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Structure of cytomatrix and nuclear matrix revealed by embedment-free electron microscopy

Barbara Gajkowska[1], **Marcin Cholewiński**[1] and **Robert Gniadecki**[2]

[1]The Laboratory of Cell Ultrastructure, Medical Research Centre, Polish Academy of Sciences, 5 Pawiński St., 02-106 Warsaw, Poland, Email: gajk@cmdik.pan.pl; [2]Department of Dermatology, University of Copenhagen, Bispebjerg Hospital, Denmark

Abstract. Embedment-free electron microscopy (EFEM) is a new method which allows the visualisation of cytoskeleton in whole-mounted cells. In this study we employed EFEM to investigate the structure of cellular scaffolds in glioma C6 cell line. The cells were extracted with Triton X-100 that dissolves phospholipids in the membranes and removes most of cytoplasmic soluble proteins. The DNA and nuclear histones were removed with DNase I and high-salt buffer, respectively. The remaining cellular frameworks were temporary embedded in diethylene glycol distearate (DGD), sectioned and observed in transmission and scanning electron microscope after the removal of DGD. The predominant structure was the extensive meshwork of 10-20 nm filaments in the cytoplasm (cytomatrix) and 15-30 nm filaments in the nucleus (nuclear matrix). The 5 nm filaments, presumably corresponding to the actin filaments, were present in the cytomatrix, but not in the nuclear matrix. Moreover, the ultrathin (3 nm) filaments, connecting other cytoskeletal components were detected. Those are possibly identical with the previously described plectin filaments. For the first time we report the occurrence of ultrathin filaments in the nuclear matrix. Thus, in a addition to the well known cytoskeletal components (microtubules, intermediate filaments, actin microfilaments) EFEM showed a new type of filaments (the ultrathin filaments) in the cytomatrix and nuclear matrix. Further immunocytochemical studies are needed to determine the biochemical identity of the filaments observed in EFEM.

Key words: embedment-free electron microscopy, cytoskeleton, nuclear matrix, cytomatrix

INTRODUCTION

The structural organization of the cell is accomplished by a network of filaments forming the cytoskeleton. Immunofluorescence staining with antibodies directed against known cytoskeletal components reveal a three-dimensional lattice of microtubules, intermediate filaments or actin microfilaments. However, it has been difficult to determine the mutual structural interactions between those filaments because in classical electron microscopy they are largely invisible since their electron density is similar to that of other soluble proteins and the embedding medium (Penman 1995). Therefore, the cytoplasm of most cells appears homogenous with scattered organelles and very few cytoskeletal components.

Special electron microscopy techniques, such as rotary shadowing electron microscopy or freeze-etching revealed quite a different cytoplasmic structure where entire cell interior was filled with a dense framework of branched filaments of different thickness (Heuser et al. 1980, Bridgeman and Reese 1984, Ris 1985, Bohn et al. 1993). This structure has been referred to as the cytomatrix or the microtrabecular network (Schliwa 1986). The classic cytoskeletal components (microtubules, intermediate filaments and microfilaments) are a part of the cytomatrix but comprise only 10-15% of its protein content (Penman 1995). Other filament types were described, for example the ultrathin 3 nm filaments interlinking other cytoskeletal components (Roberts 1987). Despite this evidence, the concept of cytomatrix and the existence of ultrathin filaments have been hotly debated. Some researchers claimed that those structures are fixation artefacts due to protein cross-linking or artificial protein aggregates formed in the extraction buffers of non-physiologic ionic strength (Schliwa 1986).

The concept of nuclear matrix has been even more controversial than that of cytomatrix. Transmission electron microscopy has not revealed any filamentous nuclear structure. Immunocytochemical techniques failed to identify the presence of the intermediate or microfilaments in interphase nuclei. First evidence suggesting that a filamentous scaffold exists in the nucleus came from the studies of Bernhard employing the EDTA regressive staining for electron microscopy (Bernhard 1969). Later, Berezney and Coffey (1974, 1977) isolated rat liver nuclear matrix by removing DNA with the nuclease and extracting soluble proteins with high-salt buffer and non-ionic detergent. Similar nuclear scaffolds were also isolated by Laemmlis group by lithium 3,5-diiodosalicylate extraction (Mirkovitch et al. 1984). Jackson and Cook (1988) prepared nuclear matrix by digesting the chromatin with nuclease and its subsequent electroelution in isotonic salt concentrations.

Recently, the group of Penman refined a technique of embedment-free electron microscopy (EFEM) (Fey et al. 1986). Whole cells are extracted with the non-ionic detergent which dissolves the membranes and allows the soluble proteins to diffuse away. DNA is removed by digestion with nuclease. What is left is the non-soluble cytomatrix and nuclear matrix. Such cellular scaffolds are embedded in a temporary medium (diethylene glycol distearate, DGD) and sectioned. DGD is removed before viewing in electron microscope which obviates the problem of a similar electron density of the resin and the filaments. Using EFEM it was able to visualise nuclear matrix as a highly branched trabecular network decorated with globular ribonucleoprotein structures (Fey et al. 1986, Nickerson et al. 1989).

In this study we applied EFEM to study the cytomatrix and nuclear matrix in glioma C6 cell line. We were able to confirm the presence of ultrathin filaments in the cytomatrix. For the first time we describe the existence of those filaments in the nuclear matrix. Moreover, structural interactions between the cyto- and nuclear matrix in nuclear envelope are described.

METHODS

Chemicals

Diethylene glycol distearate (DGD) was obtained from Polysciences Inc (Warrington, PA). Other chemicals were purchased from Boehringer Mannheim GmbH (Germany), Sigma (St. Louis, MO) and Fluka Chemie AG (Germany).

