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Authors

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COMPARATIVE SCANNING, TRANSMISSION AND ATOMIC FORCE MICROSCOPY OF THE MICROTUBULAR CYTOSKELETON IN FENESTRATED LIVER ENDOTHELIAL CELLS

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Abstract

Endothelial fenestrae control the exchange of fluids, solutes and particles between the sinusoidal lumen and the microvillous surface of the parenchymal cells. Fenestrae have a critical dimension in the order of 150-200 nm, making it necessary to use microscopes with a resolution better than the light microscope. Comparative whole-mount preparations of isolated, purified and cultured rat liver sinusoidal endothelial cells (LEC) were studied by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM). Examination of detergent-extracted LEC by SEM and TEM shows an integral cytoskeleton: sieve plates are delineated by a sieve plate-associated cytoskeleton ring and fenestrae by a fenestrae-associated cytoskeleton ring. By using microtubule altering agents we could demonstrate: (1) the architectural role of microtubules in arranging fenestrae, (2) the existence of a population of microtubules resistant against low temperature and colchicine, (3) the ability of LEC to shift the microtubule assembly-disassembly steady state under various conditions, (4) and the necessity of an intact microtubular cytoskeleton to support the increase in the number of fenestrae after cytochalasin B. Topographical examinations of AFM images revealed that sieve plates are delineated by elevated borders, probably projections of the underlying tubular cytoskeleton.

Key Words: liver, endothelium, fenestrae, sieve plates, cytoskeleton, cytochalasin B, taxol, colchicine, microtubules, fenestrae-associated cytoskeleton, electron microscopy, atomic force.

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Introduction

In 1970, the first description and electron microscopic observation of sinusoidal liver endothelial cell (LEC) fenestrae was made by Wisse [40]. The application of perfusion fixation of the rat liver revealed the presence of groups of fenestrae arranged in sieve plates. In subsequent reports, Widmann [38] and Ogawa [31] verified the existence of fenestrae in LEC by using transmission electron microscopy (TEM). The liver sinusoids can be regarded as unique capillaries which differ from other capillaries, because of the presence of fenestrae lacking a diaphragm and a basal lamina underneath the endothelium [40]. Knook *et al.* [11, 24, 25] pioneered the isolation and cultivation of LEC and they described the presence of fenestrae in freshly isolated and cultured LEC by TEM and scanning electron microscopy (SEM). In the following years, the number of reports about the ultrastructure of LEC fenestrae increased enormously [for an extended review, see reference 12].

On the basis of morphological and physiological evidence, several authors reported that the grouped fenestrae act as a dynamic filter [13, 15, 29]. Fenestrae filter fluids, solutes and particles that are exchanged between the sinusoidal lumen and the space of Disse, allowing only particles smaller than the fenestrae to reach the parenchymal cells or to leave the space of Disse [for an extended review, see reference 42]. Interestingly, the diameter and the number of fenestrae can be influenced by different agents, such as ethanol [10], serotonin [41], nicotine [16], endotoxin [14] and cytochalasin B [34]. Cytochalasin B is the most frequently described agent regulating LEC fenestrae [4, 28, 30, 34, 36]. Cytochalasin B which acts on the actin cytoskeleton increases the frequency of fenestrae enormously, as was first reported by Steffan *et al.* [34]. They found that the cytoskeleton is involved in the fenestrae dynamics [for a review, see 1]. In the last decennium, several investigators have postulated hypotheses about the dilatation and contraction of fenestrae. Briefly, the presence of an calcium-calmodulin-actomyosin system is

Abbreviations used: EGTA; ethylene glycol bis [2-aminoethylether]-N,N,N',N' tetra-acetic acid, LEC; rat liver sinusoidal endothelial cells, PIPES; piperazine-N,N'-bis [2-ethanesulfonic acid], SEM; scanning electron microscopy, TEM; transmission electron microscopy, AFM; atomic force microscopy.

postulated to be responsible for the dynamics of LEC fenestrae [17, 19]. However, it remains to be elucidated whether the LEC cytoskeleton is involved in the regulation of fenestrae diameter and how the cytoskeleton is involved in the fenestrae formation. By using whole mount TEM we described that fenestrae are surrounded by a fenestrae-associated cytoskeleton ring which alters in diameter after different treatments also known to change fenestrae diameter [6]. We also postulated that microtubules stabilize sieve plates and reported that cytochalasin B treatment changes the organization of microtubules.

It was the purpose of this study to investigate the cytoskeleton of LEC, especially with regard to microtubules and their relation to fenestrae, by using detergent-extracted whole mounts [6] of cultured LEC [5]. Atomic force microscopy (AFM) was used to obtain topographical data of the fenestrated areas and surrounding cytoplasm.

Materials and Methods

Isolation, purification and culture of LEC

Male Wistar rats (weighing approximately 250 g) were fed a standard diet *ad libitum*. The method for the isolation of LEC has been described earlier [5], and was based on the method by Smedsrød *et al.* [33]. Briefly, the liver was perfused with collagenase A (Boehringer Mannheim Biochemica, Belgium). After incubation of the fragmented tissue in the same solution, the resulting cell suspension was centrifuged at 100 x g for 5 minutes to remove the parenchymal cells. The supernatant, containing a mixture of sinusoidal liver cells, was then layered on top of a two step Percoll® gradient (25-50%) and centrifuged for 20 minutes at 900xg. The intermediate zone, located between the two density layers was enriched in LEC. LEC purity was further enhanced by selective removal through adherence of the Kupffer cells and spreading on collagen of the LEC. LEC were further cultivated in 24-multiwell plates on collagen-coated nickel grids (300 mesh) for TEM. Formvar (1%) coated nickel grids (300 mesh) were used, later coated with diluted collagen. 10 µl of Collagen-S stock solution (Boehringer Mannheim, Belgium), was diluted with 900 µl sterile water. For SEM, LEC were cultivated on collagen-coated thermanox coverslips instead of nickel

grids. Serum free LEC culture medium consisted of RPMI-1640 with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10 ng/ml endothelial cell growth factor. The cultures were estimated to have > 95% purity, since less than 5% of the cells examined by SEM and TEM were devoid of fenestrae.

