The Influence of 5-Fluorouracil on the Endothelium in Small Arteries. An Electron Microscopic Study in Rabbits

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THE INFLUENCE OF 5-FLUOROURACIL ON THE ENDOTHELIUM IN SMALL ARTERIES. AN ELECTRON MICROSCOPIC STUDY IN RABBITS

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Abstract

5-Fluorouracil (5-FU) is a widely used antineoplastic agent. 5-FU induced cardiotoxicity is a still relatively unknown side-effect of this drug. This phenomenon could be due to a direct cytotoxic effect on the endothelial cells. We tested this hypothesis in an experimental study in rabbits, by scanning or transmission electron microscopic evaluation of endothelium in small arteries (the central artery of the ear) after *in vivo* treatment with 5-FU. Both local and systemic effects of 5-FU on endothelium were studied 15, 30, 60 and 120 minutes after intra-arterial or intraperitoneal treatment. Perfusion fixation at physiological pressure and temperature was used in order to minimize damage to the endothelium during the preparation procedure. Eighteen rabbits weighing 2.5-3.0 kg were used, and 6 animals served as controls. The following parameters were evaluated: vessel wall and endothelial cell contraction, cell edema, cytolysis, occurrence of denuded areas, platelet adhesion/aggregation and fibrin formation. For the description of each parameter a scale of negative points was used. Irreversible cell damage was observed in 5-FU treated animals: disruption of the endothelial sheet and patchy exposure of the subendothelium, sometimes as a focus for thrombus formation. Our findings support the hypothesis that the thrombogenic effect of 5-FU secondary to its direct cytotoxic effect on endothelium might be one of the pathophysiological mechanisms behind 5-FU induced cardiotoxicity.

Key words: 5-Fluorouracil, cardiotoxicity, endothelium, small arteries, scanning electron microscopy, transmission electron microscopy.

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Introduction

Fluorouracil (5-FU) is an antimetabolite and one of the most commonly used chemotherapeutic agents in the treatment of cancer. It is useful, as a single drug or in combination with other antineoplastic agents, in the treatment of a variety of malignancies such as gastrointestinal cancer, breast cancer and head and neck cancer. The well known side effects of 5-FU are myelosuppression, dermatitis, mucositis, stomatitis, nausea and vomiting. Cardiotoxicity is a less commonly recognized and still relatively unknown toxic manifestation of 5-FU. Even though the first reports on the cardiac side effects of 5-FU came in the seventies (Dent and McColl, 1975), the syndrome of 5-FU induced cardiotoxicity remains a mystery. The clinical features of the syndrome include silent myocardial ischemia, angina, myocardial infarction, arrhythmia, congestive heart failure, cardiogenic chock and sudden death (Eskilsson *et al.*, 1988; Ensley *et al.*, 1989; Gradishar and Vokes, 1990). Different pathophysiological mechanisms have been suggested, such as coronary artery spasm (Burger and Mannino, 1987; Freeman and Constanza, 1988; Robben *et al.*, 1993), autoimmune-mediated injury to the cardiac cells (Stevenson *et al.*, 1977) and thrombogenic effects (Kuzel *et al.*, 1990). Another possible mechanism behind the syndrome could be a direct cytotoxic effect on the endothelial cells.