Cell culture

C6 glioma cell line (P-72-P86) was obtained initially from ATCC and cultured on plastic Petri dishes (Corning, N.Y.) in the Eagle modified Dulbeccos essential medium supplemented with 2 mM glutamine, 10% foetal calf serum and the antibiotics (50 U/ml penicillin, 50 mg/ml streptomycin) at 37°C, 5% CO2 in the humidified incubator. The cells were passaged every 2-3 days.

Cell extraction and fixation

Cells were released by trypsinisation at 37°C for 10 min. After the neutralisation of the enzyme with the culture medium the cells were centrifuged for 8 min 100 g at 4°C and washed once in cold PBS. Cell extraction was performed similarly to the previously described protocol (Nickerson et al. 1994). The material was extracted at 4°C for 3 min with the cytoskeleton buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 20 mM vanadyl riboside complex, 1 mM aminoethylbensenesulfonyl fluoride). At this step the membrane phospholipids become dissolved by Triton X-100 which enables passive diffusion of soluble proteins. After peletting by centrifugation at 500 g for 3 min, the remaining of the soluble proteins was extracted from the skeletal frameworks with the extraction buffer (10 mM PIPES, pH 6.8, 250 mM ammonium sulphate, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 20 mM vanadyl riboside complex, 1 mM aminoethylbensenesulfonyl fluoride) for 5 min at 4°C. The remaining structure was separated from the eluted soluble protein by centrifugation as above. The DNA from the chromatin was removed by incubation for 50 min at 32°C in the digestion buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 20 mM vanadyl riboside complex, 1 mM aminoethylbensenesulfonyl fluoride, 200 units/ml RNase free DNase I). Histone proteins and other soluble nuclear proteins were removed with the high salt buffer (2 M NaCl, 10 mM PIPES, pH 6.8, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 20 mM vanadyl riboside complex, 1 mM aminoethylbensenesulfonyl fluoride) for 5 min at 4°C.

Embedment-free electron microscopy (EFEM)

The extracted nuclear scaffolds were fixed in 2.5% glutaraldehyde in the cytoskeletal buffer for 40 min at 4°C. The fixed material was washed in 0.1 M. sodium cacodylate buffer, pH 7.2 and postfixed with 1% OsO_4 in the same buffer for 30 min at 4°C. After washing in the cacodylate buffer the structures were dehydrated through the increasing ethanol concentrations. The speciments for scanning electron microscopy were dried in 100% ethanol in a critical-point drier and slightly coated with gold. For transmission electron microscopy the frameworks were embedded in DGD, as described in detail elsewhere (Nickerson et al. 1994). The blocks were cut using glass knives at angle of 10° on LKB Ultracut microtome (Reichert, Germany) into 300 nm sections. The specimens were placed on carbon-coated copper and nickel grids which were additionally coated with poly-L-lysine in order to increase the adhesiveness of the sections. DGD was removed by immersing the grids in isobutanol at room temperature for overnight. For final sample preparation the grids with sections were dried in hexamethyldisilazane (HMDS), which is a solvent with very low surface tension. The samples were transferred to a 1:1 (v/v) mixture of ethanol with the dewaxing solvent for 5 min, then to three changes of 10 min each of 100% ethanol and a 1:1 (v/v) mixture of ethanol and HMDS for 5 min and finally to three changes of pure HMDS, for 10 min each time. The grids were placed on filter paper to air-dry. Transmission and scanning micrographs were taken with JEOL 1200 EX microscope at 80 kV.

RESULTS

EFEM revealed a dense filamentous meshwork filling the cytoplasmic and the nuclear areas (Figs. 1 and 2). Within the cytoplasmic territory the scaffold consists of relatively thick (10-20 nm), branched fibrils, a smaller amount of 5 nm microfilaments and short (40-200 nm length), very thin (3 nm) fibres. The former two types of filaments may correspond to the intermediate vimetin filaments and actin microfilaments, respectively. Those fibres were interconnected by the ultrathin 3 nm filaments (Fig. 3). The ultrathin filaments are similar to the plectin filaments previously observed in rotary shadowing electron microscopy (Foisner et al. 1995).

The cytoplasmic microtrabecular lattice is not homogenous but is arranged into a higher-order network apparently due to the modelling around the organelles (Fig. 4). It is conceivable that the purpose of such structures is to fasten the organelles in fixed positions within the cytoplasm and possibly to provide extra rigidity to the cells.

In the cytoplasmic processes of glioma cells, elongated 5 nm filaments supported on a 10-20 nm scaffold was observed (Fig. 5).

Figure 6 shows a high magnification of a section through the nuclear envelope. It consists of a very dense meshwork of thick filaments with very few, if any, microfilaments or ultrathin filaments. Those filaments are slightly thicker than those in the cytoplasm (15-30 nm). From the cytoplasmic side the envelope merges

