BRIEF NOTES

ACTIN-LIKE FILAMENTS IN BONE CELLS OF CULTURED MOUSE CALVARIA AS DEMONSTRATED BY BINDING TO HEAVY MEROMYOSIN

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Numerous intracellular microfilaments with a diameter of 50–70 Å have been described in the cell processes of osteoblasts and osteocytes; in addition, bundles of similar filaments have been found along the cell membrane in their cell bodies (12, 25). We have demonstrated that these structures bind specifically to heavy meromyosin (HMM) and hence can be considered actin-like.

In the osteoclast the ruffled border is the site of active resorption (5). This area is completely encircled by a clear zone (11), which was first described as being organelle-free and containing amorphous material (9). Recently, Malkani et al. (17) have shown that this area has a striated appearance, with alternating dark and light areas oriented perpendicular to the bone surface. In preliminary work, we have noticed that these darker bands consist of bundles of 50–70-Å filaments. Microfilaments with a diameter of 50–70 Å were also found under that part of the cell membrane that is not opposed to bone. In this report we describe the filaments in the clear zone and under the cell membrane of the osteoclast and demonstrate that they all bind HMM.

MATERIALS AND METHODS

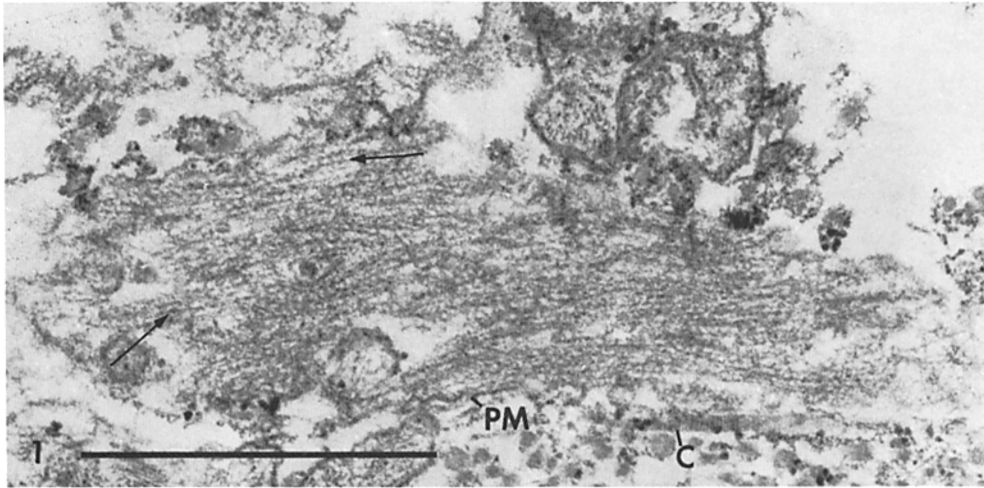
The calvaria of 14 6-day old Swiss albino mice were aseptically dissected and placed on stainless steel organ culture grids (Wilks Precision Instrument Co., Inc., Rockville, Md.) in 5-ml culture dishes. The culture medium consisted of modified BGJb tissue culture medium (Grand Island Biological Co., Grand Island, N. Y.) to which was added 5% heat-inactivated rabbit serum and 150 μg/ml ascorbic acid. In order to obtain a sufficient number of osteoclasts for study, differentiation of this cell type was stimulated by adding 1 IU/ml of parathyroid extract (Eli Lilly & Co., Indianapolis, Ind.) to the medium. The bones were placed in an incubator at 37°C in a humidified atmosphere of 5% carbon dioxide in air for 72 h. After the culture period, two calvaria were fixed immediately at 0°C and prepared for electron microscopy according to previously published techniques (11), and 12 calvaria were glycerinated according to Ishikawa et al. (14). Six of these were then incubated for 18 h in a standard salt solution (0.1 M KCl, 0.005 M $MgCl_2$, 0.006 M potassium phosphate buffer, pH 7.0) containing 4 mg/ml of HMM at 4°C. As controls, three calvaria were incubated in the standard salt solution alone, and three calvaria in a salt solution containing 4 mg/ml HMMand 0.01 M ATP. After incubation, all bones were fixed at 0°C and embedded in Epon as described earlier (11).

