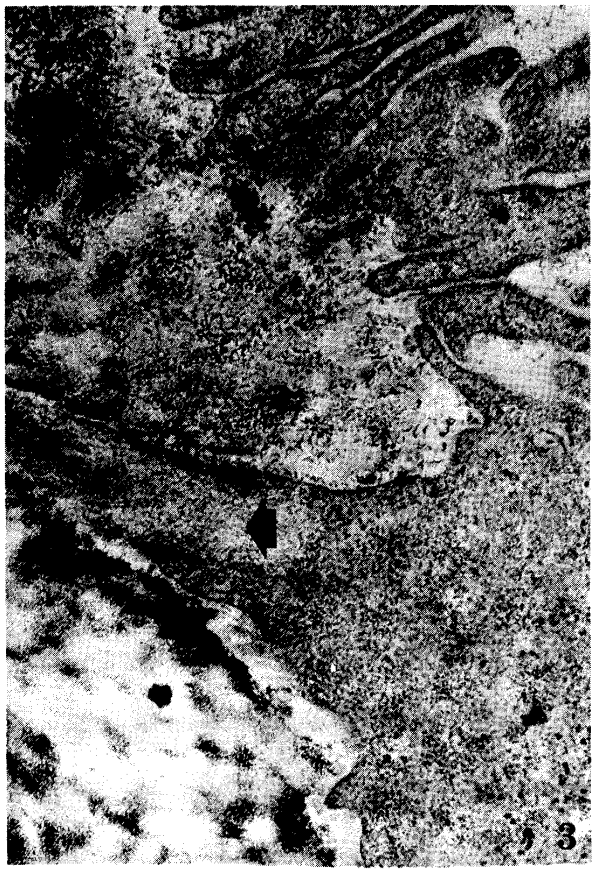

Fig. 3. Microfilaments (arrow) in a cell extension in the ruffled border region of a dentinoclast. × 36,900.

Fig. 4. Indirect immunofluorescence showing anti-actin staining produced by SMA-positive serum. Note the peripheral localization in osteoid osteocytes (arrows). Strong fluorescence was seen in the ruffled border region of a dentinoclast (open arrow) lying in a resorption lacuna in the dentine (D). × 400.

different reaction zones permitted comparison between the reaction zones within each section.

Large amounts of actin were found in the periphery of the osteoblasts and young osteocytes as well as in cell projections of these cells. Smooth muscle antibodies have not previously been used to show actin in bone cells. Actin containing microfilaments, however, have previously been demonstrated in cultured bone cells with heavy meromyosin as a specific ultrastructural marker (*King & Holtrop* 1975). They suggested that the actin played a role in the intracellular transport of small molecules.

Microtubules were also found in the actin-containing areas in matrix-producing cells. Microfilaments and microtubules are proposed to constitute the contractile part of the cytoskeleton (*Wessels et al.* 1971).

The present study showed the greatest amounts of actin in young osteocytes which are active matrix-producing cells with only a low motility. The accumulation in these cells may then indicate a function of the microfilaments in the synthesis and transport of collagen precursors or other matrix components. Actin microfilaments in myofibroblasts in granulation tissue were recently proposed to be involved in synthesis and transport of type III collagen (*Gabbiani et al.* 1976).Microfilaments are recently (*Drenckhahn et al.* 1977) suggested to be involved in transport and exocytosis in acinar cells of salivary glands.

The microfilaments and microtubules were observed in the cellular processes, which also showed tight junctions to other cellular processes from osteocytes. These findings may indicate a possible function of the microfilaments in the transport between the osteocytes. Other electronmicroscopical studies have proposed a possible transport function of the microfilaments (*Stanka* 1975) and microtubules (*Furseth* 1973) in the cell extensions.

The localization and amounts of actin in young osteoid osteocytes in the present report suggest a contribution to movements of the osteocytes within the lacunae, These amoeboid movements may have a role in movements of the extracellular fluid. A

transport function on account of amoeboid movements of the osteocytes has been proposed previously by *Bassett* (1967).

The microfilaments may then be involved in both extracellular and intracellular transport of new matrix components.

The ruffled border of some dentinoclasts in the present study showed a strong fluorescent reaction, and ultrastructurally microfilaments were found to protrude into cell projections of the clear sealing zone. The observed arrangement of microfilaments in the ruffled border of dentinoclasts corresponded with ultrastructural findings in osteoclasts as reported by *King & Holtrop* (1975). In keeping with *King & Holtrop* (1975) microtubules were not observed in the ruffled border region. The ruffled border of dentinoclasts and osteoclasts is highly motile and involved in active phagocytosis (*Göthlin & Ericsson* 1976). The actin distribution observed in the dentinoclasts of the present study could reflect the mobility of these cells.

The ruffled border with its clear zone is the active site of resorption of mineralized tissues. The clear sealing zone may have a function in making closed environments around the ruffled border in the active resorbing giant cells. This would make a reduction of pH possible, which would explain the crystal-dissolving function of these giant cells (*Schenk* 1974).

The present study shows that the microfilaments could constitute a contractile part of this sealing area of the ruffled border.

The actin microfilaments seem to be involved in a variety of biological processes and this study suggests that they are involved in matrix-production of bone as well as resorption of hard tissues.

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