Scanning electron microscopy

LEC were rinsed twice with phosphate-buffered saline (PBS). To visualize the sieve plate-associated cytoskeleton [6], LEC were extracted for 1 min at 21°C in cytoskeleton buffer, consisting of 1 mM EGTA, 100 mM PIPES, 4% polyethylene glycol 6000 and 0.1% Triton X-100 in PBS at pH 6.9. After extraction, cells were fixed with 2% glutaraldehyde in 0.1M Na-cacodylate buffer with 0.1 M sucrose for 1 hour. Postfixation was done with 1% osmium tetroxide in 0.1 M Na-cacodylate for 1 hour. SEM samples were dehydrated in graded ethanol solutions, critical point dried and sputter coated with 10 nm gold. The samples were examined with a Philips SEM 505 (Philips Eindhoven, The Netherlands) at an accelerating voltage of 30 kV.

To study the effect of colchicine or taxol, we treated the cells with 200 µM colchicine (Sigma Chemicals, C3915, Germany) [39] for 2 hours and counted the number of microtubules in relation to the number of fenestrae and sieve plates or with 10 µM taxol (Sigma Chemicals, T7402, Germany) [22] for 2 hours. In order to determine whether the microtubular cytoskeleton is involved in the increase of fenestrae caused by cytochalasin B [6], we treated LEC with colchicine or taxol as described above, followed by cytochalasin B treatment at a concentration of 20 µM for 2 hours (Sigma Chemicals, C6762, Germany) [34]. Taxol and cytochalasin B were dissolved with dimethyl sulfoxide (DMSO) and then diluted with serum free LEC culture medium. The DMSO concentration in the assay was ≤0.4% and these levels had no effect on the ultrastructure or viability of LEC as determined by EM, trypan blue and propidium iodide test. Control media also contained DMSO in the same concentration as the treated LEC and were incubated in serum free culture medium without the cytoskeletal agents.

After incubation, LEC were prepared for routine SEM investigation [5] or were used to visualize their cytoskeleton in SEM as described above.

Transmission electron microscopy

LEC were rinsed twice with PBS. In order to preserve the fine structure of the cytoskeleton, samples were slightly fixed with freshly prepared 4% formaldehyde in PBS for 1 min at 21°C. Extraction with cytoskeleton buffer for 1 min at 21°C was performed as

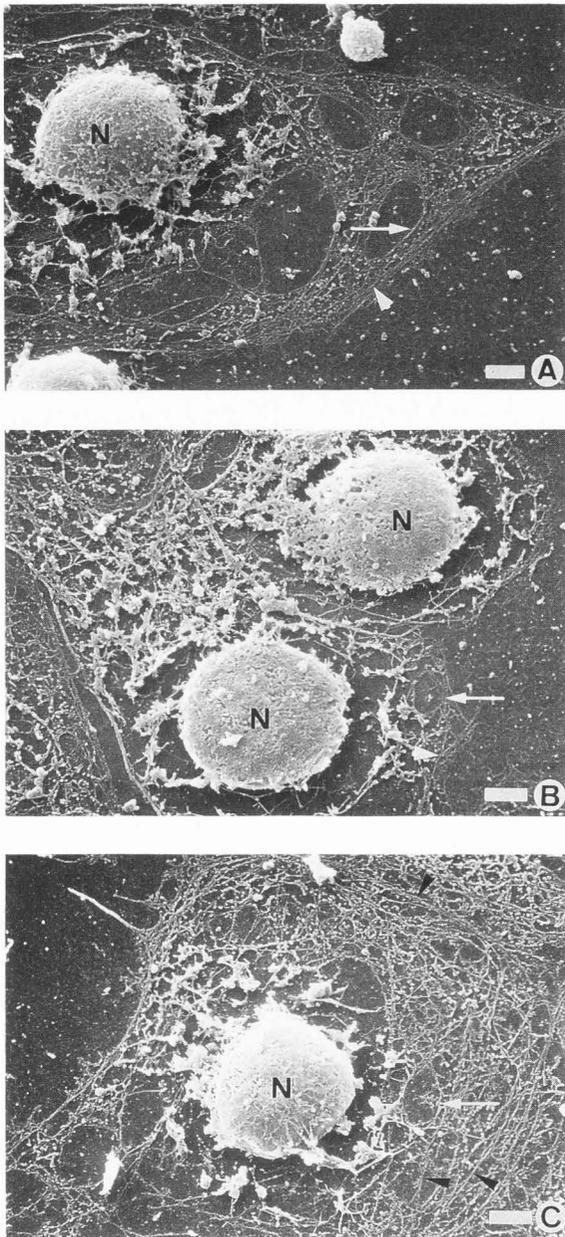


Figure 1: Scanning electron micrographs of cytoskeleton buffer extracted LEC. These cells were not prefixed before extraction. Notice the centrally located, bulging nucleus (N) and the sieve plate-associated cytoskeleton (->) composed of a circle of tubular structures. (A) Control cells reveal a normal distribution of microtubules (>), that can be recognized by their straight outline. They are scattered throughout the cytoplasm in variable directions. Notice the close relation between microtubules and sieve plates. (B) Treatment of LEC with colchicine results in the disappearance of many microtubules. However, microtubules (>) were still scattered around sieve plates. Taxol treatment of LEC (C) results in abundant microtubules (>), bars $1\mu\text{m}$.

described above. After extraction, cytoskeletons were fixed, dehydrated and critical point dried as for SEM. The specimens were examined in a Philips EM 400 (Philips Eindhoven, The Netherlands) at an accelerating voltage of 120 kV.

To investigate the effect of different agents and low temperature on the number of microtubules, LEC were treated with colchicine or taxol, as described in the previous paragraph, followed by a 2 hours incubation at 37°C or 4°C . After incubation, LEC were prepared to visualize their cytoskeleton in TEM as described above.