The endothelium is a monocellular layer which plays an important role in preventing thrombus formation and participates in vaso-regulatory mechanisms. Injury to the endothelium and exposure of underlying structures may induce platelet accumulation and activate the coagulation system, leading to thrombus formation (Mason *et al.*, 1977). Accordingly, vascular and heart endothelium are thought to be sites of considerable importance in the pathophysiology of the cardio- and vascular toxicity of various drugs. To test our hypothesis about a direct cytotoxic effect of 5-FU on the endothelial cells and, in this way, further highlight the pathophysiology of 5-FU cardiotoxicity, we carried out a study in rabbits. The aim of this study was to evaluate, by scanning and
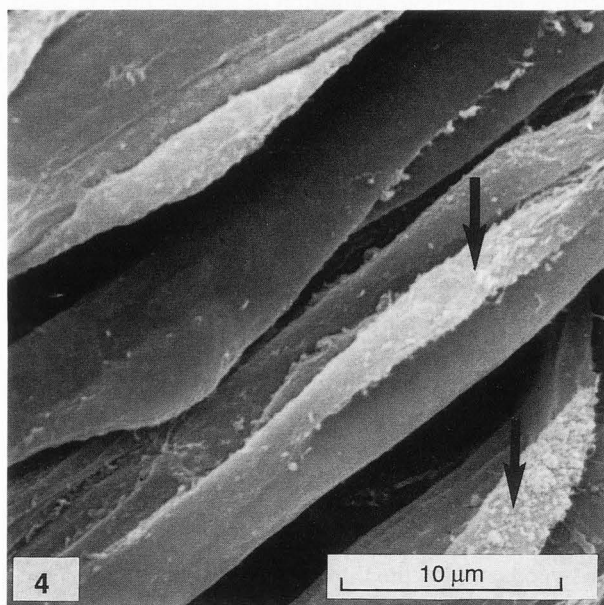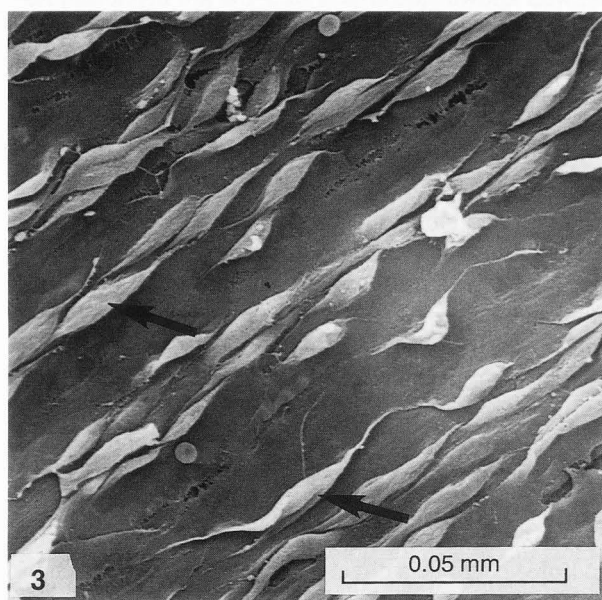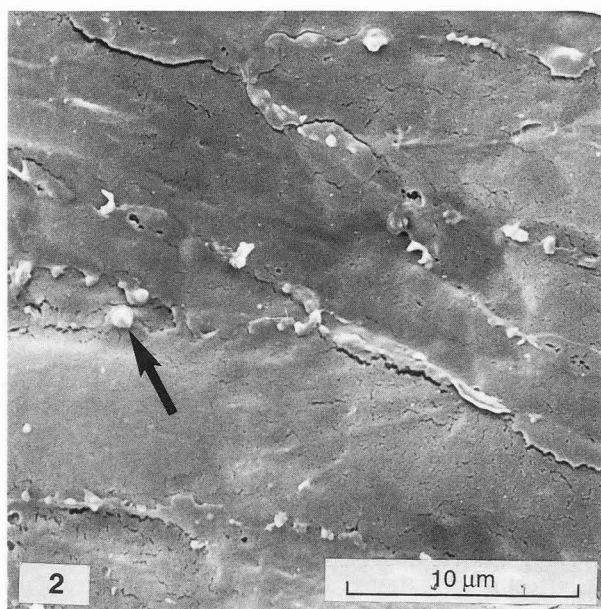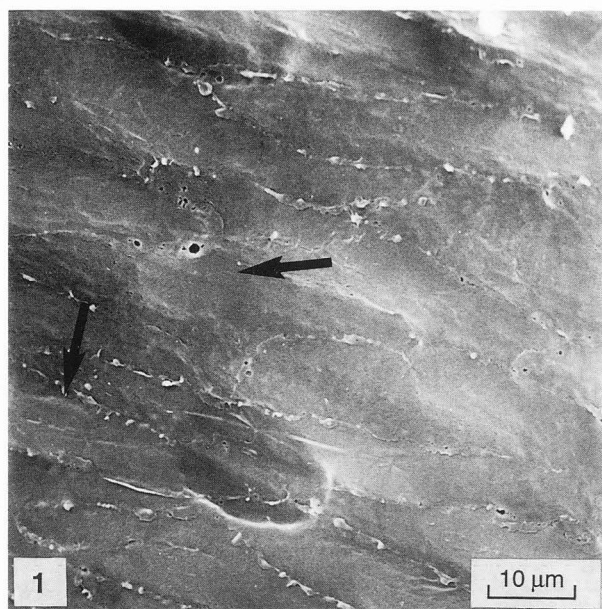
Figure 1. Relaxed arterial vessel wall. A homogeneous pattern of relaxed, flat endothelial cells arranged in the direction of the blood flow. Note the gentle protrusion of cell nuclei (arrow). The depressed area near the centre seems to be caused by the electron beam. Control.