Myosin was prepared according to the technique of Paterson and Strohman (18), and HMM was obtained by a limited digestion in trypsin (Worthington Biochemical Corp., Freehold, N. J.) according to Pollard et al. (21). A protein determination on the final product by the Lowry technique (15) gave an estimate of the concentration of HMM present. This was adjusted to 4 mg/ml with the standard salt solution. HMM was used immediately after preparation. Its activity was tested by reaction with separated filaments from rabbit muscle (13) on an electron microscope grid which was negatively stained with 1% aqueous uranyl acetate.

RESULTS

In osteoblasts and osteocytes bundles of filaments were located directly under and parallel to the plasma membrane, and these filaments were "decorated" in tissue reacted with HMM (Fig. 1), as compared to tissue reacted with HMM and 0.01 M ATP (Fig. 2). These filaments ran parallel to one another and were only rarely seen to travel into the more central cytoplasm. The cell processes of osteoblasts and osteocytes were filled with bundles of microfilaments (50–70 Å in diameter) and these were also found to bind HMM (Fig. 3).

In glycerinated tissue the osteoclast generally remained attached to the bone surface. These cells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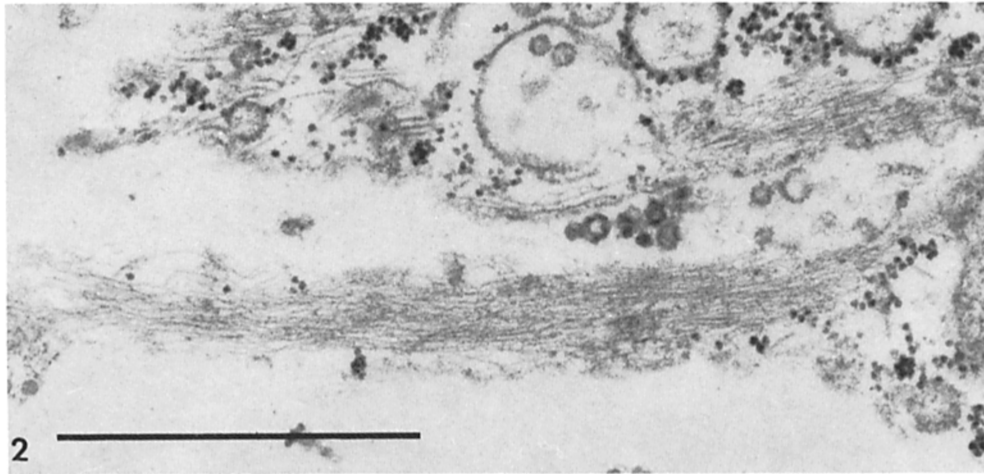
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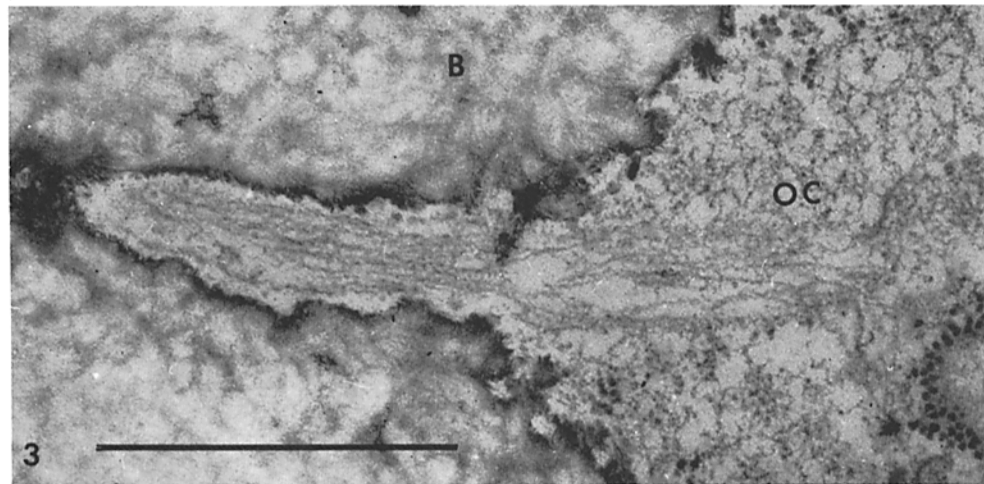
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showed decorated filaments directly under the plasma membrane on the surface of the cell away from the bone surface. These, however, were more randomly dispersed than in osteoblasts and osteocytes and appeared to show no preferential orientation to the membrane.