Atomic force microscopy

The AFM we used, is the ExplorerTM (Topometrix TMX 2000, Darmstadt, Germany). The cantilever of the AFM was positioned in the optical axis of a Leica inverted microscope. Standard silicon nitride tips (Topometrix SFM-Probes, Ref 1520-00, USA) with a spring constant of 0.032 N/m and a $4\mu\text{m}$ on $4\mu\text{m}$ pyramidal base were used. LEC cultured on cover slips were scanned in contact mode, either in air (first set of preparations) or in 0.1 M Na-cacodylate buffer supplemented with 0.1 M sucrose (second set of preparations) [9]. The first set of preparations for AFM were identical to the samples for SEM, except that they were not extracted with cytoskeleton buffer. A second set of cells, was fixed with glutaraldehyde as described above and examined with the AFM. Images, taken in sensor current mode or topographic mode, were analyzed by the Topometrix Image Analysis Software. Images of preparations, scanned in sensor current mode were directly recorded from the computer screen. The x-y-z calibration was regularly checked with a calibration grid.

Morphometric analysis

Analysis of the cytoskeleton was performed on randomly taken electron micrographs with magnifications of 5000 x and 20.000 x for SEM, calibrated with a 28.800 lines/inch grating stub. For TEM, micrographs were taken at the calibrated magnifications of 12.000 x and 44.000 x using a 54.800 lines/inch grating replica. An Ibas I computer (Kontron, Munich, Germany), with a digitizer tablet was used to count and measure the number of sieve plates, fenestrae-associated cytoskeleton rings and microtubules. For each experiment, about 15 micrographs were taken in randomly selected fields for each experimental variable and magnification. All experiments were repeated three times. Statistical analysis was performed with the Mann-Whitney U test.

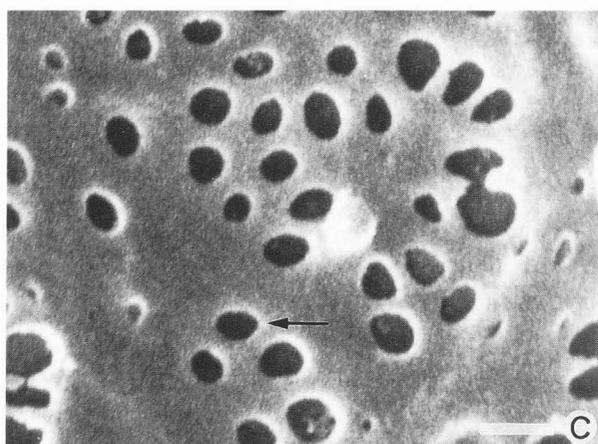
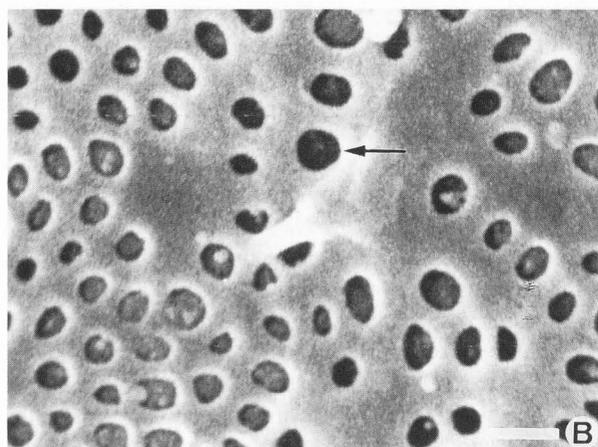
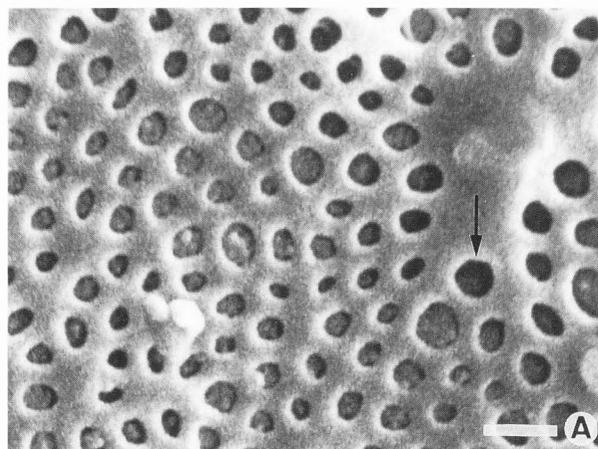


Figure 2: High-power scanning electron micrographs of intact fenestrae of non-extracted LEC. Note that the number of fenestrae (->) is higher in the cytochalasin B treated LEC (A) when compared with figures (B) (colchicine/cytochalasin B) and (C) (taxol/cytochalasin B). However, the number of fenestrae in the colchicine/cytochalasin B treated cells (B) is higher than in the taxol/ cytochalasin B treated cells (C), bars 200 nm.

LEC were treated with colchicine, the number of microtubules per area decreased significantly. However, microtubules were still present, scattered alongside the sieve plates (Fig. 1B). In the non-fenestrated areas of LEC most of the microtubules were broken or disappeared. When cells were treated with taxol (Fig. 1C) an abundance of microtubules were revealed, scattered throughout the cytoplasm and alongside the sieve plates.

As shown in Fig. 2A and Table 1, cytochalasin B treatment resulted in an increase in the number of fenestrae. Treatment of LEC with colchicine followed by cytochalasin B administration resulted also in a moderate increase of the number of fenestrae (Fig. 2B and Table 1). Taxol treatment followed by cytochalasin B administration resulted in an inhibition of the cytochalasin B effect (Fig. 2C and Table 1). The number of sieve plates was unchanged, except for the colchicine / cytochalasin B-treated LEC, where a slight difference was measured (Table 1). A redistribution of the microtubules was observed in the cytochalasin B treated cells. Most of the microtubules were lying parallel to the cell periphery [6]. In the case of the colchicine / cytochalasin B-treated LEC, we could not observe this redistribution. Most of the microtubules disappeared or were lying alongside fenestrae or sieve plates (data not shown). In the taxol / cytochalasin B-treated cells, a high number of microtubules was present (Table 1). These microtubules had the same distribution as described and illustrated in Fig. 4C-4D.

