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RESEARCH

# Epithelial-To-Mesenchymal Transition Induced by Freund's Adjuvant Treatment in Rat Mesothelial Cells: A Morphological and Immunocytochemical Study

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**Abstract** Intraperitoneal injection of Freund's adjuvant induces acute peritonitis. By the time of the Freund's adjuvant treatment the flat, simple squamous epithelial cells became rounded, cuboidal shaped, many of them have lost their connection with the neighbouring cells and detached from the basement membrane. The macrophage markers' (ED1, OX43 and CD68) expression also increased in the mesothelial cells and more mesothelin and anti-ED1 double-labelled cells were found freely present close to the surface. The cytokeratin expression of the mesothelial cells has gradually decreased. At the 5th day of the inflammation practically there was no cytokeratin labelling present in the mesothelial cells and the mesothelin expression has significantly decreased. Parallel to this mesothelial cells started to express vimentin, a characteristic mesenchymal intermediate filament protein indicating that they gradually lost their epithelial character and gained mesenchymal phenotype. These results strongly suggest that under the effect of Freund's adjuvant treatment (inflammation) mesothelial cells can undergo epithelial-to-mesenchymal transition and differentiate into phagocytotic (macrophage-like) cells. Studying the caveolae/caveolin-1 on the plasma membrane of mesothelial cells we found that the Freund's adjuvant treatment has changed the cellular distribution of caveolin-1: as the inflammation progressed strong caveolin-1 labelling was found inside of the cytoplasm (in perinuclear localization) indicating that inflammation induced the caveolae internalization. These results indicate that caveolae/caveolin-1 might play important regulatory role in signal transduction leading to trasdifferentiation.

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**Keywords** Caveolin-1/caveolae · Epithelial-to-mesenchymal transition · Mesothelial cell · Transdifferentiation

**Abbreviations**
EMT epithelial-to-mesenchymal transition

## Introduction

Epithelial-to-mesenchymal transition (EMT or transdifferentiation) is involved in a variety of normal physiological processes, including gastrulation, heart formation and palate closure during embryogenesis [1, 2] as well as pathological processes such as metastatic potential in malignancy [3, 4]. Epithelial-to-mesenchymal transition can also be induced by long-term exposure to hyperosmotic, hyperglycemic and acidic solutions used in dialysis and chronic inflammation [5]. The steps in epithelial-to-mesenchymal transition include a loss of cell-cell and cell matrix interactions [1, 6], migration, basement membrane degradation [7] and cytoskeletal rearrangement [8]. When we injected Freund's adjuvant into the peritoneal cavity of rats we found significant changes in the morphology of mesenteric mesothelial cells. Flat, simple squamous epithelial cells became rounded, cuboidal shaped, many of them have lost their connection with the neighbouring cells and detached from the basement membrane [9]. By the time of the adjuvant's treatment we could also detect a significant increase in the expression of ED1, which is a well characterized pan macrophage marker in rat [10]. The mesothelial cells were strongly labelled with nestin. (Nestin is well-known marker of multi-lineage progenitor cells; [11]. All these data strongly suggest the idea that mesothelial cells are not entirely differentiated cell and

they might undergo an epithelial-to-mesemchymal transition. Since there are few, if any *in vivo* models of EMT, our system can be used as an *in vivo* model for studying the steps epithelial-to-mesenchymal transdifferentiation.

To study the kinetic of EMT we have done detailed morphological and immunocytochemical analysis focusing on the reorganization of the cytoskeleton. By the time of the inflammation (Freund's adjuvant treatment) we followed the changes in the expression of cytokeratin (epithelial intermedier filament protein) and vimentin (a mesenchymal cell-specific intermedier filament protein). We found that as the inflammation progressed the cytokeratin is gradually disappeared from the cytoplasm of the mesothelial cells and replaced by vimentin. Since caveolae—the flask-or omega-shaped plasma membrane invaginations—are abundantly present on the plasma membrane of mesothelial cells we were also interested in whether the number and the cellular distribution of caveolae are changing during inflammation. Our immunocytochemical results showed that by the time of the inflammation the cytoplasmic caveolin labelling became stronger. When we studied the cells electron microscopically we found numerous multivesicular bodies in the cytoplasm of the mesothelial cells indicating that caveolae were pinching off from the plasma membrane. These data support the idea that caveolin-1/caveolae might be involved in regulation of signal transduction leading to transdifferentiation. At the peak of the inflammation (5–7 days) there was an extensive cellular proliferation, migration, structural reorganization and loss of mesothelial cell adhesion. By the 11th day the inflammation passed off, mesothelial layer became continuous, the integrity of these cells were evident, new basement membrane was created. The procedure, however, was not entirely reversible: although the mesothelial cells retrieved their original squamous morphology, they still expressed ED1, vimentin,