In optimally preserved material, clear zones could be found peripheral to the ruffled border of the osteoclast and in close apposition to the bone surface. The bone under the clear zones showed no signs of disruption, unlike that under the ruffled border. At low magnification, the clear zones consisted of alternating dark and light striations which were oriented perpendicular to the bone surface. At higher magnification, the light areas appeared to contain amorphous material (Fig. 4). The darker bands consisted of bundles of filaments with a diameter of 50–70 Å which were oriented perpendicular to the cell membrane at the bone surface (Fig. 4). These filament bundles were usually found in association with short cell processes, which, in turn, were located at points of irregularity on the bone surface. In cross section, the filamentous bundles appeared as discrete, roughly circular areas surrounded by the amorphous material (Fig. 5). The structures in these circular areas are cross sections of filaments, as distinct from granular material, because they were only seen when the plane of section was parallel to the bone surface. Furthermore, at points where the cut was at a slightly oblique angle, filamentous structures could be recognized (arrows, Fig. 5). In the HMM-treated specimens, these clear zones appeared rich in decorated filaments (Fig. 6), but the pattern of dark and light banding could not be demonstrated.

In glycerinated specimens which were not reacted with HMM or those incubated with HMM and 0.01 M ATP, the filaments in all three cell types were not decorated (Fig. 2). At low magnifications, it also seemed that the filament bundles were less densely stained than those which were treated with HMM. This difference was most apparent in the osteoclast clear zone.

DISCUSSION

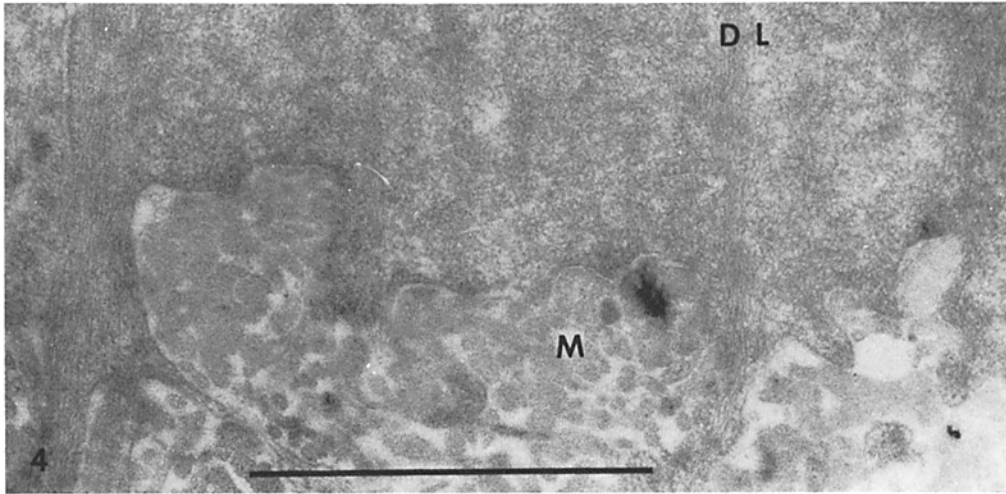
Microfilaments are present under the cell membrane and scattered through the cytoplasm in osteoblasts and osteocytes (12, 25). This report demonstrates that the filaments directly under and parallel to the cell membrane bind HMM and therefore can be considered actin-like. Filaments of similar structure, normally found scattered through the cell bodies, could not be demonstrated in glycerinated specimens. It is possible that many of these less organized filaments were lost or disrupted in the glycerination procedure. In addition, the processes of these cells are filled with bundles of microfilaments running parallel to the long axes of the processes, and these also bind to HMM. Microfilaments have been implicated in the process of cytoplasmic streaming in cell-free extracts of *Amoeba proteus* (20) and *Chaos carolinensis* (23). In nerve axons, microfilaments are suggested to represent a mechanism for axoplasmic transport (3, 6). There are indications that bone cell processes serve a transport function: cell processes of osteoblasts and osteocytes are connected by membrane structures which are probably gap junctions (12, 25, 26), facilitating a close intercellular communication between one cell process and another. Since the filaments which fill the processes are actin-like, it is suggested that these processes may function to transport small molecules from one cell to another and hence from the bone surface to sites deep in the bone and vice versa.