Transmission electron microscopy

Examination of extracted control LEC (Fig. 3A) showed results comparable to SEM (Fig. 1A). Sieve plates were delineated by dense cytoplasmic arms (Fig. 3A and 3B). Microtubules could be recognized at higher magnification within this border (Fig. 3B). Three different spatial organizations of fenestrae were found. Some were arranged in sieve plates, others were oriented linearly and a third group was formed by fenestrae lying single in the cytoplasm (Fig. 3A). It seems that microtubules determine these different patterns, because they closely delineate the fenestrae (Fig. 3A-3B). Incorporation of a fixative in the extraction method

Results

Scanning electron microscopy

The extraction of LEC without formaldehyde treatment, showed a well preserved cytoskeleton (Fig. 1A-1C). In control cells (Fig. 1A), microtubules could be recognized by their diameter and straight outline. Typical sieve plates were encircled by tubular structures; apparently sieve plate-associated cytoskeleton [6]. When

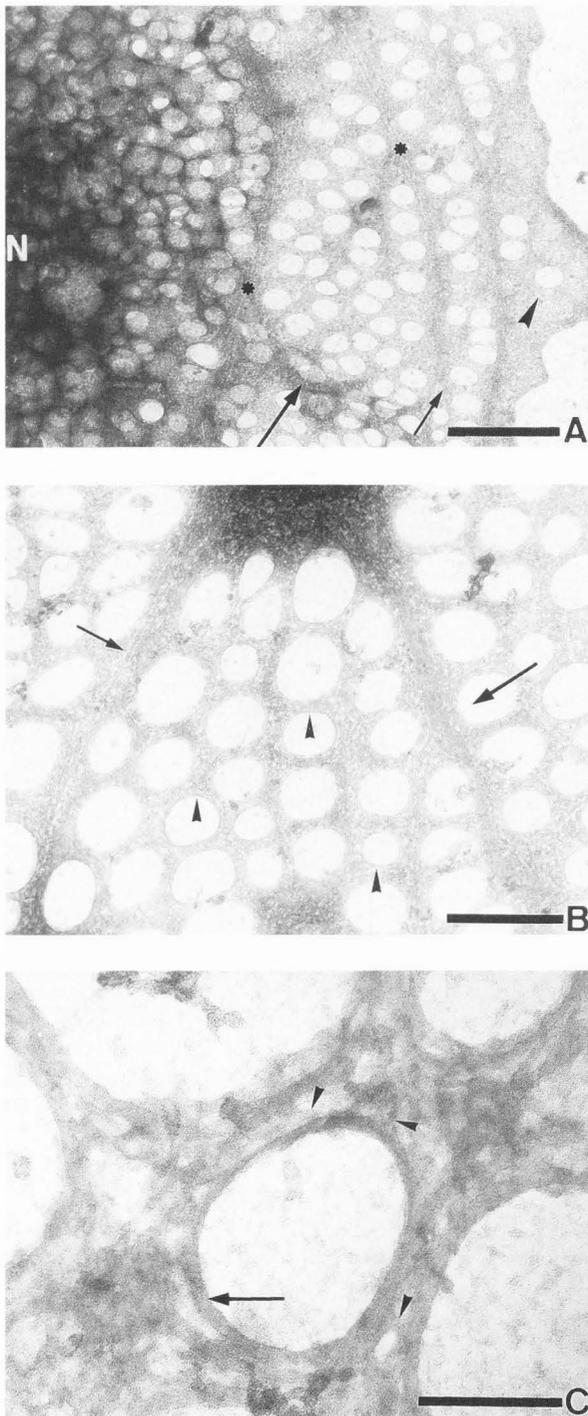


Figure 3: Transmission electron micrographs of whole mount formaldehyde prefixed, cytoskeleton- buffer extracted control LEC. (A) Low magnification showing the cell nucleus (N) and extracted cytoplasm. Three different organizations of fenestrae were found, some are arranged in sieve plates (large arrow), others linearly (small arrow) and some are lying single in the cytoplasm (>). Note also the microtubules which scatter throughout the cytoplasm (*), bar 1000 nm. (B) Higher magnification showing a part of a sieve plate indicated by large arrows. Sieve plates are defined by a darker border. Note the long tubular structures that are running close to the sieve plate (small arrow). Inside the sieve plate, numerous fenestrae-associated cytoskeleton rings (>) can be observed, bar 500 nm. (C) Higher magnification of a fenestra, showing that the fenestrae-associated cytoskeleton ring (->) is connected to the cytoskeleton elements (>) surrounding the fenestrae, bar 100 nm.

(Fig. 4A). In the non-fenestrated cytoplasm most of the microtubules disappeared, but when present, they now showed a zigzag pattern (Fig. 4B). When cells were treated with taxol and 4°C, the number of microtubules quadrupled as compared to the control (Table 2), whereas microtubules scattered throughout the cytoplasm in variable directions (Fig. 4D). Accumulation of microtubules could be observed along sieve plates (Fig. 4C). During experiments on the effect of temperature, we found that cold (4°C) incubation of LEC resulted in a lower number of microtubules per area as compared with 37°C (Table 2). However, taxol treated cells revealed an equal number of microtubules at all temperatures (Table 2). We also observed a difference in the number of fenestrae when incubated at different temperatures (Table 2). Measurements revealed that the number of fenestrae decreased significantly when incubated at 4°C in control, colchicine and taxol-treated cells. In contrast, the number of sieve plates was constant, except for the taxol treated cells.

Atomic force microscopy

LEC prepared for SEM without extraction were examined by AFM (Fig. 5A). The cell surface displayed fenestrae arranged in sieve plates at high magnification. These sieve plates are well delineated by an elevated border. This border probably corresponded to the underlying tubular structures as observed in SEM (see Fig. 1) and TEM (see Fig. 3A, 4A and 4C). Height measurements between the lowest point of the sieve plates and the elevated border, reveal that the sieve plates lie approximately 200 nm lower than the surface of the nearby cytoplasm. In addition, measurements on the elevated borders reveal a width of 176 ± 33 nm (n

enabled the visualization of the fenestrae-associated cytoskeleton ring (Fig. 3C). This ring is approximately 30 nm thick and is clearly connected to the surrounding cytoskeleton by branching structures.