Figure 2. The relaxed vessel with a regular sheet of endothelial cells which sometimes feature microvilli at their edges ("edgevilli," arrow). Endothelial cells overlap at cell borders. Control.

Figure 3. Contracted endothelial cells lying on top of a slightly folded wall. The cells generally seem to preserve their connection to each other, but gaps are seen at some cell borders. Contracted cell (arrow). Control.

Figure 4. Contracted vessel wall with contracted endothelial cells and, in some cells, microvilli (arrow), varying in number and shape from one cell to another. The cell borders are difficult to identify but there is no evidence of cell detachment or denuded areas. Control.
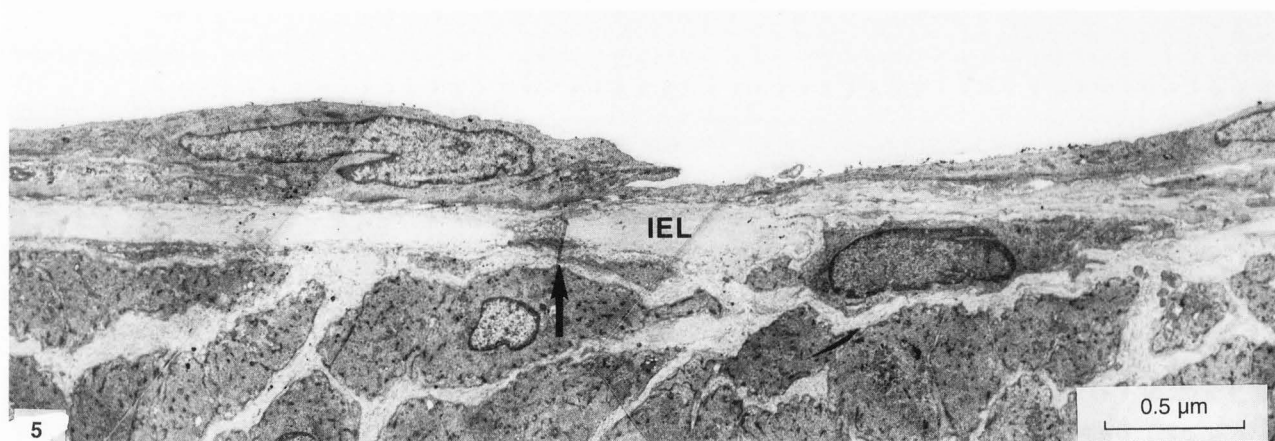
Figure 5. A single-layered endothelial cell sheet rests on the internal elastic lamina (IEL). The cell edges overlap each other. The region of the endothelial cell nucleus protrudes into the vessel lumen. The opening in the internal elastic lamina (IEL), lying under the site of overlapping cell edges, seems to create a channel (arrow) between the vessel wall media and the lumen of the vessel. Control.

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transmission electron microscopy, the endothelium in small arteries after *in vivo* treatment with 5-FU.

It has previously been shown that the technical procedure used in preparing vessels for histological and electron microscopy is of utmost importance and that relaxed fixed vessels are required for a correct evaluation of endothelium (Wieslander, 1987). The preparation of unfixed vessels, removed from the animal, by placing them in fixative, causes contraction of the vessel in both length and diameter and is therefore unacceptable. Damage to the endothelium can be the result not only of cytotoxic drug effects but also of unphysiological conditions during the preparation procedure, e.g., inappropriate pressure or temperature at perfusion fixation. Thus, perfusion fixation "*in vivo*" is a preferable and more correct method (Wieslander, 1987).

We report here on experiments in rabbits, showing the early effects of 5-FU on the endothelium of small arteries.

Material and Methods

Animals

Eighteen male rabbits, weighing 2.5-3.0 kg, were kept on a standard pellet diet and given water ad libitum. They were anaesthetized with pentobarbital (Mebumal 60 mg/ml, ACO Läkemedel, Sweden) administered in a marginal ear vein; anaesthesia was maintained by intermittent injections of the same compound.

