

RINGS OF INTERMEDIATE (100 Å) FILAMENT BUNDLES IN THE PERINUCLEAR REGION OF VASCULAR ENDOTHELIAL CELLS

Their Mobilization by Colcemid and Mitosis

STEPHEN H. BLOSE and SAMUEL CHACKO. From the Laboratory of Pathology, Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174

Intermediate-sized (100 Å) filaments have been reported in many cell types *in vivo* (1, 4, 24, 27) and *in vitro* (2, 12, 17, 25). These filaments are normally sparse and scattered throughout the cytoplasm. Intermediate-sized filaments can be induced by treatment of cells with the drugs colchicine and Colcemid (5, 12, 17, 28). The 100 Å filaments induced by these drugs are concentrated into bands or large pools in the cytoplasm with no specific location (5, 12, 17).

Vascular endothelial cells have been shown to contain cytoplasmic filaments which measure ~100 Å in diameter (13, 20, 21, 23, 26), and some workers have interpreted these filaments as "thick" filaments (9, 20, 30). This investigation was carried out to study the nature of the cytoplasmic filaments in endothelial cells. In this communication, we wish to report the natural occurrence of bundles of 100 Å filaments (unrelated to the thin-actin and thick-myosin filaments) that completely encircle the nucleus in vascular endothelial cells *in vitro*, their similar location *in vivo*, and their mobilization by Colcemid and mitosis *in vitro*.

MATERIALS AND METHODS

Endothelial cells were obtained from thoracic aortas and portal veins of young adult guinea pigs as described before (3). Approximately $1-5 \times 10^8$ cells were cultured in 60-mm Cooper dishes (Falcon Plastics, Div. of Bio-Quest, Oxnard, Calif.) and fed on alternate days with Eagle's minimal essential medium supplemented with 15% fetal calf serum, 1% L-glutamine solution, and 1% Pen-Strep (all from Grand Island Biological Co., Grand Island, N. Y.). Endothelial cells for electron microscopy were grown on a collagen substratum in carbon-coated Cooper dishes. The cells in culture dishes were fixed *in situ* at room temperature with 2% glutaraldehyde in 0.075 M cacodylate buffer with 4.5% sucrose and 0.48 mM CaCl_2 at pH 7.4 for 30 min, and then washed with three changes of 0.05 M cacodylate buffer with 6% sucrose over 2 h. Cells were postfixated with 2% osmium tetroxide in 0.05 M cacodylate buffer for 20 min. They were then stained with saturated aqueous uranyl acetate

for 20 min, dehydrated in graded alcohols over 15 min, and embedded in Epon 812. The plastic culture dishes were separated from embedded cells in liquid nitrogen. Selected cells were marked under the phase microscope, and blocks were trimmed around the cells to be sectioned. Thin sections (~700 Å) were prepared and stained with lead citrate. Whole blood vessels were processed for electron microscopy according to Somlyo et al. (24).

Endothelial cells examined by polarized light were grown on collagen-coated glass cover slips (24 × 40 mm). The cover slips were removed from the culture dishes, inverted, and sealed to a glass slide with sterile Vaseline. The slide was kept at 37.5°C with an air curtain (Sage Instruments, White Plains, N. Y.) while on the microscope stage.

Cultured endothelial cells were briefly glycerinated (4 h, 50% glycerol in 0.05 M standard salt) *in situ* and prepared for decoration with heavy meromyosin subfragment-1 (S-1)¹ after the method of Ishikawa, Bischoff, and Holtzer (18). Dividing endothelial cells in various stages of mitosis were marked under the phase microscope and processed for electron microscopy as previously described.

Cultures for studying the effect of Colcemid were incubated with 10^{-6} M Colcemid for 24 h. Cycloheximide at the concentration of 0.1 mM was used to block protein synthesis (5). In examining the cultures treated with Colcemid, a 537-nm interference filter was used to prevent photoconversion of Colcemid to its inactive form lumicolcemid (16).