LEC treated with colchicine and incubated at 4°C, showed a decrease in the number of microtubules (Table 2). Only straight microtubules were lying in the neighborhood of sieve plates and linearly arranged fenestrae

Table 1. The effect of cytochalasin B on the number of microtubules, fenestrae and sieve plates, after colchicine and taxol treatment.

Parameter	n of <i>Microtubules</i> per 10 μm^2	n of <i>Fenestrae</i> per 10 μm^2	n of <i>Sieve plates</i> per 100 μm^2
Control	2.3 \pm 1.3	61.4 \pm 12.1	8.1 \pm 1.8
Colchicine	2.6 \pm 1.1	44.4 \pm 7.3***	8.7 \pm 2.6
Taxol	16.9 \pm 7.0***	26.4 \pm 12.9***	7.6 \pm 1.8

Note: Data on the number of microtubules, fenestrae and sieve plates obtained by SEM. LEC were first treated with colchicine or taxol for 2 hours, followed by cytochalasin B treatment for 2 hours. The control LEC were only treated with cytochalasin B. The number of fenestrae and sieve plates were counted on non-extracted LEC, while the number of microtubules were counted by using detergent-extracted LEC. Results are expressed as mean \pm S.D.. Data are obtained from 3 experiments. Notice the significant difference in number of microtubules and fenestrae after the different treatment (*** $p \leq 0.001$ Mann-Whitney U test, two-sided).

Table 2. Effect of temperature on the number of microtubules, fenestrae and sieve plates, after colchicine and taxol treatment.

Parameter	n of <i>Microtubules</i> per 10 μm^2	n of <i>Fenestrae</i> per 10 μm^2	n of <i>Sieve plates</i> per 100 μm^2
<i>at 37°C:</i>			
Control	8.3 \pm 1.9	34.5 \pm 10.0	6.9 \pm 1.9
Colchicine	2.4 \pm 1.2***	35.3 \pm 8.8	7.1 \pm 2.1
Taxol	15.6 \pm 2.6***	38.3 \pm 15.1	9.1 \pm 3.6
<i>at 4°C:</i>			
Control	2.9 \pm 0.9	22.3 \pm 6.4	7.5 \pm 2.7
Colchicine	1.1 \pm 0.8**	24.3 \pm 6.4	6.9 \pm 1.4
Taxol	13.7 \pm 4.2***	28.8 \pm 6.4**	6.7 \pm 1.9

Note: Morphometric data on the number of microtubules in the fenestrated areas, studied by whole mount TEM. LEC were treated with colchicine or taxol for 2 hours, followed by a two hours incubation at 37°C (upper row) or 4°C (lower row). Results are expressed as mean \pm S.D.. Data are obtained from 3 experiments. Notice the significant difference in number of microtubules, fenestrae and sieve plates after colchicine and taxol treatment (*** $p \leq 0.001$ or ** $p \leq 0.01$, * $p \leq 0.05$ Mann-Whitney U test, two-sided).

= 100). Examination of wet-fixed LEC results in comparable observations with the ones obtained after critical point drying and gold sputtering. Sieve plates are well delineated by an elevated border, suggesting the underlying tubular cytoskeleton (Fig. 5B).

Discussion

The cytoskeleton, microtubules in particular, are thought to play an important role in cell architecture, as expressed in shape, organization of organelles and vesicle traffic [23]. As for LEC, it has been demonstrated that microtubules take part in intracellular trafficking of vacuolar transport [21] and general cyto-architecture [36]. In our previous reports [6, 8] we described that microtubules have a close topographical relation with

sieve plates and the fenestrae-associated cytoskeleton. This observation made us anxious to study the alterations of LEC microtubules under different experimental conditions, using microtubule altering agents.

Colchicine prevents further polymerization of microtubules in cells [39], whereas taxol stabilizes or enhances the polymerization of microtubules [22]. Our results confirm the disruption of microtubules by colchicine (Fig. 1B) and the polymerizing ability of taxol (Fig. 1C). However, after treatment with colchicine, a low number of microtubules was still found scattered alongside sieve plates (Fig. 1B). The presence of microtubules after colchicine (Fig. 1B and Table 2) or cold treatment (Fig. 4A-4B and Table 2) demonstrates that a fraction of the microtubules is stable and insensitive against depolymerizing conditions, which has also been demonstrated