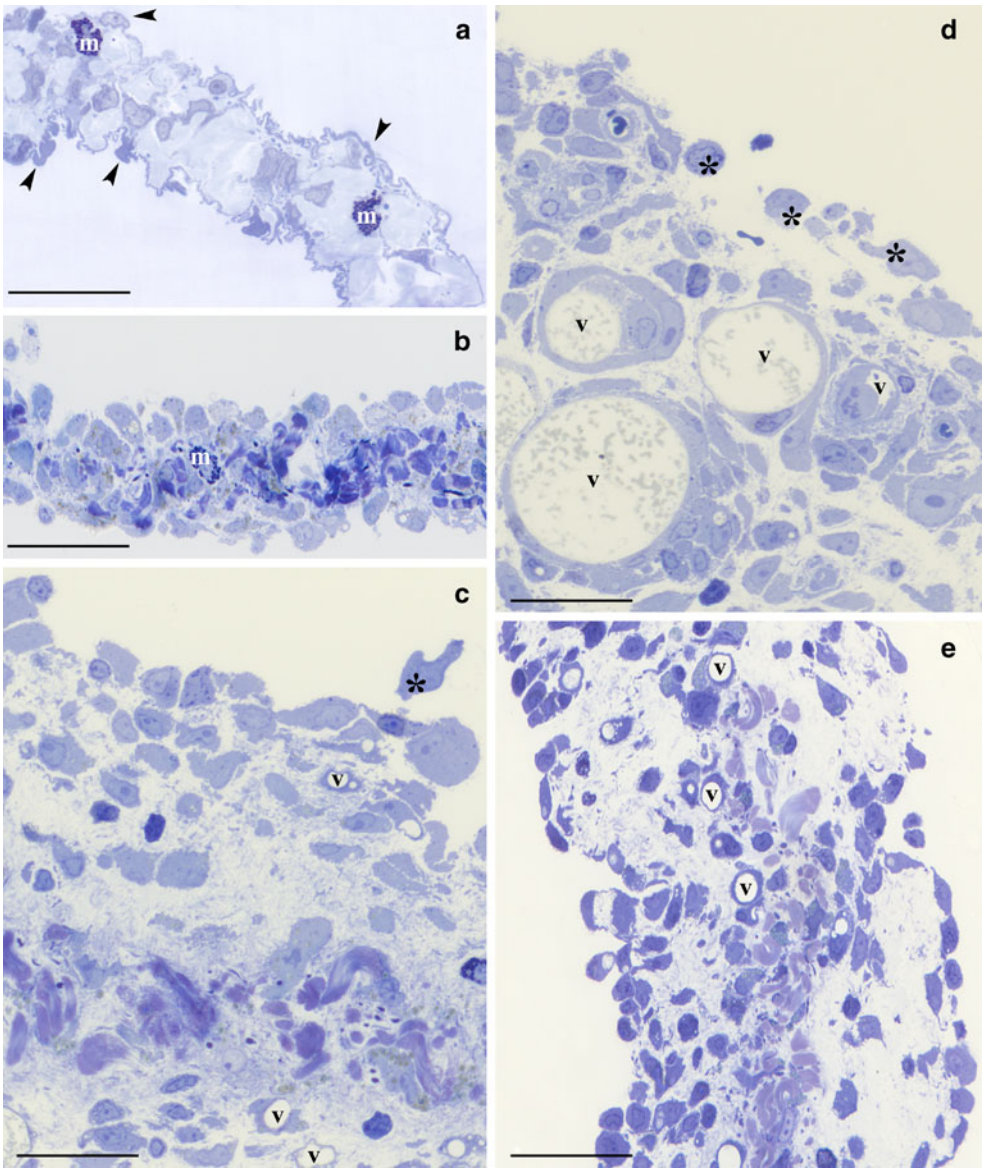



**Fig. 1** Light microscopic pictures of mesentery isolated from control and treated rats. On the control toluidine-blue stained semithin sections (**a**) the mesothelial cells are seen as elongated, flat cells, they can be identified according their nuclei (*arrowheads*), their cytoplasm is hardly seen and appears as wavy lines. The connective tissue between the two layers of mesothelial cells contains blood vessels (arterioles and capillaries) and collagen fibres. There are only few cells (mainly fibrocytes, mast cells and granulocytes) present in this layer. 3 days after the Freund's adjuvant treatment the mesothelial cells become rather cuboidal or round shaped. The basement membrane is hardly seen (**b**). On the 5th and 7th days of inflammation many free cells are present close to the surface of mesentery (*) and the connective tissue layer become highly vascularised (**c**, **d**, **e**). On both surfaces of mesentery cells are present in many layers indicating a significant cell migration toward the surface. v: blood vessels; m: mast cells. Bars: 50 μm