Small catheters were introduced into the central arteries of one or both ears. These catheters were used for the administration of 5-FU and perfusion fixation of the vessels. In the control group, the central arteries of only one ear were used, the other ear was used for injections of pentobarbital and was omitted from the study.

Artery diameters were 0.6-1 mm. Six rabbits received no treatment and served as controls.

Twelve rabbits were treated in two different ways with 5-FU (Fluracedyl 50 mg/ml, Nycomed, Norway) at a dose of 25 mg/kg. Six animals (group A) were injected with 5-FU intraperitoneally (i.p.) and the other six rabbits (group B) intra-arterially (i.a.) using the central artery of one ear. In the group of rabbits treated i.p., vessel specimens were taken after 30 minutes, 60 minutes and 120 minutes, whilst in the other group, treated i.a., after 15 minutes, 30 minutes and 60 minutes following 5-FU injections. The slow drug absorption rate from the peritoneum into the vascular space was the reason for taking the first sample later in this group (Iwamoto *et al.*, 1984)

Perfusion fixation

In order to minimize vascular spasm and secure a high and uniform blood flow, the rabbits were kept on a thermostatically regulated heating-pad at a temperature of about 38.5°C. With the same aim, small amounts of lidocain (Xylocain 10 mg/ml, Astra, Sweden) were applied locally at 5 minutes and immediately prior to fixation.

Perfusion fixation was performed in group A at 30 minutes, 60 minutes and 2 hours after treatment with 5-FU, at physiological pressure (120 mm Hg) and temperature (38.5°C), and continued for 15 minutes. The animals were killed seconds before the start of the procedure. In group B, samples were also taken at 15 minutes. The fixation solution was composed of 400 ml 0.2 mol/l phosphate buffer + 50 ml 50% glutaraldehyde + 90 ml 20% dextran T 70 + 455 ml distilled water. Vessel specimens were taken from central arteries approximately 2 cm distal to the catheter tip and kept immersed overnight in the fixative.

Table 1. The scores for the phenomena observed in the control vessels. The scores are the sums of negative points in each group and express the severity and extent of damage to the endothelium.

No.	Vessel contrac- tion	Endothe- lial cell contraction	Denuded areas	Endothe- lial cell edema	Endothe- lial cytolysis	Platelet adhesion	Platelet aggre- gation	Fibrin forma- tion	Erythro- cytes	Micro- villi	Edge- villi	Total Negative Score
1	-0.5	-0.25	0	-1.5	0	0	0	0	0	-0.25	-0.5	-3
2	0	0	0	0	-1.5	0	0	0	0	0	-2	-3.5
3	-0.5	-1.75	0	-0.5	-0.75	0	0	0	0	0	0	-3.5
4	-1.5	-1.5	0	-2.5	-3	0	0	0	0	-0.25	-0.25	-9
5	0	-0.25	0	0	-3	0	0	0	0	0	-0.25	-3.5
6	0.25	1	0	0.5	0.75	0	0	0	0	0.5	1	4

There were three groups of vessel specimens: those obtained from the central artery of the ear of animals treated with i.p. injection of 5-FU, group A; those from the untreated ears of rabbits injected with 5-FU i.a., group BI. The third group, BII, included vessel specimens obtained from the central artery of the ear used for i.a. 5-FU injections. In groups A and BI, the systemic effect of 5-FU on the endothelium was studied while in group BII, the local effect of 5-FU on the vessel endothelium was estimated.

Preparation for scanning electron microscopy (SEM)

Specimens for SEM were fixed in 2.5% glutaraldehyde (in 0.15 M cacodylate buffer, pH 7.3) for 12 hours, followed by postfixation in 1% osmium tetroxide in 0.15 M cacodylate buffer for one hour. After dehydration in a graded series of ethanol and critical point drying, the specimens were sputter-coated with gold and examined in a Philips 515 SEM scanning electron microscope operated at an accelerating voltage of 20 kV. Four standard magnifications were used: 500, 1000, 2500 and 5000. Each vessel was photographed at three random locations in the proximal, central and distal part of the sample.

Preparation for transmission electron microscopy

The samples were fixed and dehydrated in ethanol in the same manner as for SEM preparations. The samples were then embedded in Vestopal W or Epon. Ultrathin sections were cut and stained with lead citrate or uranyl acetate, and examined in a JEOL 2000X transmission electron microscope (TEM).