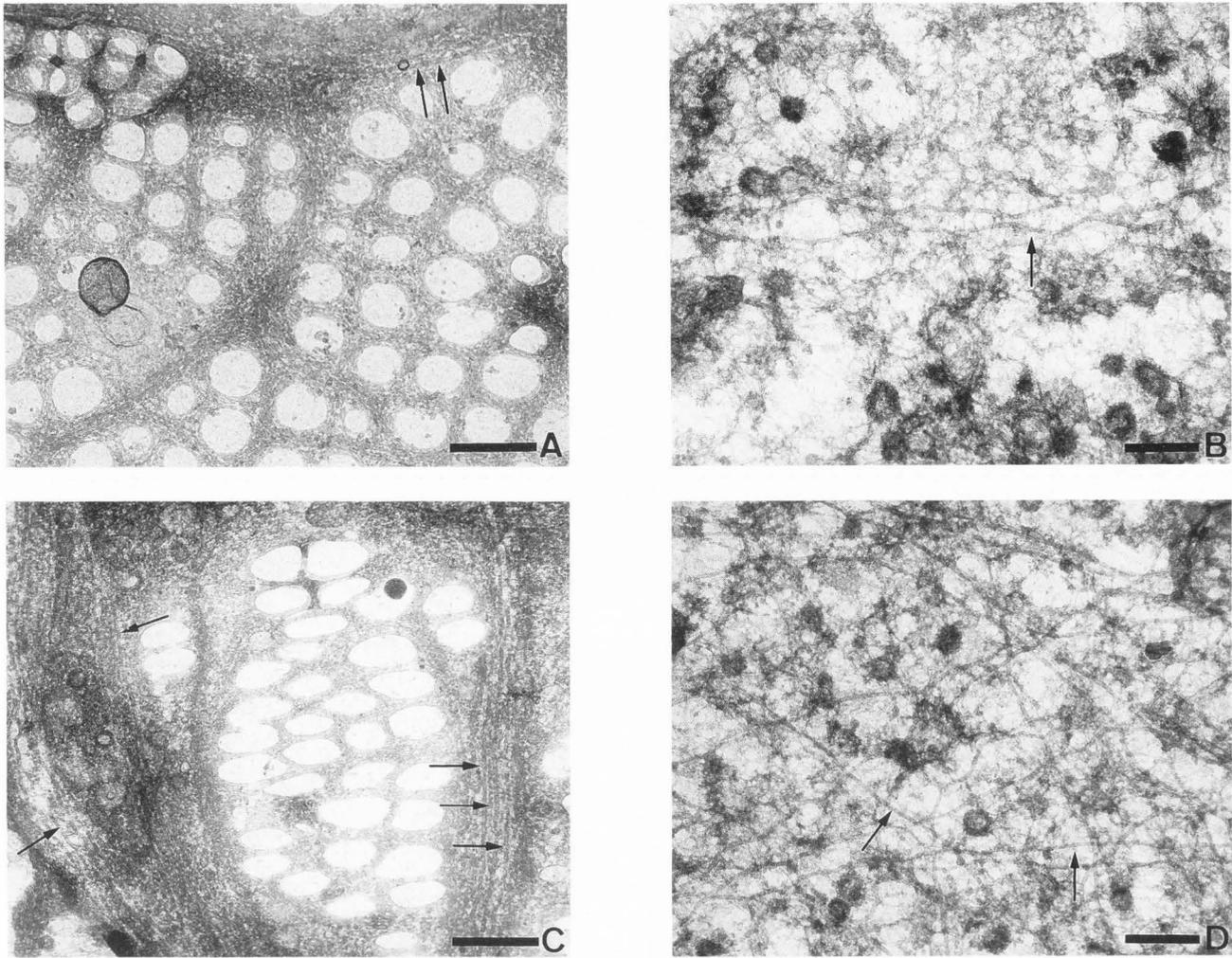


Figure 4: Transmission electron micrographs of whole mount formaldehyde prefixed, cytoskeleton buffer extracted LEC. Figures A (bar 500 nm) and B (bar 250 nm) represents LEC treated for 2 hours with colchicine followed by a 2 hours incubation at 4°C. Figures C (bar 500 nm) and D (bar 250 nm), are LEC treated with taxol for 2 hours followed by a 2 hours incubation at 4°C. Note that the occurrence of microtubules is low in the colchicine/4°C treated cells as compared to the taxol/4°C treated cells. (A) only straight (->) microtubules in the neighborhood of sieve plates and fenestrae were preserved, in the non-fenestrated areas (B) a low number of microtubules (->) could be observed which lost their straight outline. (C) Taxol/4°C treatment revealed an accumulation of parallel microtubules (->) around sieve plates. (D) However, in the non-fenestrated areas straight microtubules scatter throughout the cytoplasm in variable directions.

in other cell types [27, 32]. These data illustrate that stable microtubules lie around sieve plates or linearly arranged fenestrae, which demonstrates the intrinsic architectural role of these microtubules in maintaining LEC structure [6, 7, 8]. Taxol with or without cold treatment resulted in an unusual abundance of parallel arranged microtubules along sieve plates (Fig. 1C and 4C). Parallel arrangements of newly assembled microtubules were only found in the fenestrated areas (Fig. 4C). The occurrence of ordered microtubular arrays

under influence of taxol was also described in other cells [22]. LEC treated with taxol show a doubling of the number of microtubules, whereas a four fold loss was found after colchicine treatment (Table 2). These measurements demonstrate the ability of LEC to shift the microtubule assembly-disassembly steady state under various conditions. The disassembly of the LEC-cytoskeleton under external stimuli, for instance after contact with invading cancer cells, probably leads to an increased permeability of these cells. The loss of cytoskeletal elements in endothelial cells [20] has been

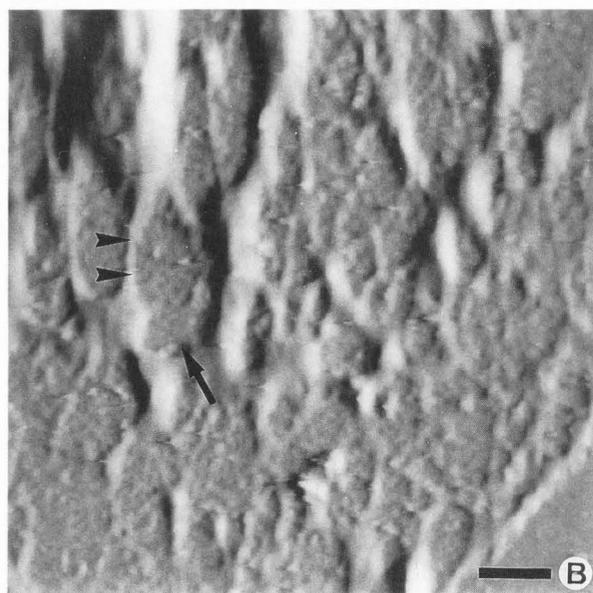
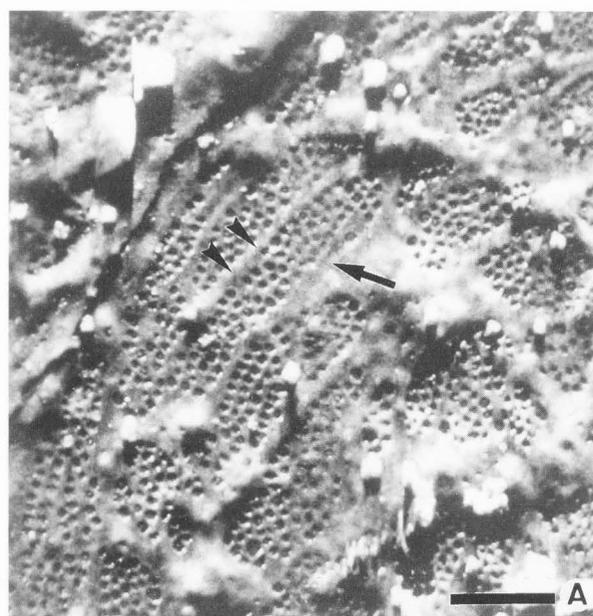