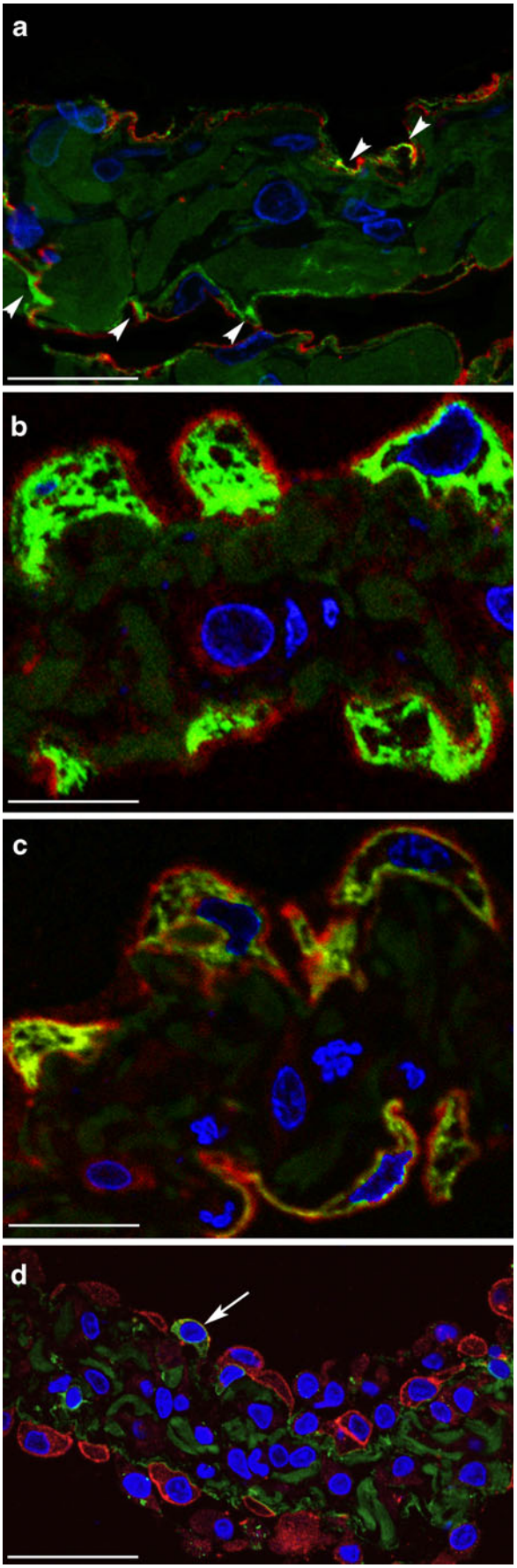



**Fig. 2** Changes in the cytokeratin expression. (**a**) Although in the mesothelin labelled cells (*red*) cytokeratin (*green*) can be detected (*arrowheads*), in the non-treated cells the cytoplasmic distribution of cytokeratin is difficult to see. (**b**) After 24 hours of the Freund's adjuvant injection cytokeratin appears as a delicate cytoplasmic network; (**c**) 48 hours treatment results in a decreased cytokeratin expression. (**d**) On the third day of inflammation the majority of the mesothelial cells are cytokeratin negative and only a few cells express cytokeratin (*arrow*). Nuclei are labelled by DAPI (*blue*). Bars: a: 22μm; b and c: 18μm; d: 34μm

cytokeratin was missing from their cytoplasm and there was only few caveolae present on plasma membrane.

## Material and Methods

*Material* To induce inflammation 1 ml complete Freund's adjuvant (Sigma, Steinheim, Germany) was injected into the peritoneal cavity of Sprague–Dawley male rats (200 to 400 g). After 1, 2, 3, 5, 7, 11 days mesentery was isolated from control and treated animals.

*Antibodies* polyclonal anti-caveolin-1 antibody (1:100) was purchased from Transduction Laboratories (Lexington, England). To label mesothelial cells anti-rat mesothelin (1:200) was purchased from Immuno-Biological Laboratories (Co LTD, Japan). Anti-pan cytokeratin antibody (Lu5, 1:200; from BMA Biomedicals, Augst, Switzerland), anti-vimentin antibody (V9, 1:100; from Millipore Chemicon, USA) and anti-E-cadherin (1:75; BD Transduction Laboratories, USA) were used to follow the changes in the expression of the cytoskeletal proteins. To identify the macrophage character ED1 antibody (a generous gift from Prof. Dr. C.D. Dijkstra; Dep. Cell Biology, Medical Faculty, Vrije University, Amsterdam, The Netherlands). For confocal microscopy Alexa Fluor 488, 594 (Molecular Probes, Leiden, The Netherlands) conjugated second antibodies (1:100) were used. The nuclei were labelled by DAPI (Vectashield DAPI mounting medium).

*Experimental procedures* mesentery was isolated from both control and Freund's adjuvant injected animals. The samples were fixed either in 2% glutaraldehyde (GA) in Millonig's phosphate buffer or 4% formaldehyde (FA) in 0.1 M phosphate buffer (PBS), pH7.4 (1 h, room temperature). The fixation was followed by washing in PBS, and the adipose tissue was removed from the surface of the mesentery. The GA-fixed material was proceeded to electron microscopic embedding, while the FA-fixed samples were used for immunocytochemistry.

*Immunocytochemistry* The FA-fixed samples were stored until further processing in 1% formaldehyde at 4°C. For semithin cryosectioning and immunolabelling the fixed samples were washed with 0.05 M glycin in PBS, infiltrated