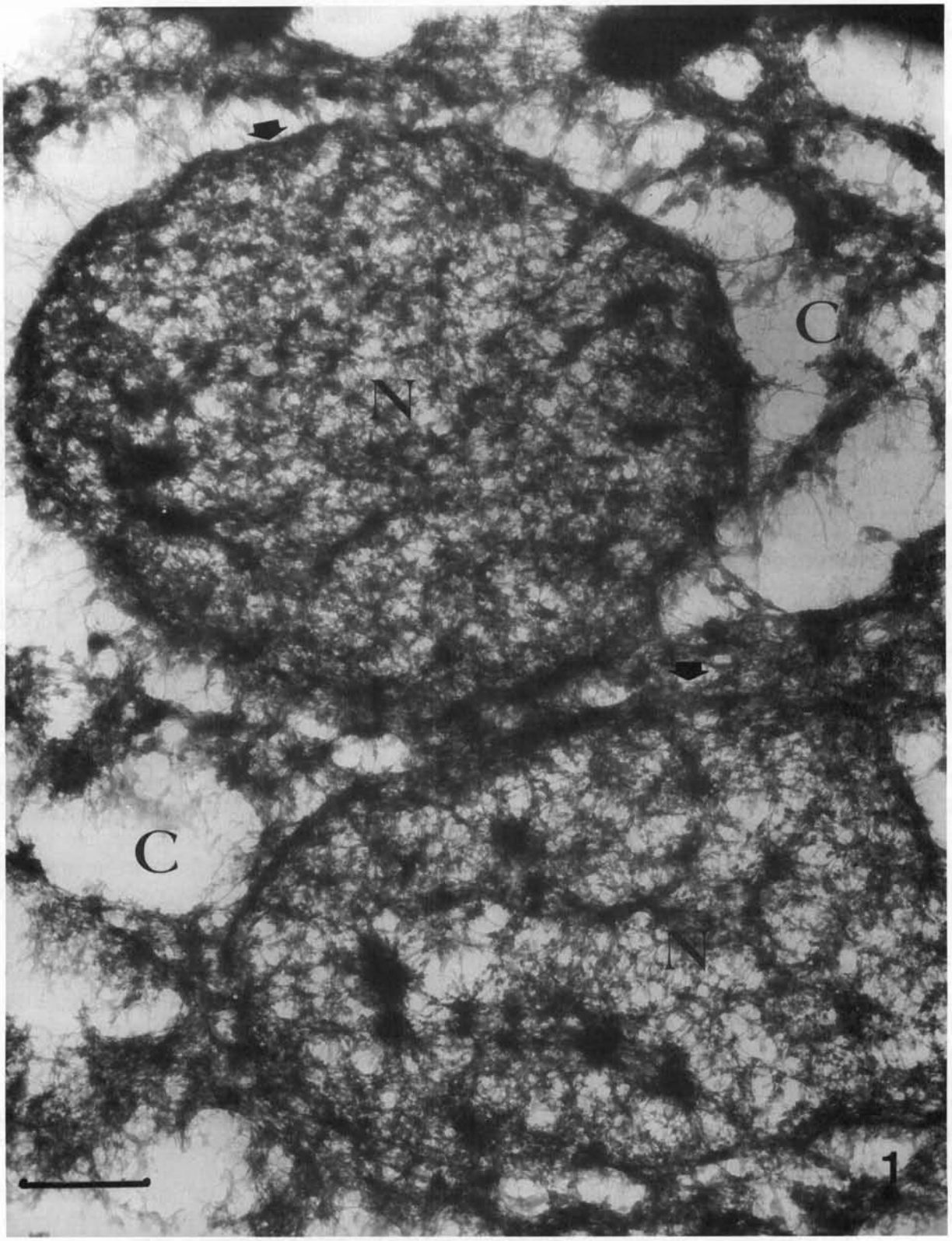

Fig. 1. Transmission embedment-free electron micrographs of cellular scaffolds of glioma C6 cell line. The cells were grown on Petri dishes, trypsinised and extracted as described in Methods. N, nuclear scaffold; C, cytomatrix; arrow, nuclear envelope. Magnification (22.500. Bar = 1 μm.