Figure 5: (A) Atomic force image of a critical point dried and gold sputtered LEC, bar 2 μm . This high resolution image of a part of the LEC cytoplasm clearly shows fenestrae in sieve plates (->). Note that these sieve plates are well delineated by elevated borders (>). This border probably corresponds to the underlying tubular structures as observed in SEM (see Fig. 1) and TEM (see Fig. 2A, 3B and 3C). (B) Atomic force image of a wet-fixed LEC, bar 2 μm . This image shows the same details as in figure A, indicating that the SEM preparation did not cause preparations artifacts. Note also that the sieve plates (->) are well delineated by an elevated border (>), suggesting the underlying tubular cytoskeleton.

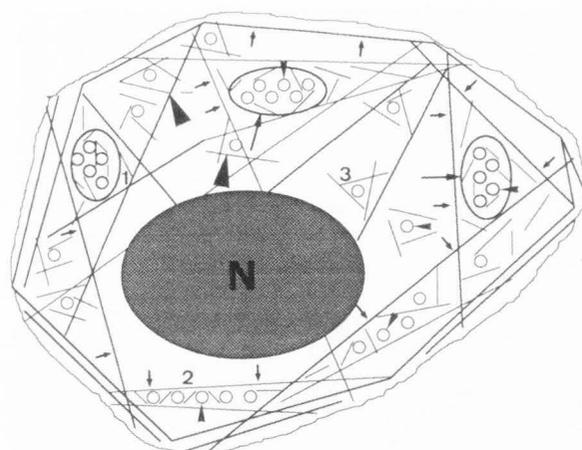


Figure 6: Proposed model of LEC fenestrae and its associated cytoskeleton, based on whole mount cytoskeletal preparations. The drawing shows the following cytoskeletal components: microtubules (small arrows), sieve plate-associated cytoskeleton (large arrows), fenestrae-associated cytoskeleton (small arrowheads), and a centrally located nucleus (N). Three different organizations of fenestrae were found, some were arranged in sieve plates (1), others linearly (2) and some were lying single in the cytoplasm (3). Additionally, in the neighborhood of sieve plates and fenestrae, microtubules always were running nearby the sieve plate- or fenestrae-associated cytoskeleton. From the microtubules small interconnecting structures (large arrowheads) seem to cross-link the surrounding cytoskeleton.

considered as a possible cause of the increased mobility of cancer cells [35].

Cytochalasin B is well known to increase the number of fenestrae [34]. Our results confirm this increase of fenestrae (Table 1 and Fig. 2A). When LEC were treated with colchicine, followed by cytochalasin B, we could also observe a moderate increase of the number of fenestrae. However, taxol treatment followed by cytochalasin B, resulted in an inhibition of the cytochalasin B effect (Fig. 2C and Table 1). We can conclude that the assembly of microtubules, resistant against depolymerization, blocks the effect of cytochalasin B. In addition, removal of microtubules leads to an inhibition of the de novo formation of fenestrae. It seems, therefore, that an intact and functional microtubular cytoskeleton is necessary for the increment of fenestrae by cytochalasin B.

When LEC were incubated at 4°C, the number of fenestrae decreased in normal, colchicine or taxol

treated cells (Table 2). We do not have an explanation for this phenomenon. However, Fratté *et al.* [18] reported that the first preservation damage of livers stored for 2 hours at 4°C occurred in LEC. The reduction in fenestrae is probably one of the first signs of cold storage. It seems that this reduction in fenestrae is independent of microtubules. This was illustrated when LEC were treated with taxol and incubated at 4°C or 37°C. The number of microtubules was equal in both groups, but the number of fenestrae decreased when incubated at 4°C (Table 2).

It was reported that measurements on isolated microtubules by AFM showed a width of 80-110 nm [37]. Measurements on the elevated borders around sieve plates (Fig. 5A) reveal a width of approximately 180 nm. We postulate that these elevated borders are projections of the underlying tubular cytoskeleton. Further studies on LEC-cytoskeleton by AFM are necessary to prove this statement. However, at the moment no literature data are available about AFM and detergent-extracted cells. Probably, AFM studies on the naked cytoskeleton will suffer from technical problems; such as shadowing and lateral deformation of structural details [9]. Fenestrae visualization has hitherto been restricted to electron microscopy [40, 42], due to their limited size. We consider, therefore the possibility of visualizing fenestrae and the projections of the underlying cytoskeleton by AFM as an important achievement, particularly because preparation steps beyond fixation are not needed [9].

In this study, we used detergent-extracted whole mounts of LEC visualized by SEM (Fig. 1) and TEM (Fig. 3-4) based on a modification of the method of Bell *et al.* [2, 3, 26]. In addition, non-extracted LEC were visualized by SEM (Fig. 2) and AFM (Fig. 5). Based on the data obtained in this report and in our previous studies [6, 7, 8] we can conclude that whole mount TEM is a powerful method for studying the organization of the LEC-cytoskeleton. We demonstrated the possibility to visualize the fenestrae-associated cytoskeleton by SEM using prefixation before the extraction [6, 7, 8]. However, the best results obtained on fenestrae-associated cytoskeleton was obtained by TEM (Fig. 3) [6, 7, 8]. With this method it was possible to observe the closely spaced branching filaments which interconnect the fenestrae-associated cytoskeleton and microtubules (Fig. 3C) [6]. Whole mount SEM can be used to visualize the LEC-cytoskeleton. However, a lower resolution is obtained because of the gold-coating of the cytoskeleton and the lower resolving power of the SEM. For example, it was impossible to resolve closely spaced filaments which interconnect the surrounding cytoskeleton. In addition, it was impossible to count the number of microtubules in relation to the number of fenestrae-

associated cytoskeleton rings in SEM-preparations when prefixation before the detergent-extraction was used. Prefixation enhances the retention of cytoskeletal proteins which mask other cytoskeletal structures, i.e. microtubules, which lie deep in the cell [3]. When SEM was used, we counted microtubules on detergent-extracted LEC without prefixation, and fenestrae on non-extracted LEC (Table 1). It is clear that whole mount TEM is a more convenient method to depict the entire cytoskeleton without restriction in resolving power (resolution) or penetration power (masking of cytoskeleton proteins).