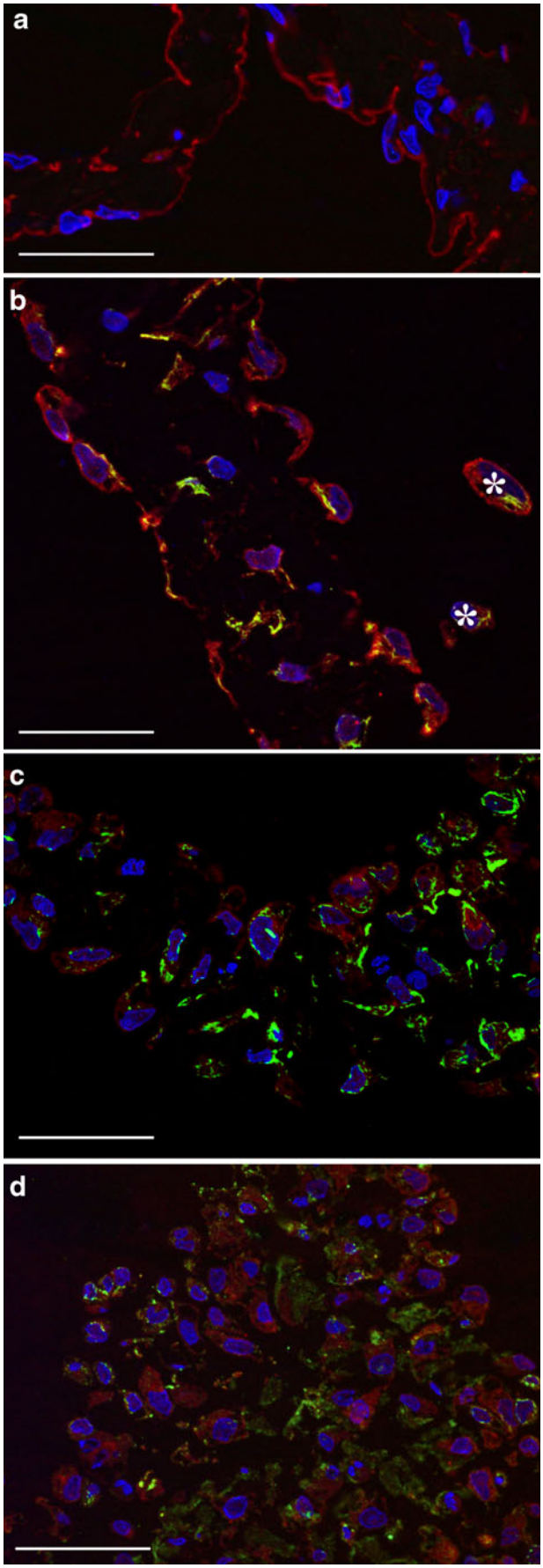



**Fig. 3** Expression of vimentin in mesothelial cells. (**a**) Non-treated mesothelial cells do not express vimentin. The cells are labelled only with mesothelin (*red*). (**b**) At the first day of inflammation mesothelial cells start to express vimentin: this intermediate filament protein can be detected in a few cells (*green*), most of the cells, however, are vimentin negative. A couple of double labelled free cells can also be visible (*). 48 and 72 hours after the Freund's adjuvant treatment the expression of vimentin is increasing (**c**, **d**). Most of the mesothelin labelled cells (*red*) are vimentin positive (*green*). Many cells labelled with anti-vimentin are present in the connective tissue as well. Nuclei are labelled by DAPI (*blue*). Bars: a: 28μm; b and c: 26μm; d: 32μm

gradually with gelatine at 37°C (2%, 5% and 12% in PBS). The 12% gelatine (containing mesentery) was solidified on ice and cut into small blocks. For cryoprotection blocks were infiltrated overnight with 2.3 M sucrose at 4°C and afterwards mounted on aluminium pins and frozen in liquid nitrogen. The 0.8 μm thick frozen sections were cut by Leica Ultracut S ultramicrotome (Vienna/Austria). To pick up the sections 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used (15). Sections were incubated with the first antibodies at 4°C (overnight), after washing this was followed by 1 h incubation (at room temperature) with the second antibody. In all cases we used double labelling immunocytochemistry to detect mesothelin.

Bio-Rad Radiance 2100 Rainbow confocal microscope was used for confocal images.