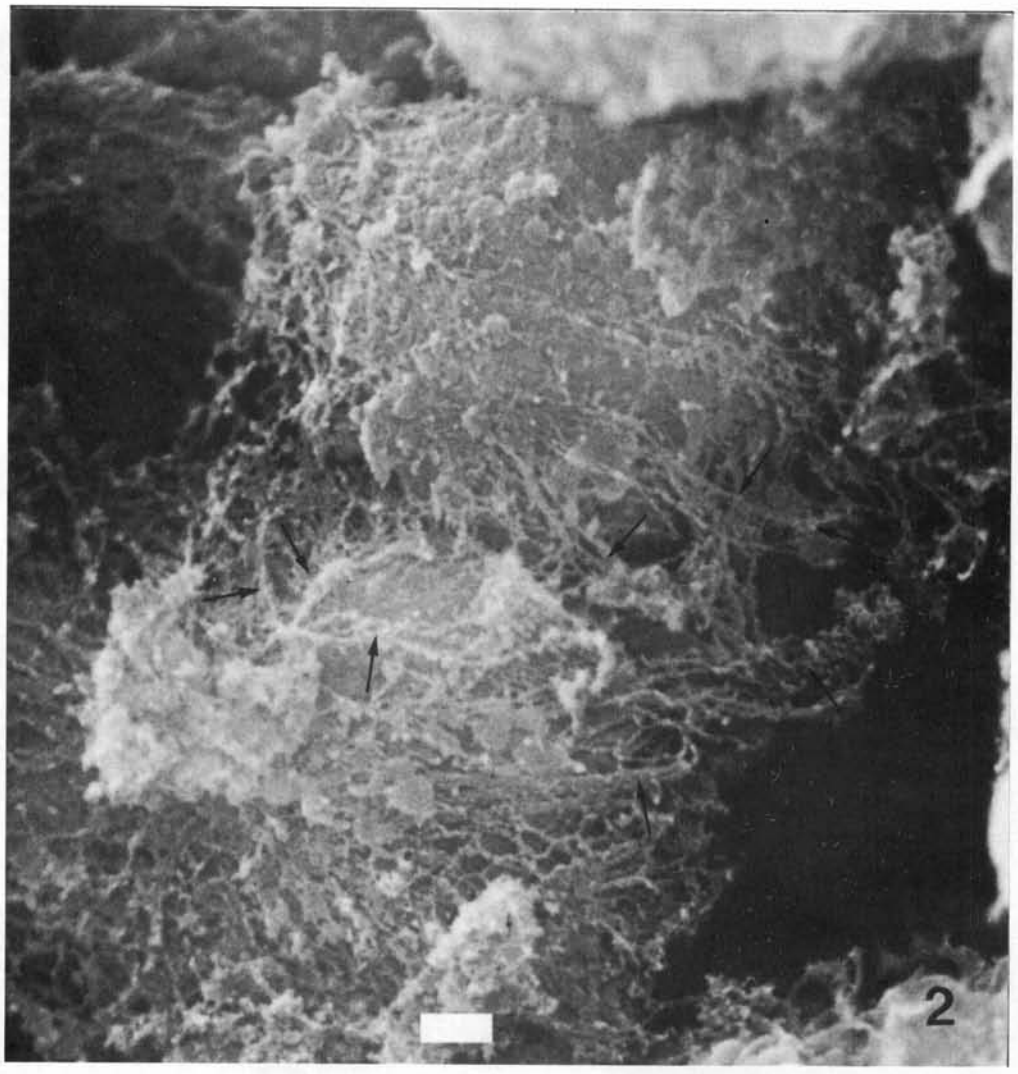

Fig. 2. Scanning electron microscopy of extracted whole glioma C6 cells. Note dense cytomatrix extending into the cytoplasmic processes (arrow). Magnification x 15.000. Bar = 1 μm.

with the 10-20 nm filamentous network. Sparse microfilaments connecting nuclear envelope with the rest of cytomatrix could be seen (Fig. 3).

On the side facing the interior of the nucleus, the envelope meshwork merges with the internal nuclear scaffold. The latter consists of thick, densely woven, branched filaments. On high-power images the ultrathin filaments could be detected. Similarly to the cytomatrix, they connected the adjacent 15-30 nm filaments (Figs. 6 and 7).

DISCUSSION

In the present study we investigated the ultrastructure of cytomatrix and nuclear matrix of C6 glioma cells. This cell line provides an ideal model for the studies on cytoskeleton, since its biochemical composition and structure has been investigated extensively (Koszka et al. 1985, Röser et al. 1991, Bohn et al. 1993, Foisner et al. 1995). The immunofluorescence studies revealed an extensive fibrillar network of vimetin intermediate filaments and 5-7 nm actin microfilaments. A recent whole-mount rotary shadowing electron microscopy study revealed the presence of ultrathin filaments (150-200 nm in length, 3 nm in diameter), cross-linking vimetin filaments and composed mainly of plectin (Foisner et al. 1995). Those filaments are likely to constitute a fourth class of cytoskeletal filaments, in addition to the well known microtubules, intermediate filaments and microfilaments. The existence of ultrathin filaments