In the osteoclast, 50–70-Å filaments, reactive to HMM, were demonstrated under the cell membrane away from the bone surface. However, unlike those found in the other bone cell types, these seemed fewer in number, not necessarily

FIGURE 1 Filament bundle directly under the plasma membrane (*PM*) of a glycerinated osteoblast incubated with HMM. Collagen can be seen in the extracellular space (*C*). In favorable areas, regular projections can be seen "decorating" the filaments (arrows). Note that the filaments are arranged parallel to one another and to the plasmalemma. Bar represents 1 μm. × 48,250.

FIGURE 2 Subplasmalemmal filament bundle of a glycerinated osteoblast. This cell was incubated with HMM and 0.01 M ATP. Note the lack of decorations on the filaments and their thin and pale appearance in comparison with those in Fig. 1. Bar represents 1 μm. × 48,250.

FIGURE 3 Filament bundles in a glycerinated osteocyte (*OC*) incubated with HMM project from the cell body into a process. Bone (*B*) Bar represents 1 μm. × 48,250.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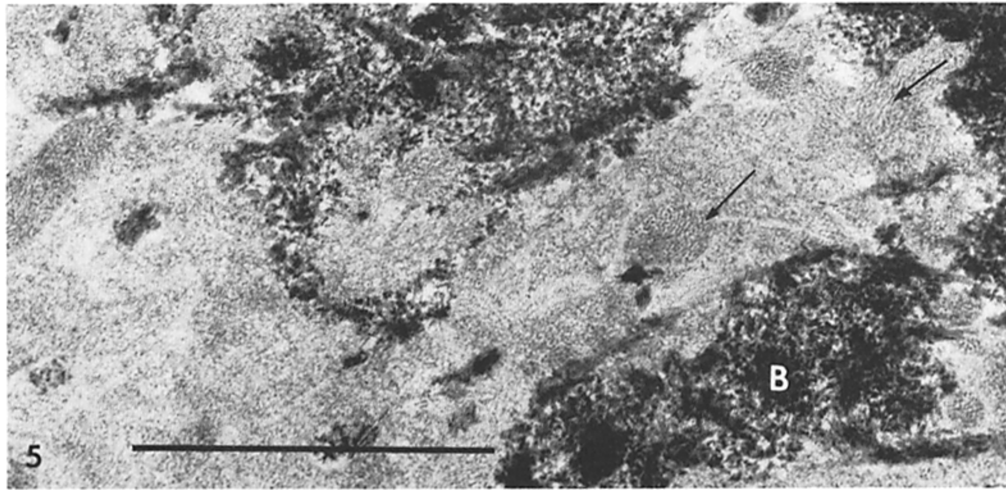
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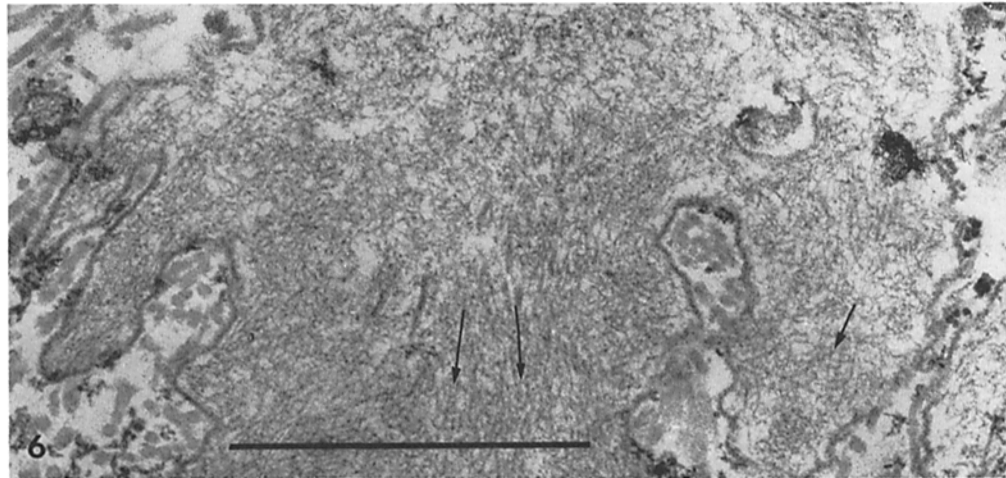
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arranged in bundles and with no preferential orientation to the membrane. Similar observations have been made in motile cells distant from a surface of attachment, as in cultured neuroblastoma cells (4) and in resting macrophages (22). The osteoclast has been shown to be a highly motile cell by microcinematography (7, 8) while the osteoblast and osteocyte are not. It is possible that such a difference in organization of filaments under the cell membrane is related to the motile activity of the membrane.