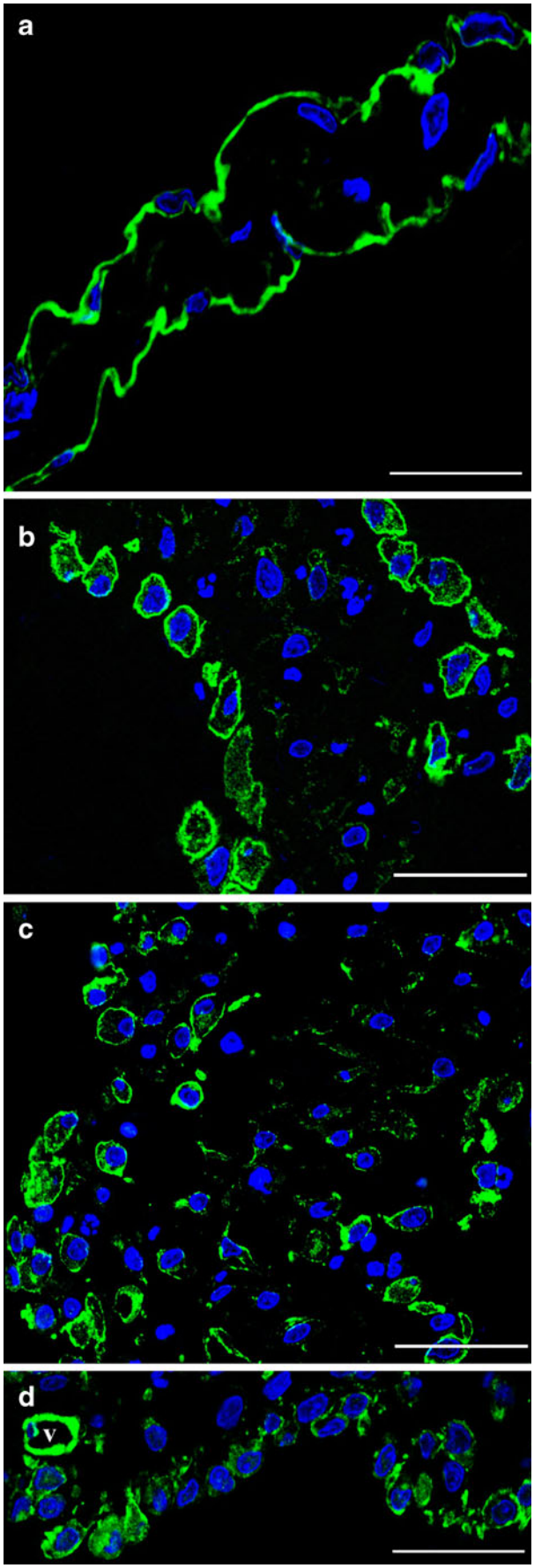
*Electron microscopy* The GA-fixed samples were washed in Millonig's buffer, which was followed by washing in 0.1% cacodylate buffer, and post-fixation in 1% $OsO_4$ (1 h at 4°C in 0.1% cacodylate buffer, pH7.4). A pre-embedding staining with 1% uranyl acetate in distilled water was performed for 1 h at 4°C. The samples were dehydrated and embedded in araldite. The semithin sections were stained with toluidine blue. Ultrathin sections were contrast stained with lead citrate.

*Quantitation methods* To follow EMT quantitatively 150 mesothelin positive cells were counted and checked how many of them were double labelled with mesothelin/cytokeratin, mesothelin/vimentin as well as mesothelin/E-cadherin. The ratio of the double labelled cells was expressed as the percentage of the total mesothelin positive cells.

## Results

### Morphological Changes Induced by Freund's Adjuvant Treatment

Mesothelial cells present on the surface of the *control* (non-treated) rats' mesentery are elongated, typical simple squamous cells. They rest on a thin basement membrane. In toluidine-blue stained semithin sections they can be identified according their nuclei, their cytoplasm is hardly seen and appears as wavy lines




◀ **Fig. 4** Cellular distribution of caveolin-1. Control mesothelial cells show strong caveolin-1 labelling (*green*) but the cellular distribution of caveolin-1 is not well visible (**a**). On the second day of inflammation the caveolin-1 labelling on plasma membrane is still very strong, but caveolin-1 can be detected in the cytoplasm of the morphologically changed mesothelial cells as well (**b**). 5 and 7 days after the Freund's adjuvant treatment more and more caveolin-1 can be found in the cytoplasm of the mesothelial cells (**c**, **d**). Nuclei are labelled by DAPI (*blue*). v: blood vessel. Bars: a: 24μm; b: 26μm; c and d: 32μm

(Fig. 1a). The connective tissue between the two layers of mesothelial cells contains blood vessels (arterioles and capillaries) and large amount of collagen fibres. There are only few cells (mainly fibrocytes, mast cells and some granulocytes) present in this layer (Fig. 1a). 3 days after the Freund's adjuvant injection the mesothelial cells became rather cuboidal, many of them seem to detach from the surface of the mesentery. The basement membrane is hardly seen, tightly packed bundles of collagenous fibres could be found in the connective tissue layer (Fig. 1b). As the inflammation is progressed (5 and 7 days), many free cells were found on the surface of the mesentery indicating that the intracellular junctions of these cells were disrupted (Fig. 1c, d, e). The thickness of the mesentery has significantly increased; collagen fibres' bundles—as a core—were seen in the middle of the mesentery. Numerous cells appeared in the connective tissue layer they seem to migrate to the surface (Fig. 1b, c, d). The round shaped morphology of the cells present on the surface suggests that they have lost the apical-basolateral polarity of epithelial cells and they adopted a migratory and invasive phenotype. The connective tissue layer became highly vascularised (Fig. 1d, e), many cells were present around the blood vessels indicating that there should be a significant migration of the cells from the blood vessels as well (Fig. 1d). Dividing cells were often found in the connective tissue, in the blood vessels as well as on the surface of the mesentery. The peak of the activity occurred by day 7 (Fig. 1e). At this time there was an extensive cellular proliferation, migration, structural reorganization, loss of epithelial cell adhesion and continuity. The connective tissue between the 2 layers of mesothelial cells was highly vascularised (Fig. 7e).