RESULTS

Cultured endothelial cells were flat and polygonal, and formed epithelioid colonies as previously described (3). At 10–14 days when the cultures approached confluence, many endothelial cells contained a phase lucent ring that completely encircled the nucleus (Figs. 1 A and 7 A). Polarization microscopy revealed these rings to be bire-

¹ S-1 was the generous gift of Dr. Frank Pepe, Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

fringent (Figs. 1 B, C, and 7 B, C). Occasionally, a second ring encircled the Golgi region (Fig. 2 A, B). *En face* sections of these cells were made and examined with the electron microscope. A bundle of intermediate-sized (100 Å) filaments was found in the perinuclear region (Figs. 3 and 4). These filament bundles correspond to the phase lucent rings seen in Figs. 1 A and 7 A. These rings are in a plane parallel to the substratum. Smaller numbers of filaments could be found leaving the larger perinuclear bundle and coursing out into the cytoplasm (Fig. 4). The region occupied by the filament bundles was devoid of other cytoplasmic organelles. Each bundle consisted of 200–500 filaments in cross section (Fig. 5). The individual filaments measured ~100 Å (99 ± 13 Å) in diameter, and through-focus series of electron micrographs revealed the filaments to have a less dense core.

The 100 Å filament bundles in cultured endothelial cells remained intact after glycerol extraction and, unlike the actin filaments (60 Å in diameter), the 100 Å filaments did not decorate with S-1 to form arrowhead complexes (15, 18).

Sections of the aorta fixed *in situ* were examined with the electron microscope to determine whether the 100 Å filaments arranged in a similar pattern were present *in vivo*. Fig. 6 shows the perinuclear distribution of bundles of 100 Å filaments *in vivo*. Thick (150 Å diameter) myosin filaments were never observed, though filaments that resemble actin (60 Å in diameter) were occa-

sionally present on the attachment side of cultured cells (13) and abluminal side *in situ* (23).

In view of the fact that Colcemid produces pools of intermediate filaments distributed throughout the cytoplasm in the absence of protein synthesis (5), we were interested to find out the effect of Colcemid on naturally occurring bundles of 100 Å filaments. Cultures containing cells with phase lucent rings of birefringent fibrils around the nucleus were treated with Colcemid (10^{-6} M). The position and shape of the fibrils were followed with phase, polarization, and electron microscopy. The phase lucent rings "coiled" into an eccentrically placed phase lucent "cap" (Figs. 7 A, 8 A, and 9 A) adjacent to the nucleus. In some cases, the cap displaced the nucleus from the center of the cell (see Fig. 9 A). These cells when viewed with the polarized light still demonstrated the birefringent nature of the cap (Figs. 7 B, C, 8 B, C, and 9 B, C). Electron microscopy of these cells revealed the cap to consist of disorganized aggregates of 100 Å filaments (Fig. 10 A, B). The cap formation was observed in all the endothelial cells (over 1,000 cells examined) when incubated with Colcemid for 24 h. In another experiment, the endothelial cells were preincubated with cycloheximide (0.1 mM) for 16 h, then treated with Colcemid. This was done to determine whether protein synthesis was required for the mobilization of these filaments. This experiment showed that the cycloheximide did not block the "coiling effect" of Colcemid.

FIGURE 1 (A) Living vascular endothelial cells near confluence at day 14 of culture. Many of these cells contained a phase lucent ring (arrows) that encircles the nucleus (N). Polarization microscopy of the same cells in Fig. 1 A (B and C at opposite compensator settings) revealed these rings to be birefringent. Phase microscopy done with a Zeiss 40 × phase 2 water immersion lens. Bar is 40 μm. × 420.