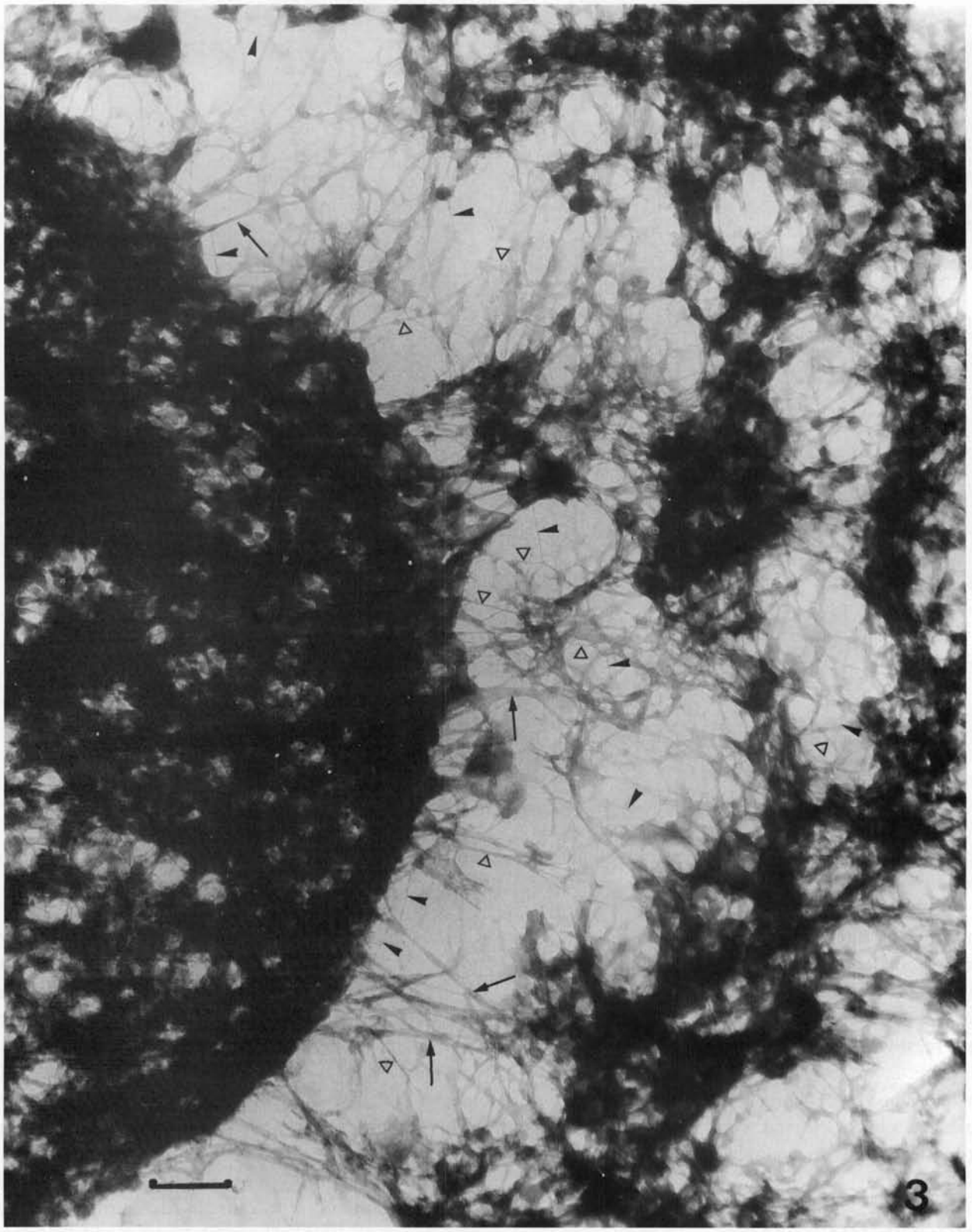

Fig. 3. Transmission EFEM microphotograph showing a fragment of cytomatrix adjacent to the nucleus. Arrows–thick, 10-20 nm filaments; open arrowheads, 5 nm microfilaments; arrowheads, 3 nm ultrathin filaments. Magnification x 60.000. Bar = 200 nm.

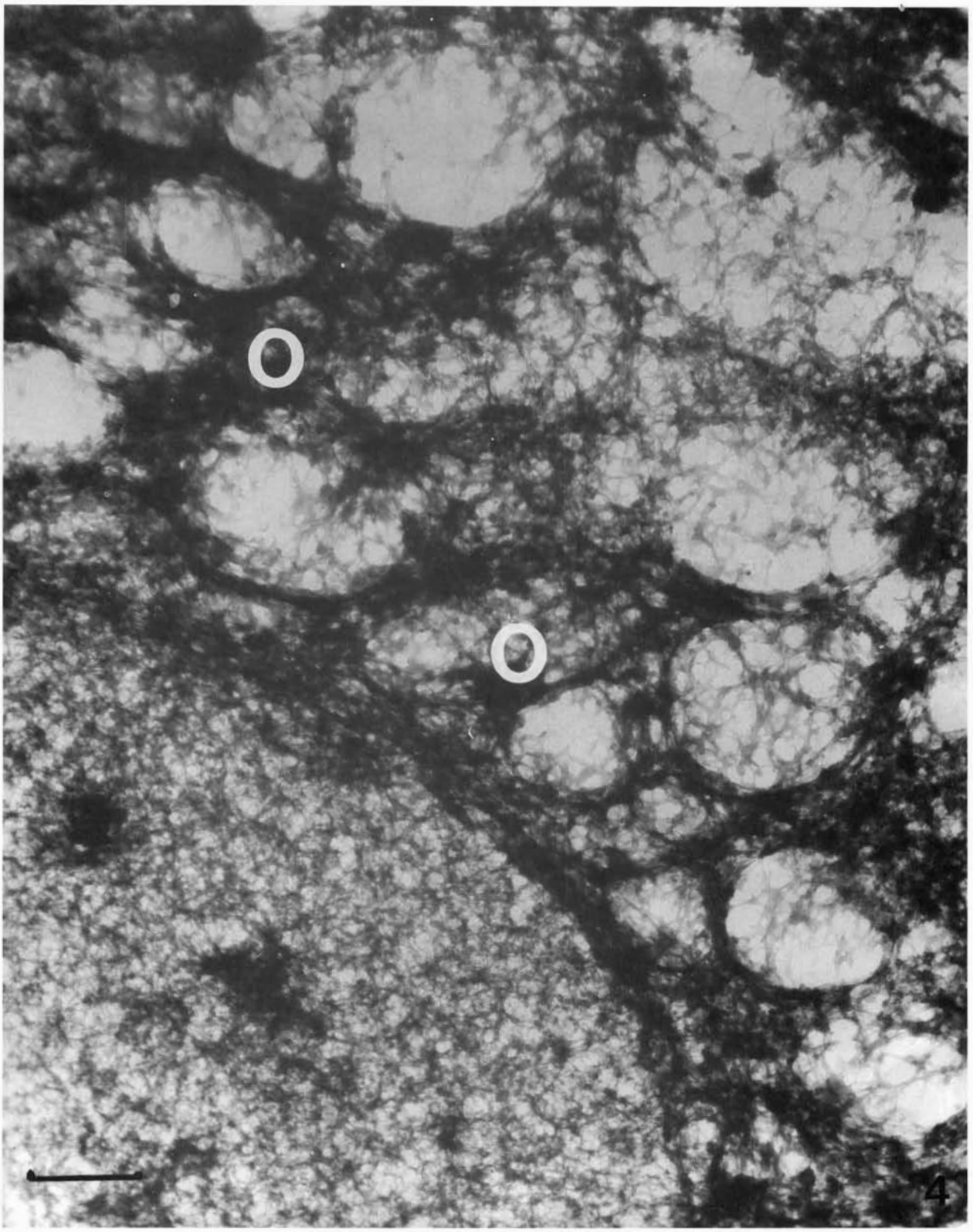

Fig. 4. Transmission micrograph of a resinless section of extracted glioma cells. Note a mesh in a net pattern due to a modelling of cytomatrix around the spaces occupied by cytoplasmic organella (O). The organella have been removed during extraction process. Magnification x 36.000. Bar = 500 nm.