## Epithelial-To-Mesenchymal Transition in Vivo

The morphological changes and down-regulation of cytokeratin in mesothelial cells could be indicative of an epithelial-to-mesenchymal transition. To determine the nature of the cells we analyzed the expression of the intermediate filament proteins cytokeratin and vimentin as markers of transdifferentiation. Cytokeratins are intermediate filament proteins, typical epithelial markers, while the expression of vimentin intermediate filament protein is characteristic of mesenchymal cells. Our confocal immunofluorescence microscopical results clearly show that the expression of cytokeratin has markedly decreased by the time of inflammation (Fig. 2). In control (non-treated) mesothelial cells because of the flat morphology

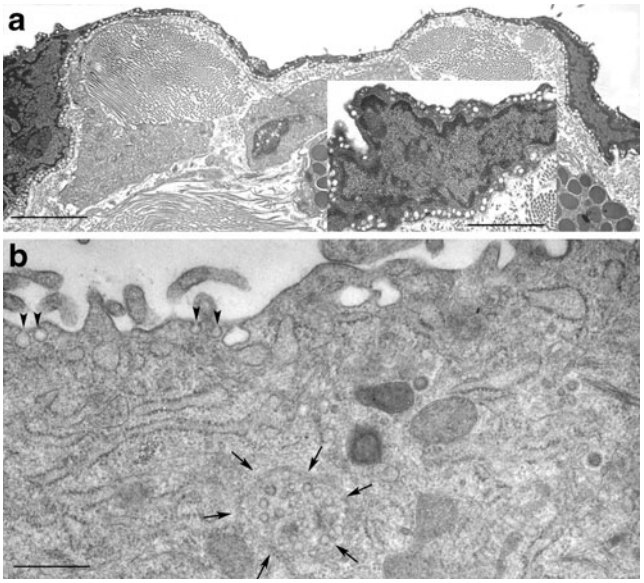



**Fig. 5** Cytoplasmic distribution of caveolae in mesothelial cells. On the electron microscopic picture of control cells lots of "empty holes" are visible on the plasma membrane (**a**). Insert shows one mesothelial cell with higher magnification: many caveolae are seen on both the basal and apical surfaces. 3 days after the Freund's adjuvant treatment, only a few caveolae are present on the plasma membrane (*arrowheads*). At the same time multivesicular bodies are found in the cytoplasm (*arrows*). Nuclei are labelled by DAPI (*blue*). Bars: a: 7μm; insert: 1.2μm; b: 0.3μm

the cytoplasmic distribution of the cytokeratin is difficult to see (Fig. 1a) After 24 h of the Freund's adjuvant injection cytokeratin forms a delicate cytoplasmic network which is still characteristic of many mesothelial cells layering the mesentery (Fig. 2b). There was a markedly lower level of cytokeratin expression in the mesothelial cells of the 48 h-treated samples (Fig. 2c). At the 3rd day of the inflammation only a few mesothelial cells were found to express cytokeratin (Fig. 2d). The expression of vimentin has change reversely: by the time of inflammation the cytokeratin was gradually replaced by vimentin (Fig. 3a, b, c and d). Our quantitative data (Table 1) strongly support our confocal microscopical results.

## The Effect of the Freund's Adjuvant Induced Inflammation on the Intracellular Distribution of Caeolae/Caveolin-1

Our confocal immunocytochemical results revealed that mesothelial cells show very strong anti-caveolin-1 labelling indicating that these cells have numerous caveolae on their plasma membrane (Fig. 4a). Since the control, non-treated mesothelial cells are flat; the exact cellular distribution of this labelling is difficult to interpret. Studying these cells