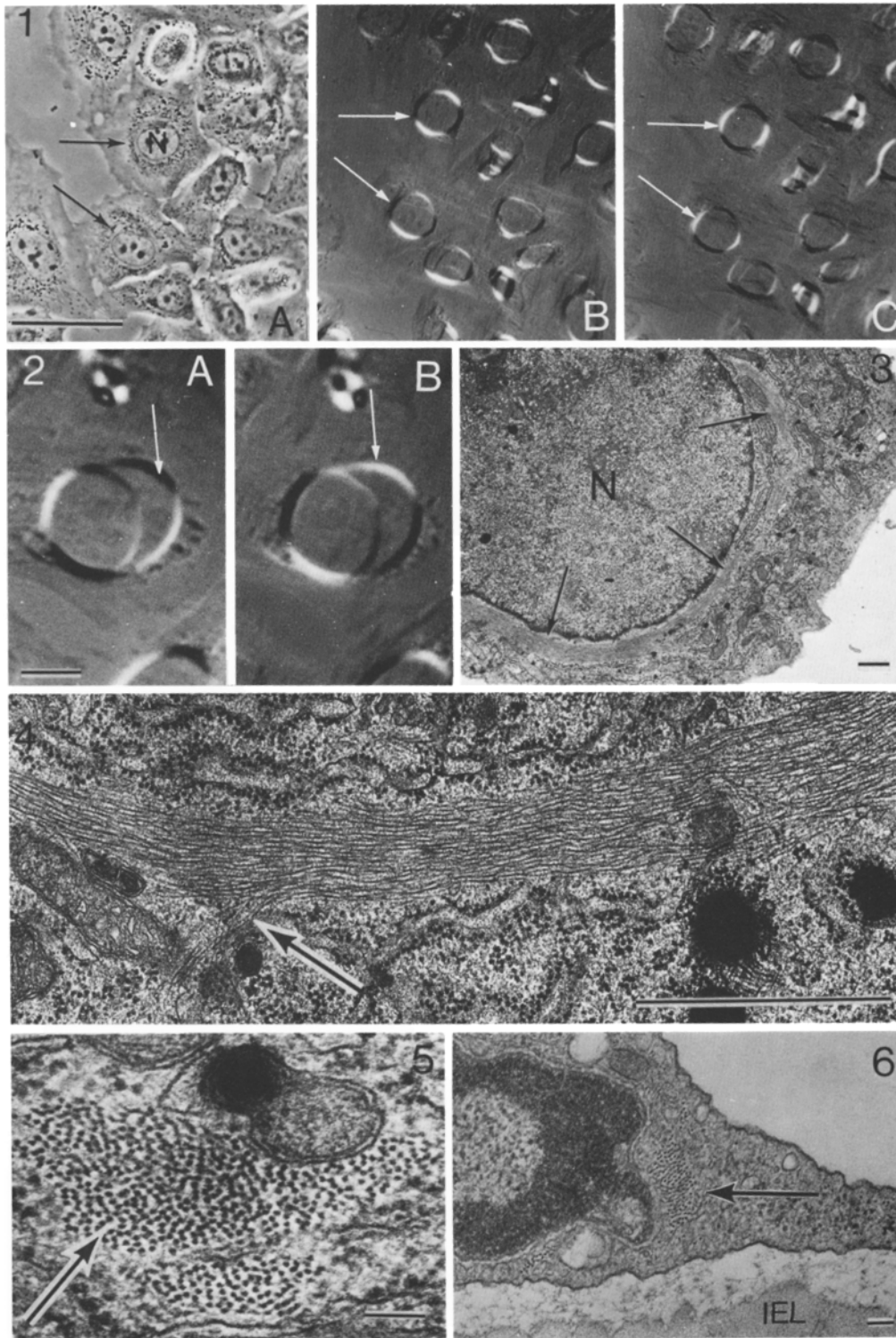
FIGURE 2 Polarization microscopy of vascular endothelial cells (A and B at opposite compensator settings). Occasionally, a second ring (arrows) was found to encircle the Golgi region. Bar is 8 μm. × 1140.

FIGURE 3 Electron micrograph of cultured endothelial cells sectioned *en face*. The arrows point to the perinuclear bundle of 100 Å filaments surrounding the nucleus (N). Bar is 1 μm. × 4800.