Evaluation of specimens

The authors independently evaluated all specimens using the following parameters: vessel-wall contraction, contraction of endothelial cells, occurrence of denuded areas, endothelial cell edema, endothelial cell cytolysis, platelet adhesion and aggregation, fibrin formation, microvilli formation and the occurrence of erythrocytes.

Scores

For the description of each parameter, a scale of negative points (0.25-3.0) was used. The points expressed the ratio between the area covered with damaged and normal endothelium respectively. The scores for denuded areas, platelet accumulation and fibrin formation were multiplied by three and those for endothelial cytolysis by two, because these reactions were judged to be more severe than e.g., endothelial cell contraction.

Results

Control group

Scanning electron microscopy. The *in vivo* perfusion fixation method at physiological pressure and temperature produced relaxed vessel samples in the majority of cases. The relaxed vessels had unfolded walls, with a homogeneous endothelial sheet, consisting of oval, flat, regular endothelial cells with slightly protruding nuclei (Fig. 1). The endothelial cells were slightly overlapping at their edges and were oriented in the direction of the blood flow. Many cells presented microvilli at their edges, so-called "edgevilli" (Fig. 2). Only in one case was there apparent contraction in the vessel wall with regular folds and ridges. In some areas, the contracted vessel wall was covered with contracted endothelial cells (Fig 3), but the occurrence of contracted endothelial cells could even be observed in completely relaxed vessels. The contracted endothelial cells had a preserved connection to each other and disruption of the endothelial sheet with formation of denuded areas was rare. Occasionally the endothelial cells appeared to be swollen, but edema was observed only in minor areas of the samples. In some areas the swelling was relatively severe, leading to cytolysis usually expressed as irregularity of the cell wall and as the formation of microvilli (Fig. 4). However, cytolysis could be observed even in cells without evidence of edema and could appear in different manifestations. There was no evidence of platelet
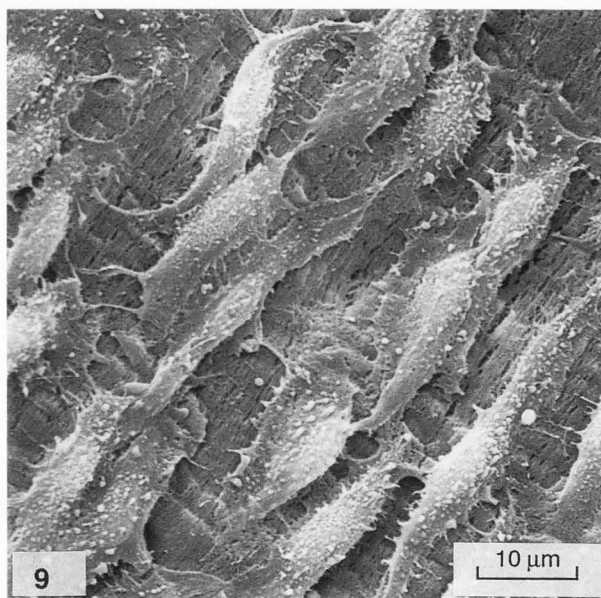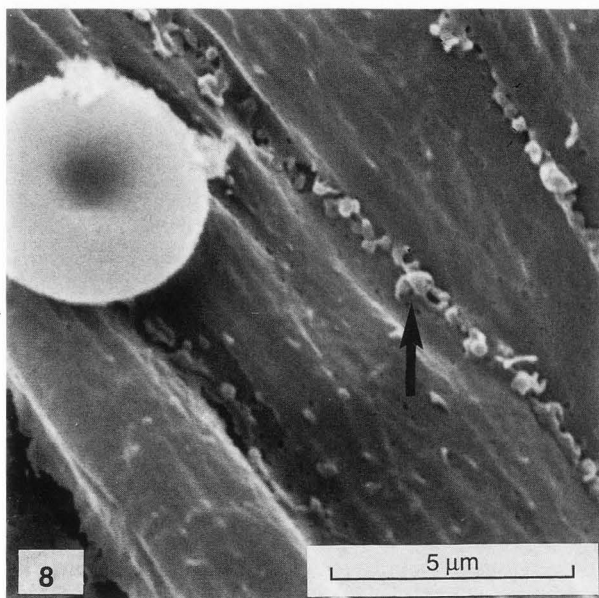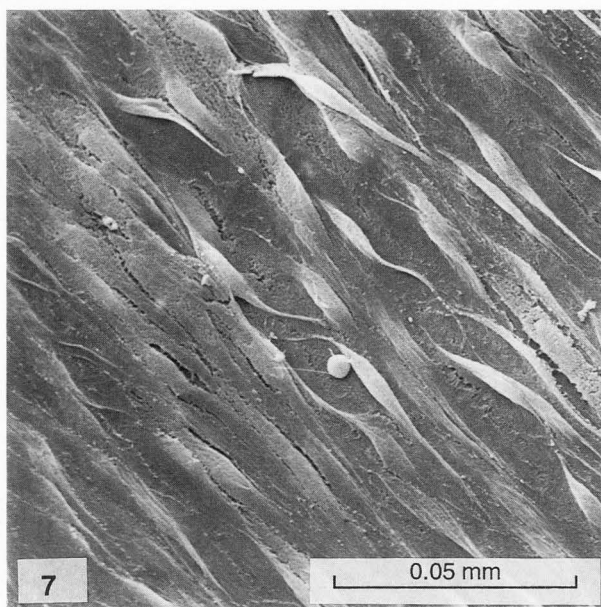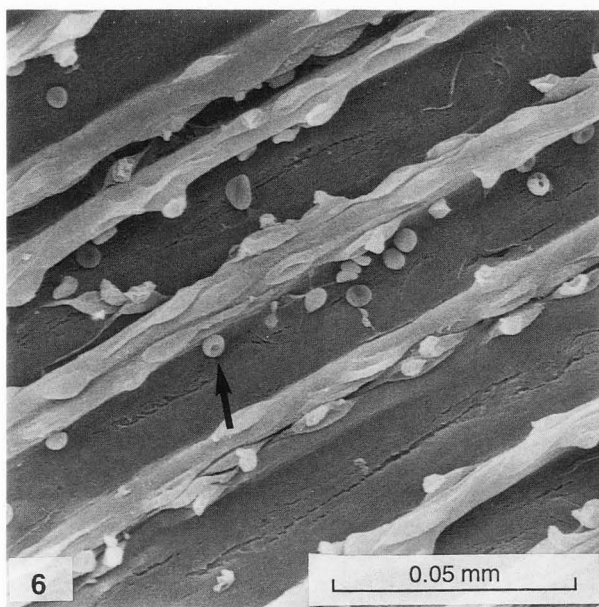
Figure 6. A contracted arterial vessel wall with a pattern of regular folds. Contracted endothelial cells lying on top of the folds. Erythrocytes close to ridges (arrow). 60 minutes after i.p. 5-FU injection.