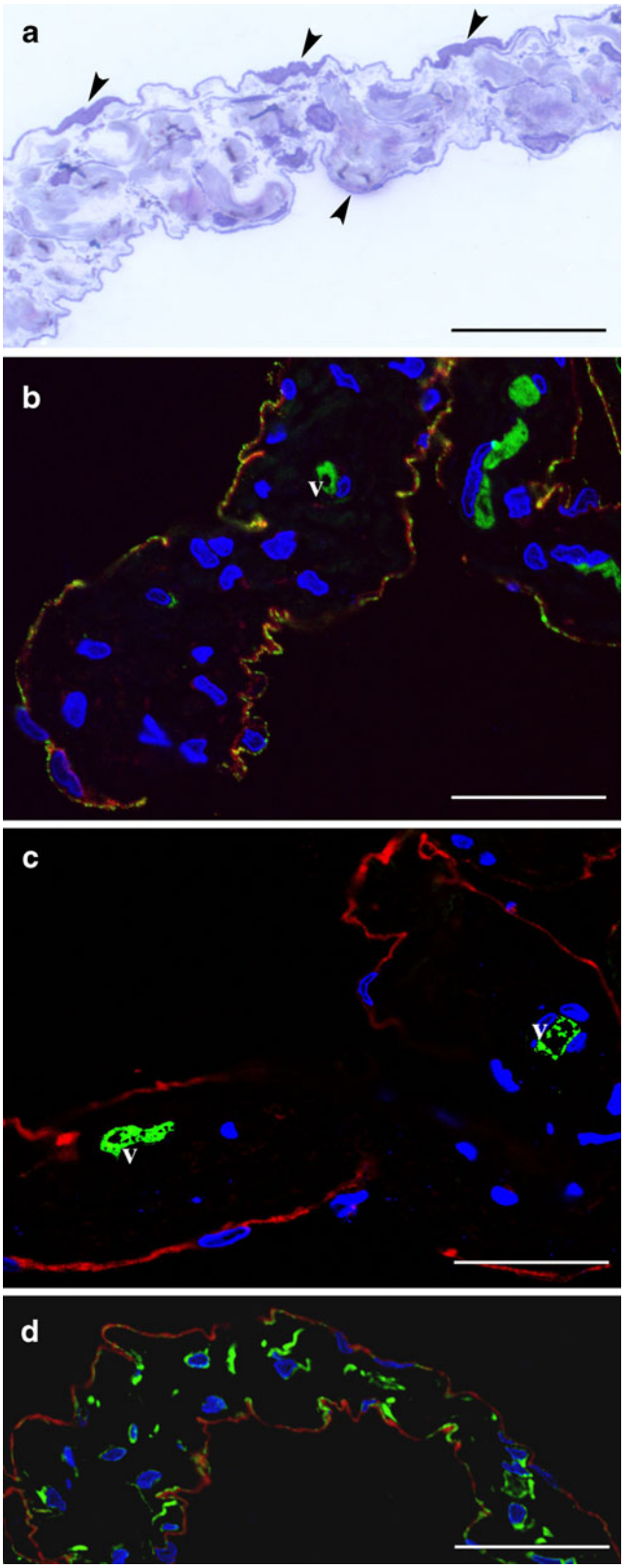



**Fig. 6** By the 11th day the inflammation is passed off. On the toluidine-blue stained semithin section (**a**) the shape of mesothelial cells is similar to that of the non-treated cells (arrowheads: nuclei of mesothelial cells). Although they still express ED1 macrophage marker (**a**, *green*) and vimentin (**d**, *green*) but we cannot detect cytokeratin (**c**, *green*) in their cytoplasm. Mesothelial cells are labelled with mesothelin (red) and nuclei with DAPI (*blue*). v: blood vessels. Bars: a: 50μm; b and c: 30μm; d: 36μm

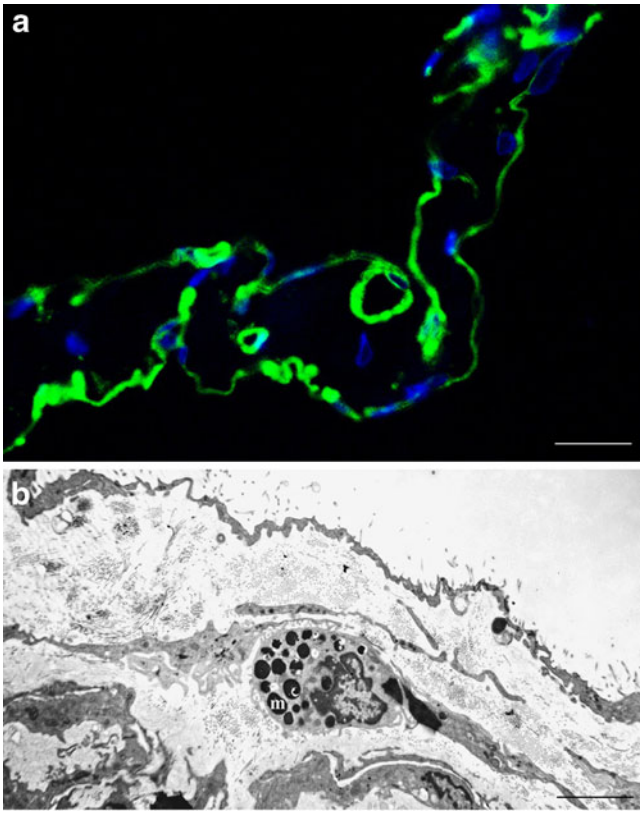



**Fig. 7** 11 days after the Freund's adjuvant intraperitoneal injection: caveolin-1 detection. (**a**) Although the mesothelial cells show caveolin-1 labelling, (*green*) but the number of caveolae on plasma membrane is significantly lower than in control cells (**b**). Nuclei are labelled by DAPI (*blue*). m: mast cell. Bars: a: 22μm; b: 8μm

electron microscopically we found many omega- or flask-shaped plasma membrane invaginations on the plasma membrane of these cells (Fig. 5a and insert). As the morphology of the mesothelial cells have been changing the cellular distribution of caveolin-1 labelling is better seen. By the time of the inflammation we found an increasing cytoplasmic caveolin-1 labelling (Fig. 4b, c, d) indicating that caveolae were internalized. Our electron microscopic data showed that the number of the surface-connected caveolae decreased and multivesicular bodies (containing caveolae-like structures inside) appeared in the cytoplasm, suggesting that caveolae were indeed internalized (Fig. 5b).

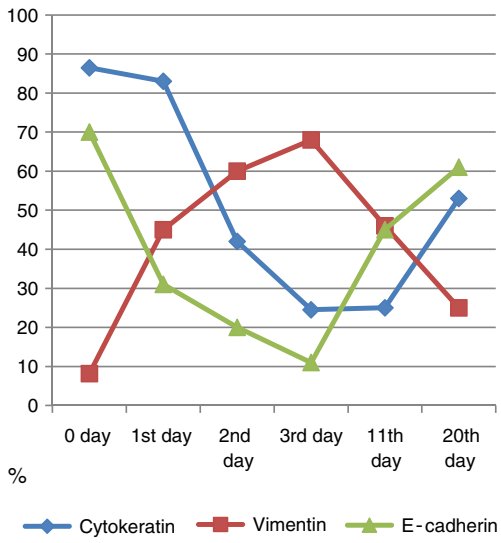
Is the Transdifferentiation of Mesothelial Cells a Reversible procedure?