FIGURE 4 *En face* electron micrograph of the perinuclear 100 Å filaments. A small number of filaments could be found leaving the perinuclear bundle (arrow) and coursing into the cytoplasm. Bar is 1 μm. × 38,000.

FIGURE 5 Electron micrograph of cultured endothelial cell in cross section. The filaments of the perinuclear bundle (arrow) measure ~100 Å in diameter. Bar is 0.1 μm. × 90,000.

FIGURE 6 Electron micrograph of a section through the thoracic aorta fixed *in situ*. The endothelial cells also have a perinuclear bundle of 100 Å filaments (arrow). IEL, internal elastic laminae. Bar is 0.1 μm. × 44,000.



The presence of intermediate-sized filaments encircling the nucleus raises the following question: what happens to these filaments during mitosis? Cultures during the logarithmic phase of growth (3) were kept in an incubator built on an inverted phase microscope. The atmosphere was maintained at 37.5°C and 5% CO₂ in 95% air. Cells in various stages of mitosis were marked and processed for electron microscopy. Figs. 11 and 12 show the appearances of the filament bundles in anaphase and telophase, respectively. Time-lapse polarization cine-microscopy showed that the filament bundles remain throughout the entire mitotic process. The filament bundles appeared to be passively pulled between daughter cells. During mitosis, the intermediate-sized filaments did not disappear when large numbers of spindle apparatus microtubules assembled.

DISCUSSION

The present study makes use of endothelial cells that normally contain 100 Å filaments concentrated as a ring in the perinuclear region both in vivo and in vitro. Neither the function of these filaments nor the reason for their unique location in the perinuclear region is clear. Though these filaments are reported to be present in differentiating muscle, it is unlikely that they are related to the thick (myosin) or thin (actin) myofilaments (1, 17). Unlike the 60 Å actin filaments, the 100 Å filaments in endothelial cells seen in this report did not decorate with S-1. This is in accord with previous studies on 100 Å filaments (12, 18).

The effect of Colcemid or colchicine on other cell types is twofold: firstly, it prevents the polymerization of the tubulin dimer (16) into cytoplasmic and mitotic spindle microtubules, and, secondly, it causes an apparent increase in concentration of 100 Å filaments (6, 14, 17, 22, 28). From these observations, some investigators formed an implicit hypothesis that microtubules and 100 Å filaments may come from the same protein precursor pool (14, 19, 28). It is interesting to note in our studies that 100 Å filament bundles do not disappear during mitosis when large numbers of microtubules assemble. From this morphological evidence, it is possible that in endothelial cells the 100 Å filaments and tubulin do not share a common pool of precursor proteins.

DeBrabander et al. (8) have proposed that the effect of colchicine and vinblastine causes an in-

creased synthesis of 100 Å filaments. On the other hand, Croop and Holtzer (5) and Goldman and Knipe (12) have blocked protein synthesis with cycloheximide and still observed the formation of 100 Å filament pools by Colcemid or colchicine. In the endothelial cells, naturally occurring rings of 100 Å filaments disorganize and coil into a juxtannuclear cap in the presence of Colcemid.² This phenomenon occurs in the absence of protein synthesis. Goldman (11) has shown that cap formation (consisting of 100 Å filaments) in BHK-21 cells occurs when the cytoplasmic microtubules break down in the presence of colchicine. We cannot entirely rule out the possibility that mobilization of the 100 Å filaments from the perinuclear ring to the juxtannuclear cap was caused by loss of integrity of the cytoplasmic microtubule system. However, after looking at numerous endothelial cells, we have been unable to see microtubules associated with the filament rings. Therefore, Colcemid may exert a direct effect on these bundles. Working with the 100 Å neuro-filament, Yen et al. (29) and Davison and Huneus (7) have shown that the 100 Å filament protein subunit is distinct from tubulin. Furthermore, Shelanski et al.³ have shown that purified 100 Å neurofilament protein does not bind the antitubulin colchicine. The 100 Å filaments in endothelial cells may be different in spite of the fact that they are similar in diameter to neurofilaments. In endothelium, Colcemid may exert its effect by binding to the 100 Å filament protein or proteins associated with the filaments. Uehara, Campbell, and Burnstock (27) have proposed that tropomyosin may be associated with the 100 Å filaments. Tropomyosin might then play a role in maintaining these filament bundles in a ring configuration. Since the antitubulin vinblastine is known to cause the precipitation of tropomyosin (10), Colcemid *might* operate in a similar manner by removing and precipitating tropomyosin from the filament bundles, causing the ring to coil into the juxtannuclear cap. Work is now in progress to determine whether tropomyosin is indeed associated with 100 Å filaments in endothelial cells.