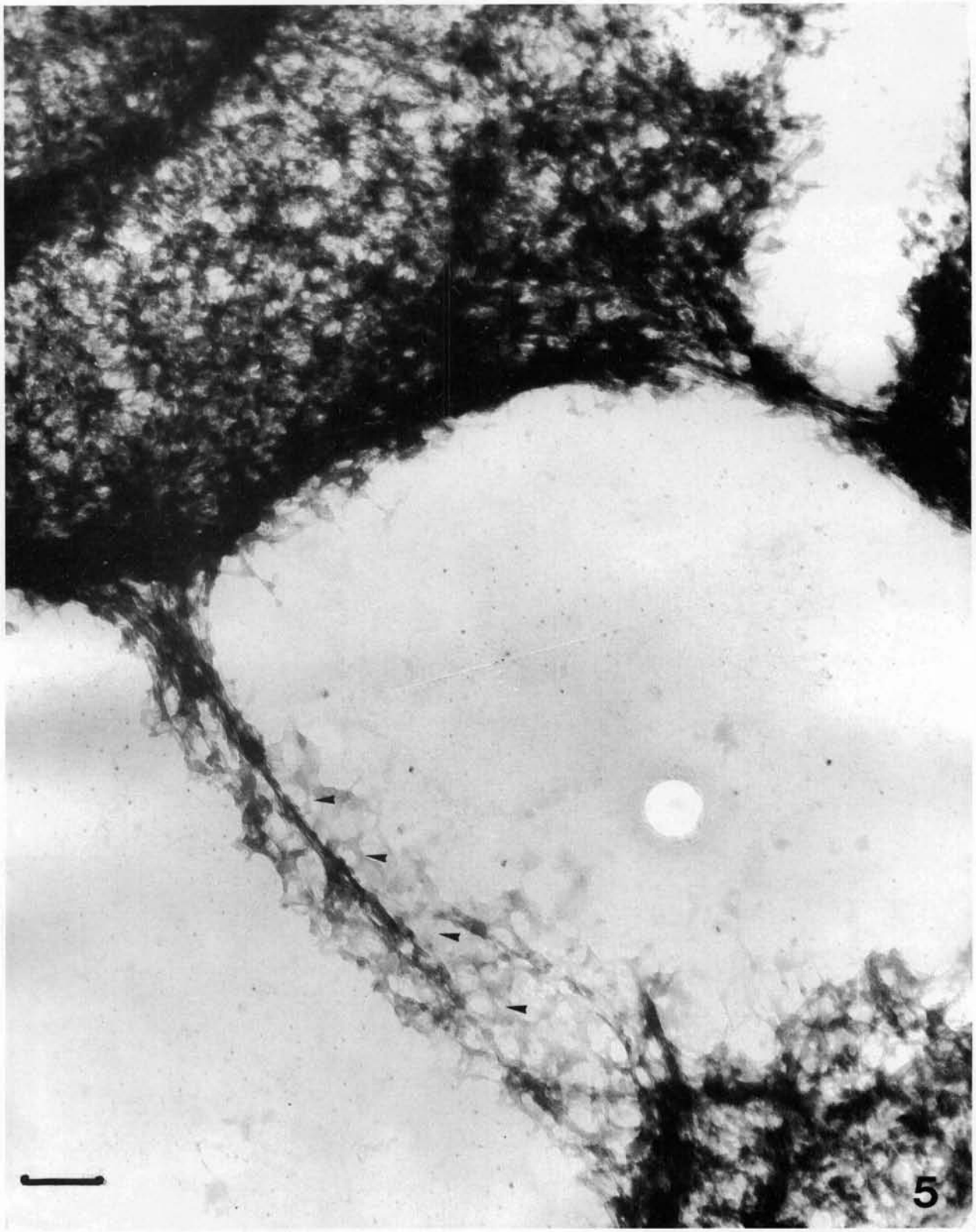

Fig. 5. Transmission EFEM micrograph showing the structure of cytomatrix within the cytoplasmic process. Note a central bundle of microfilaments (arrowheads) supported by a network of 10-20 nm filaments. Magnification x 60.000. Bar = 200 nm.