Bell *et al.* [2] postulated the possibility of introducing artifacts by using aldehydes. Without prefixation prior the extraction, we could clearly observe precipitates as remnants of fenestrae in SEM [6]. In addition, fixation with 4% formaldehyde for 1 minute at 21°C resulted in well preserved cytoskeleton rings around fenestrae [6, 7, 8]. These results demonstrated that structures which are present, but not well preserved could be visualized when prefixation prior the crude detergent-extraction was used. We can conclude, that prefixation markedly improves the preservation of the cytoskeleton. However, we must be aware about the different results which can be obtained when different preparative methods are been used [26].

In general, a model for the microtubular cytoskeleton of LEC can be proposed as presented in figure 6: (1) Sieve plates and linearly arranged fenestrae are stabilized by microtubules, (2) LEC possess a stable population of microtubules resistant against cold and colchicine, (3) LEC have the ability to shift the microtubule steady state of assembly under various conditions, (4) And it seems that an intact microtubular cytoskeleton is necessary to promote the increase of fenestrae under influence of cytochalasin B.

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Discussion with Reviewers

K.R. Robinson: What is your hypothesis regarding the role of F-actin in fenestrae and sieve plate dynamics, since inhibition of G-actin polymerization shows dramatic effects on fenestrae number? Could F-actin be the "small interconnecting structures" mentioned in the legend for Fig. 6, or "branching structures" seen in the whole-mount preparations?

P.B. Bell Jr: Do you have any ideas as to the nature of the short filaments that you observe connecting microtubules to the fenestrae-associated cytoskeleton and interconnecting the cytoskeletal filaments?

Authors: At the moment we have no idea how filamentous actin is involved in the fenestrae dynamics. However, it has been postulated by others [4, 34] that the inhibition of actin polymerization leads to the fusion of lipid bilayers, resulting in the formation of fenestrae. The small branching structures are fine and short interconnecting filaments, which have a thickness of approximately 8 nm [6]. In detergent-extracted cells, the reported thickness of microfilaments is 5 to 10 nm [26]. These branching structures fulfil therefore the morphologic characteristic of filamentous actin [6]. However, at the moment we have no data available about the immunological identification of these structures.

K.R. Robinson: Does the size of the fenestrae (mean size or distribution) change with the experimental

treatments? The appearance of the fenestrae in the SEMs of Fig. 2 suggests this.

Authors: Different reports show conflicting data about the alterations in fenestrae diameter after treatment with cytochalasin B, i.e. decreased [4], increased [30] or unchanged [34]. However, all reports confirm the increase in the number of fenestrae when LEC were treated with cytochalasin B [4, 6, 30, 34]. In our hands, fenestrae diameter decreases 3-5% when LEC were treated with cytochalasin B (unpublished results). Unfortunately, we have no data available about the alterations in fenestrae diameter after taxol / cytochalasin B and colchicine / cytochalasin B-treatment.

K.R. Robinson: Would an alternative, ultrathin metal coating strategy such as 1nm chromium deposition as employed by Apkarian and others, enhance the visualization of fine detail of the cell surface as well as extracted preparations? This might be useful for instance, in delineating the fenestrae-associated cytoskeleton ring or obtaining more accurate measurements of fenestral dimensions.

Authors: Of course, structural surface information is limited by the quality of the metal film deposited onto it. Coating of the sample with a thin layer of chromium or tungsten enhances the visualization of fine structural details of the cytoskeleton when observed by SEM [3]. Our experience is that gold-coating with a Balzers sputtering device (Type 07120/160) induces melting artifacts caused during sputtering. However, we have no experience with LEC-cytoskeletons coated with a thin layer of chromium or tungsten using advanced sputtering methods.

K.R. Robinson: What is the potential for aldehyde fixation to alter the cell surface morphology? Have you performed any living-cell studies using the AFM?

Authors: AFM allows high-resolution imaging of biological samples under wet conditions. When living cells were scanned, images with artifacts were obtained, mainly caused by the softness of the samples. When cells underwent a short aldehyde fixation, the image quality improved. It seems that aldehyde fixation increases the rigidity of cells, resulting in a better image acquisition (Hoh JH and Schoenenberger CA (1994) Surface morphology and mechanical properties of MDCK monolayers by atomic force microscopy. *J Cell Sci* 107: 1105-1114). This phenomenon was also discussed in our previous study [9]. Further studies will be conducted to find the optimal conditions for visualizing living LEC by AFM.

R.M. Albrecht: Our experience has been that extraction of cellular and cytoskeletal associated material in order

to clean the cytoskeletal elements sufficiently to permit good observation by SEM can cause partial disruption of the cytoskeleton. (ie. the cleaner you get it the less there is of it). The extraction required for whole mount TEM is less rigorous and affords better retention of the overall structure since all the cytoskeletal associated material need not be completely removed. Did the authors carry out any comparisons along these lines? Have they tried observing the cytoskeletal organization of unextracted, stained cells by HVEM or IVEM and compared this to TEM and SEM observations of extracted cells?

Authors: We agree with these statements, which are consistent with our experience. When LEC were slightly fixed, followed by detergent-extraction, a better retention of the overall cytoskeleton was observed in TEM [6]. When these samples were observed by SEM, cytoskeletal structures which lie deep in the cell couldn't be observed (see also discussion). Previously we discussed the visualization of the cytoskeleton and the different results when preparation procedures for whole mount-SEM and TEM are used [6]. Additionally, in an attempt to elucidate the possible effects of detergent-extraction on the LEC-cytoskeleton, we tried to visualize the cytoskeleton of fixed-LEC in a cryo-EM study (see also this volume, F. Braet & P. Frederik). We found evidence that the fenestrae are again delineated by cytoskeleton rings in cryo-preparations, in accordance with results obtained with detergent-extraction.