² We have also found that the cap will form in the presence of colchicine (10⁻⁶ M) and vinblastine SO₄ (10⁻⁶ M) (unpublished observations).

³ Shelanski, M. L., S. Albert, G. H. DeVries, and W. T. Norton, 1971. Isolation of filaments from brain. *Science (Wash. D. C.)*. **174**:1242-1245.

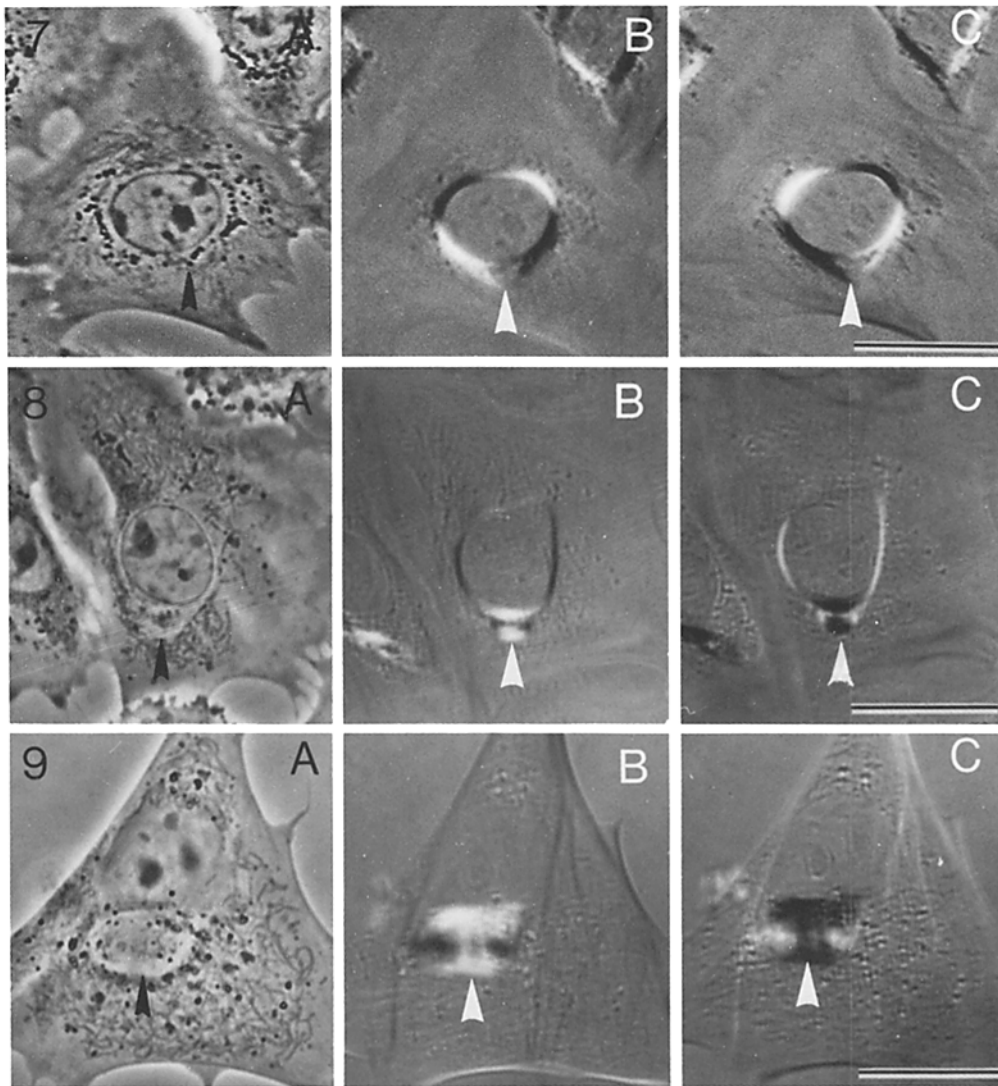


FIGURE 7 Living endothelial cells several minutes after being incubated with 10^{-6} M Colcemid. Arrowheads point to the perinuclear ring. (A), phase contrast; (B and C), polarized light at opposite compensator settings. Bar is $20\ \mu\text{m}$. $\times 960$.

FIGURE 8 Living endothelial cell at $t \approx 19$ h of incubation with 10^{-6} M Colcemid. The ring is coiling into an eccentrically placed cap (arrowheads). (A), phase contrast; (B and C), polarized light at opposite compensator settings. Bar is $20\ \mu\text{m}$. $\times 960$.

FIGURE 9 Living endothelial cell at $t \approx 24$ h of incubation with 10^{-6} M Colcemid. The perinuclear ring has completed coiling into the cap (arrowheads). Polarization microscopy (B and C) still demonstrates the birefringent nature of the cap. (A), phase contrast; (B and C), polarized light at opposite compensator settings. Bar is $20\ \mu\text{m}$. $\times 960$.

SUMMARY

Vascular endothelial cells cultured from guinea pig aorta or portal vein contain naturally occurring

bundles of $100\ \text{\AA}$ (diameter) filaments that completely encircle the nucleus. These rings are phase lucent and birefringent when examined with the light microscope. Perinuclear bundles of $100\ \text{\AA}$