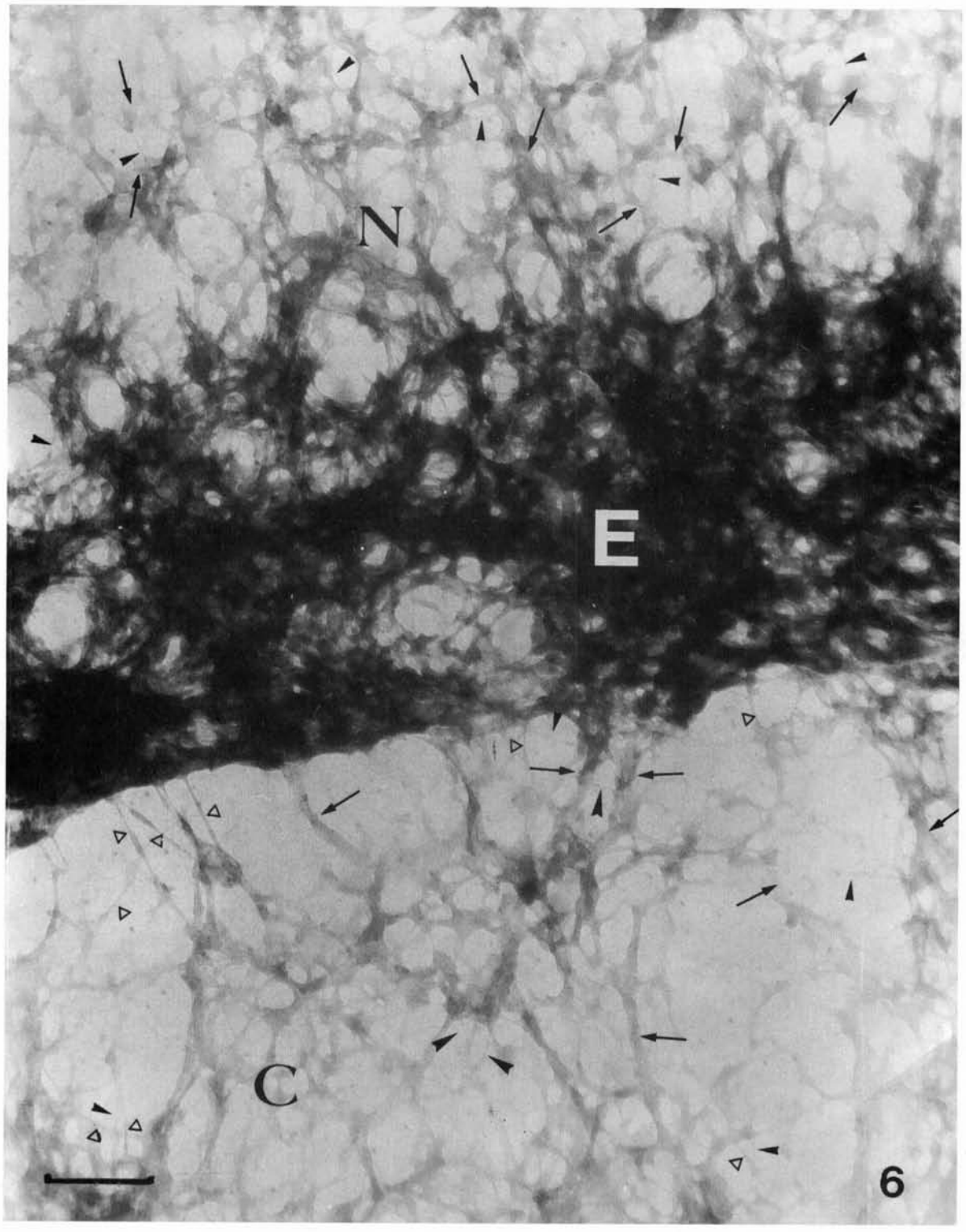

Fig. 6. Transmission EFEM in the region of nuclear envelope. On the cytoplasmic side (C) the cytomatrix is mainly composed of 10-20 nm branched filaments (arrows), the 5 nm microfilaments (open arrowheads) and the 3 nm ultrathin filaments (solid arrowheads). The nuclear envelope (E) contains very densely packed, branched filaments and is devoid of microfilaments or the ultrathin filaments. The inner nuclear matrix (N) contains predominantly the branched filaments, somewhat thicker that those seen in the cytomatrix (15-30 nm thick, arrows) interconnected by the ultrathin filaments (arrowheads). Magnification x 90.000. Bar = 200 nm.