Osteoclasts which are actively engaged in the resorption of bone have a characteristic structure at the bone-cell interface known as the ruffled border. This membrane is highly motile (7, 8) and actively phagocytosing (5, 16). Encircling the ruffled border is an area which is organelle-free and densely packed with some amorphous material. This so-called clear zone rests directly on the bone surface and, since the bone beneath it is relatively undisturbed, one must assume that it is not directly involved in the process of resorption. Malkani et al. (17) observed that these structures have a striated appearance, with alternating dark and light bands perpendicular to the bone surface and the plasmalemma. In the present paper, we demonstrated that the dense bands actually consist of bundles of 50–70-Å filaments which are reactive to HMM and apparently pass through the cytoplasm and terminate on or near the membrane in short cytoplasmic processes directed toward indentations on the bone surface.

Filament concentrations similar to those found in the clear zones but less extensive appear within 60 s of the establishment of a contact between two moving fibroblasts in vitro (10). Similar areas of specialization have also been noted between fibroblasts and a substrate (1). It has been suggested that such localized microfilament concentrations represent cell-cell or cell-substrate adhesions or some integral part of the contact inhibition phenomenon (10). Similar filament bundles have also been reported in cultured fetal rat cells (2), cultured chick embryo fibroblasts (19), and phagocytizing cultivated macrophages (22) at points of apparent attachment of a cell to a surface. In chick embryo fibroblasts these filaments were shown to be actin-like (19). The suggestion has also been made that such filament networks might be involved in the stabilization of existing adhesions (2, 19). This idea is supported by data which show that cytoplasmic viscosity increases significantly concomitant with increases in cytoplasmic microfilaments (20, 23). Such filament networks may be viscous enough to maintain the membrane in a quiescent state on a surface of attachment. In the macrophage, a complex organization has been described in the subplasmalemmal region of the attached cell surface, consisting of a filamentous network and microtubules passing through microchannels to the membrane on the glass surface (22). Microtubules could not be demonstrated in the area of the clear zones in our optimally preserved specimens, but since they were fixed in the cold and microtubules are unstable under these conditions, it is likely that they may have been degraded if, indeed, they were present. Perdue (19) described microfilaments actually passing through the membrane at points of attachments in cultured embryonic fibroblasts. This could not be demonstrated at the interface between the clear zones and the bone surface.

The nature of the amorphous material which is seen in the light areas of the clear zone is unknown but it could represent G-actin. This monomeric actin has been shown to exist as an amorphous material in the acrosomal process of certain echinoderm sperm (24). G-actin has been isolated

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FIGURE 4 Detail of a clear zone in an osteoclast. The area consists of dark (*D*) and light (*L*) bands oriented perpendicular to the bone surface. The darker bands are bundles of filaments (50–70 Å in diameter) which are associated with short processes at the bone surface. Electron-dense mineral has been removed from the bone, leaving the matrix components (*M*). Note that cell processes containing microfilaments enter irregularities of this bone surface. Bar represents 1 μm. × 54,000.

FIGURE 5 Detail of an osteoclast clear zone sectioned parallel to the bone surface (*B*) at the bone-cell interface. The filamentous bundles (arrows) appear as discrete, roughly circular areas surrounded by amorphous material. Note the filamentous nature of the dense material in the circular areas in oblique section (arrows). Scale represent 1 μm. × 48,250.

FIGURE 6 Clear zone of a glycerinated osteoclast reacted with HMM. Note the decorations on the filaments of the short process which inserts at the bone surface (arrows). Bar represent 1 μm. × 48,250.

from these structures and will polymerize into filaments which are biochemically and morphologically identical to muscle actin.

SUMMARY

A variety of intracellular filaments (50–70 Å in diameter) found in bone cells was shown to bind specifically to HMM. Because of this property, these filaments are probably biochemically similar to muscle actin.

In osteoblasts and osteocytes, these reactive filaments were oriented in bundles parallel to the plasma membrane and filling the cell processes.

In the osteoclast the filaments along the cell membrane were not so highly organized. In the clear zone, the quiescent part of the cell adjacent to the motile ruffled border, organized filament bundles were oriented perpendicular to the cell membrane and terminated in short processes at the bone surface. These filaments were also reactive with HMM.

The possible significance of the filaments with respect to the physiology of bone cells is discussed.

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