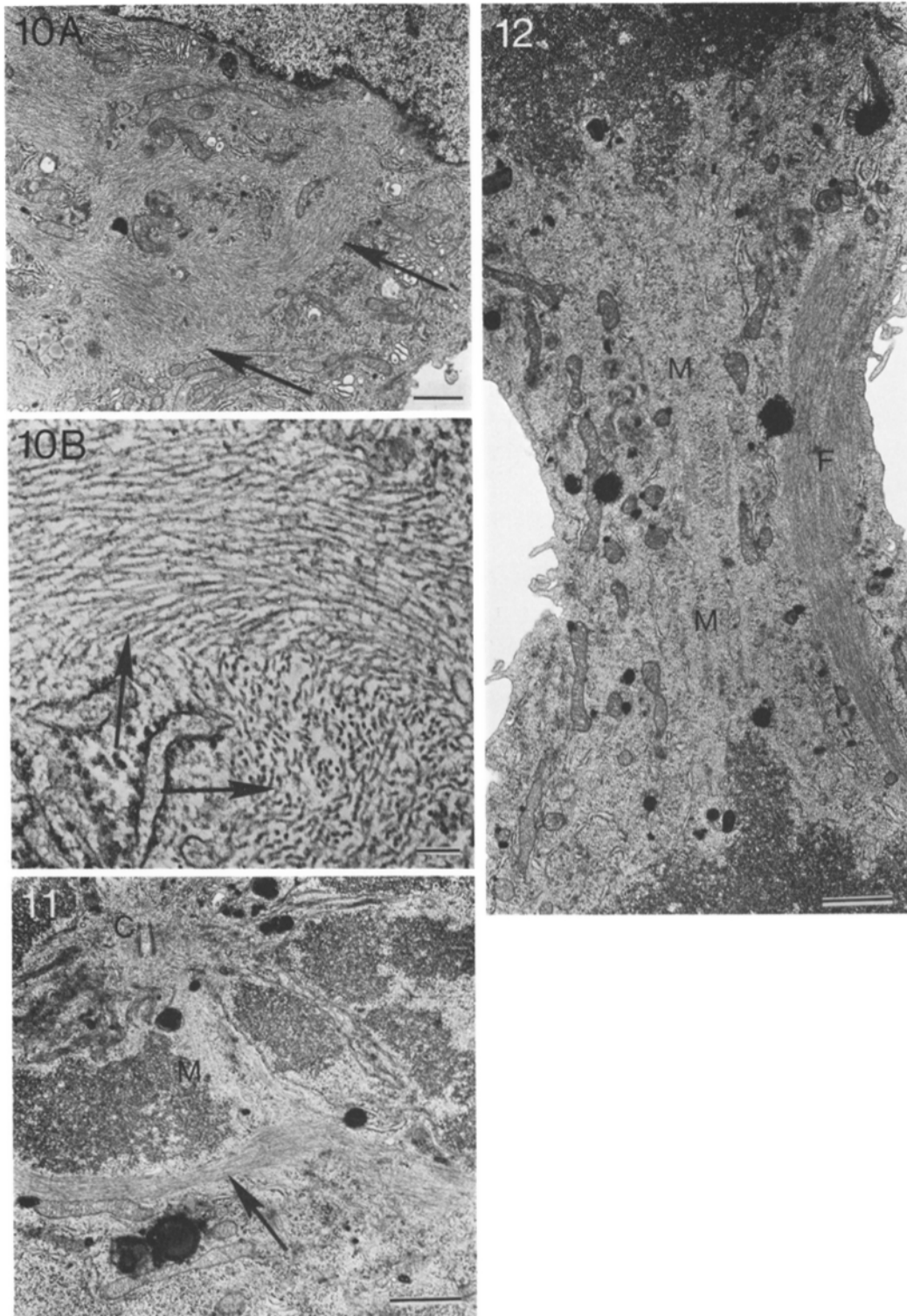


FIGURE 10 Electron micrographs of cultured endothelial cells incubated for 24 h in the presence of 10^{-6} M Colcemid. The perinuclear ring coiled into a juxtannuclear cap that consisted of disorganized arrays of 100 Å filaments (arrows). (A) Bar is $1 \mu\text{m}$. $\times 7110$. (B) Bar is $0.1 \mu\text{m}$. $\times 67,500$.

FIGURE 11 Electron micrograph of endothelial cell in anaphase. The 100 Å filament bundles (arrow) did not disappear when the microtubules (M) of the mitotic spindle assembled. C, centriole; bar is $1 \mu\text{m}$. $\times 11,000$.

FIGURE 12 Electron micrograph of endothelial cell in telophase. During mitosis, the 100 Å filament (F) bundles are passively pulled into the daughter cells. M, microtubules of the mitotic spindle. Bar is $1 \mu\text{m}$. $\times 10,560$.

filaments were also seen in endothelial cells in vivo, indicating that they are a normal cytoplasmic component. These filaments did not decorate with S-1, and were not disrupted by glyceination. With these cells, experiments were designed to answer the following questions: (a) does Colcemid have an effect on these naturally occurring bundles? and (b) do these filaments remain during cell division? Endothelial cells grown in the presence of Colcemid were followed over 24 h. The perinuclear ring coiled into a juxtannuclear cap that consisted of disorganized arrays of 100 Å filaments. This "coiling" effect was not blocked by cycloheximide, an inhibitor of protein synthesis. In another experiment, dividing cells were examined. During division the bundle of filaments is passively pulled in half into the daughter cells. These bundles did not disappear during mitosis when mitotic spindle microtubules assemble. These studies suggest that Colcemid may exert a direct effect on 100 Å filaments, independent of microtubules. Since these filaments do not disappear during mitosis, it is possible that in these cells the 100 Å filaments and tubulin do not share a common pool of precursor proteins.

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