It is supposed that transdifferentiation is a complex and generally reversible process. To study the possible reversibility of this epithelial-to-mesenchymal transition of mesothelial cells we followed morphologically and immunocytochemically the inflammation till the day of 11. By this time the destruction and rearrangement of the mesentery was resolving. Mesothelial cells re-gained the flat, squamous morphology, their integrity was evident; a new submesothelial basement membrane was created (Fig. 6a). Studying the epithelial as well as the mesenchymal markers, however, we found that the epithelial characters are not completely recovered. The mesothelial cells still expressed macrophage marker (ED1; Fig. 6b), vimentin was obviously present in these cells (Fig. 6d), while cytokeratin expression was still down-regulated (Fig. 6c). Although the recovered mesothelial cells were labelled with caveolin-1 (Fig. 7a) our electron microscopic data clearly showed that the number of caveolae present on the plasma membrane was still significantly lower than in control cells (Fig. 7b).

## Discussion

We have previously demonstrated that peritoneal injection of Freund's adjuvant results in marked changes in the morphology of the mesothelial cell lining the surface of rat mesentery: flat squamous epithelial cells became rounded, cuboidal shaped, they have lost their apical-basolateral polarity, many of them tend to detach from the basement membrane, and the cell-cell interactions seemed to be lost. Freund's adjuvant treatment induced an extensive cellular proliferation, migration, structural reorganization, and loss of cell continuity (Katz et al). All these morphological signs resemble to the steps of epithelial-to-mesenchymal transition (EMT) or transdifferentiation. During transdifferentiation epithelial cells lose their cell-cell interaction through

**Table 1** As the inflammation progresses the number of the cytokeratin and E-cadherin positive mesothelial cells is continuously decreasing. Parallel to this we found a significant increase in the number of vimentin and mesothelin double labelled cells. When the mesothelium starts to recover, the number of the cytokeratin and E-cadherin positive mesothelial cells is increasing, and significantly fewer vimentin and mesothelin double labelled cells were detected



down regulation of E-cadherin and ß-catenin [1, 6], cells migrate, basement membrane is degraded through matrix metalloproteinase-2 (MMP-2) [7], and cytoskeleton is rearranging [8]. Epithelial-to-mesenchymal transition can be induced by long-term exposure to hyperosmotic, hyperglycemic and acidic solutions used in dialysis and chronic inflammation [5]. Since Freund's adjuvant causes massive inflammation and inflammatory cytokines initiate mesothelial transdifferentiation [12], our morphological (light microscopical) data suggest the idea that Freund's adjuvant treatment can really induce epithelial-to-mesenchymal transition.

In order to prove that Freund's adjuvant treatment really induce epithelial-to-mesenchymal transition we have done a detailed immunocytochemical analysis studying the expression of cytokeratin (an epithelial intermediate filament protein) and vimentin (an intermediate filament protein characteristic of mesenchymal cells). We found that as the inflammation progressed the cytokeratin is gradually disappeared from the cytoplasm of the mesothelial cells. Parallel to this the vimentin expression is gradually increased indicating the mesothelial cells gradually lost their epithelial character and underwent a transition from epithelial to a mesenchymal phenotype. By the time of the Freund's adjuvant treatment the mesothelial cells started to express macrophage markers as well (ED1 and OX43, CD68) [9]. Caveolae—the flask-or omega-shaped plasma membrane invaginations—are abundantly present on the plasma membrane of mesothelial cells [13]. We were interested in whether the number and the cellular distribution of caveolae are changing during inflammation. Our immunocytochemical results showed that by the time of the inflammation the cytoplasmic caveolin-1 labelling became stronger. When we studied these cells electron microscopically we found numerous multivesicular bodies in their cytoplasm indicating that caveolae were pinching off from the plasma membrane and translocated to the cytoplasm. Since epithelial-to-mesenchymal transition is a complex process (involving restructuring of the cytoskeleton, cell membrane and cell-cell junction) many signalling molecules and signalling pathways should be involved in this procedure. As regulators of the actin cytoskeleton and cadherin junction, the Rho family of small GTPases is commonly implicated in these processes [14]. Rac1 and Cdc42 are involved in the establishment and maintain of epithelial intercellular adhesions [15, 16]. p38MAP pathway is required for TGF-ß-mediated epithelial-to-mesenchymal transition and cell migration [17], PI3kinase/Akt may contribute to dissolution of tight junctions [18]. Many of these molecules are known to localize in caveolae [19]. Caveolin-1, the major molecular component of caveolae is known to bind various signalling molecules [20] and can regulate their activity. Thereby caveolae can function as organizers of signal transduction molecules [19], signalling organelles [21]. Caveolin-1 has been shown to bind TGF-ß receptor-I [22] and the receptor can be internalized by caveolae [23]. Our observations demonstrating that the cytoplasmic distribution of caveolin-1 is changing during Freud's adjuvant induced mesothelial EMT strongly suggest that caveolin-1/caveolae are involved in regulation of signal transduction leading to transdifferentiation.

By the 11th day the mesothelial cell layer became continuous, the integrity of these cells were evident, new basement membrane was created. Although the mesothelial cells retrieved their original squamous morphology, they still expressed ED1 and vimentin, cytokeratin was missing from their cytoplasm and there was only few caveolae present on their plasma membrane.

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