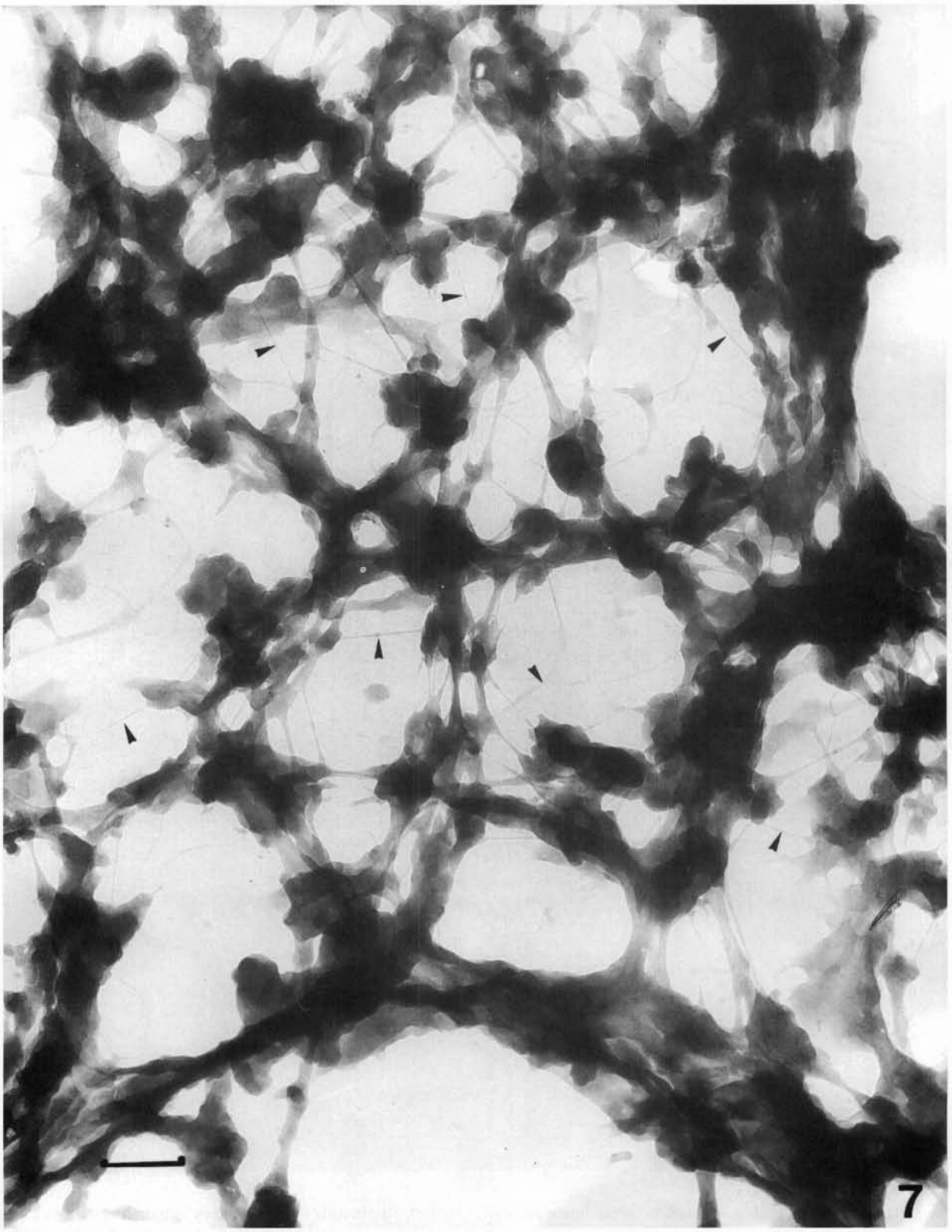

Fig. 7. Transmission EFEM of nuclear matrix. Note ultrathin filaments (arrowheads) connected with filaments in nuclear matrix. Magnification x 120. Bar = 200 nm.

could be confirmed in our study employing a different technique - EFEM. In contrast to the rotary shadowing microscopy where formaldehyde and glutaraldehyde cells are used, the cells for EFEM are extracted from soluble proteins, chromatin and phospholipids before fixation. This reduces the possibility of obtaining artefactual structures due to accidental cross-linking of soluble proteins into macromolecular conglomerates.

EFEM enabled us to investigate the ultrastructural details of the interface between the nuclear envelope and the cytomatrix. The nuclear envelope consisting of a very tight meshwork of 15-30 nm branched filaments merges on the cytoplasmic side with the intermediate filament network. This feature is also visible in light microscope after fluorescent staining with the anti-vimetin antibody (Foisner et al. 1995). Microfilaments seem to interact with the nuclear scaffold only sporadically.

The general structure of the nuclear matrix resembled that of the cytomatrix. One of the differences was the predominance of short, thick, 15-30 nm filaments forming a relatively dense, homogenous network. This structure is similar to the fibrous component visualised by the EDTA regressive staining (Monneron and Bernhard 1969), the filament system obtained after electroelution of enzymatically degraded DNA (Jackson and Cook 1988), or finally the core filaments described by Penmans group in other cell types (Nickerson et al. 1995, Nickerson et al. 1997, Wan et al. 1999). Interestingly, the ultrathin interconnecting filaments similar to these seen cytomatrix were also part of the internal nuclear lattice.

The biochemical identity of nuclear matrix filaments has not been satisfactorily resolved. Typical cytoplasmic proteins such as vimetin, desmin, actin, are not present in the nucleus (Nickerson et al. 1995). Other proteins, such as topoisomerase II, RNA polymerase II, several protein kinases, e.t.c. have been detected by immunogold labelling of nuclear scaffolds. This has led many researchers to consider that the nuclear matrix represents a functional feature, apparent only after chromatin removal and stabilisation or cross-linking of various enzymatic and regulatory proteins (Pederson 1998). However, according to the competing view, the nuclear filaments exist autonomously and are built of various structural proteins such as the matrins in the internal nuclear lattice and the lamins in the nuclear envelope (Fey and Penman 1988, Berezney et al. 1995). These considerations are important when studying the nature of the nuclear ultrathin filaments. There is some indirect evidence that these filaments may be primarily structural. Immunofluorescence analysis of Feisner et al. (1995) of C6 glioma cells revealed the presence of plectin, but not actin or vimetin, in the nucleus. Thus it is conceivable that although different proteins constitute intermediate filaments in cytomatrix and nuclear matrix, the same protein (plectin) is involved in cross-linking and stabilisation of those filaments. Immunogold studies that are feasible in EFEM could resolve this issue. However, we cannot exclude the possibility that the observed ultrathin filaments are not truly autonomous structures but occur due to protein condensation in the high-salt buffer. In such a case they could constitute a morphologic manifestation of assemblies of some regulatory proteins, putatively involved in the maintenance of matrix homeostasis. This issue is difficult to resolve with the currently available electron microscopy techniques. Even the recently refined EFEM techniques (Nickerson et al. 1997, Wang et al. 1999) still require chromatin extraction and chemical modification of the non-soluble proteins such as formaldehyde cross-linking or amine modification that may lead to the occurrence of artefacts.

In conclusion, EFEM can be considered to be a useful technique for visualisation of cytomatrix and nuclear matrix. In contrast to the traditional views considering the nucleus as an organelle having an autonomous matrix component, we show that a continuous, dense filamentous network is present within the cells without clear-cut morphological differences between the nuclear matrix and the cytomatrix. Moreover, we confirm the existence of the highly controversial ultrathin fibrils cross-linking other filaments in the cytoplasm and reveal the presence of a similar structure in the nuclear matrix.

ABBREVIATIONS

DGD - diethylene glycol distearate
EFEM - embedment-free electron microscopy

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