Figure 7. A relaxed arterial vessel wall presenting a mixture of "normal" and slightly contracted endothelial cells. 30 minutes after i.a. 5-FU injection, systemic effect.

Figure 8. The vessel intima with endothelial cells presenting microvilli at their edges (edgevilli). In the corner, an erythrocyte (arrow) is present. 120 minutes after i.p. 5-FU injection.

Figure 9. The intima of the relaxed arterial vessel wall with contracted endothelial cells creating denuded intercellular gaps. Massive microvilli formation especially over the cell nuclei. Some cells seem to be undergoing cytolysis and are partly detached from the underlying structures. Platelets or fibrin are not to be seen. 15 minutes after i.a. 5-FU injection, local effect.
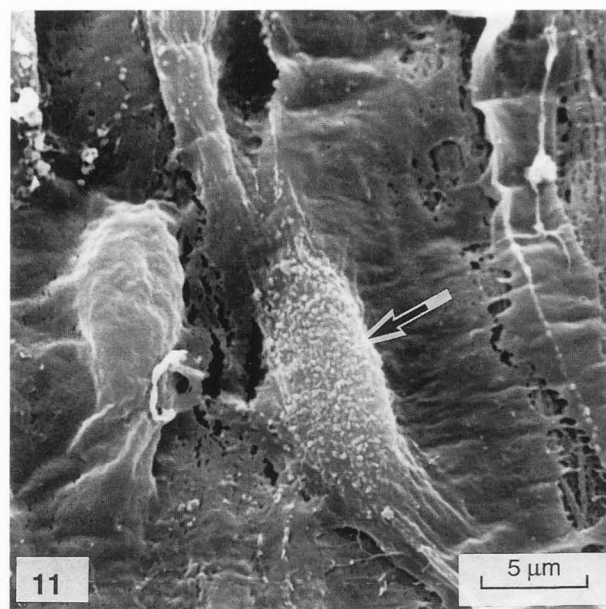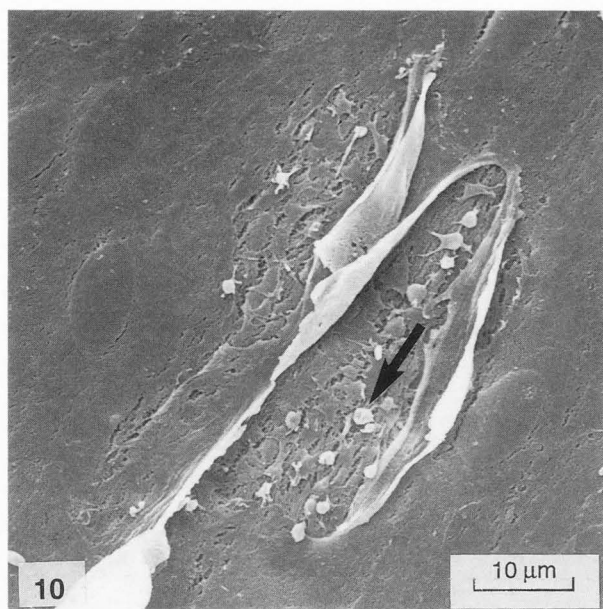
Figure 10. The site of a local complete disruption of the endothelial sheet, revealing underlying structures forming a denuded area. Platelet adhesion in the damaged area indicates an *in vivo* phenomenon. Relaxed artery (arrow). 30 minutes after i.a. 5-FU injection, systemic effect.

Figure 11. Contracted endothelial cells in a relaxed vessel. The intercellular spaces form denuded cracks and fissures. One cell (arrow) shows massive diffuse microvilli formation. 15 minutes after i.p. 5-FU injection.

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adhesion, aggregation or fibrin formation in our material. The occurrence of erythrocytes was rare.

The severity and extent of damage to the endothelium was described by the mean of scores. Each phenomenon observed was estimated separately and then, the sum of negative points was registered for each sample (Table 1). The negative scores were low in these control samples, varying from -3.0 to -9.0 (0-60) due to the fact that no severe damage such as platelet accumulation or fibrin formation was seen.

Transmission Electron Microscopy. The micrographs show the arteries in longitudinal section. Most samples in our material presented unfolded, relaxed vessel walls, showing the endothelial cells resting on top of the internal elastic lamina. The spindle-formed relaxed endothelial cells were arranged in a single cell layer. The cells were closely connected to each other and overlapped at their edges (Fig. 5). No disarrangement or disruption of the endothelial cell layer, exposing the underlying structures, was seen. One of the most characteristic features of the endothelial cells was the protrusion of their nuclei into the lumen of the vessel (Fig. 5). The nuclear membranes were regular and the nucleoplasm homogeneous. Nucleoli were observed in most of the nuclei. Despite the protrusion of the nuclei, the endothelial cells were rather flat and of regular shape

and size, with no pronounced variation from one cell to another. However, some irregularities in the cell membrane, in the form of the discrete, finger like protrusions from the cell surface (microvilli), were observed. The microvilli could be seen anywhere on the surface of the cytoplasm but were most highly concentrated in the areas adjacent to the cell borders and therefore, assessed to be equivalent to the edgevilli seen in scanning electron micrographs. Except for a very few microvilli, there were no manifestations of cytoplasmic changes suggesting cell lysis, neither were cell features compatible with cell edema to be seen. As with SEM, in material evaluated by TEM, there was no evidence of platelet accumulation or fibrin formation. There were few erythrocytes. The endothelial cells were adjoined at their basal surfaces to the internal elastic lamina (IEL): a continuous, non-cellular sheet separating the endothelium from the underlying connective tissue. The IEL generally presented a homogeneous layer, occasionally becoming thinner and discontinuous, creating irregularly shaped holes in the membrane (Fig. 5). These holes seemed to occur in close proximity to endothelial cell borders, lying just under the sites of overlapping cell edges and creating channels between the lumen of the vessel and the adjacent tissue.

There was a smooth muscle cell layer underneath and closely appositioned to the internal elastic lamina.
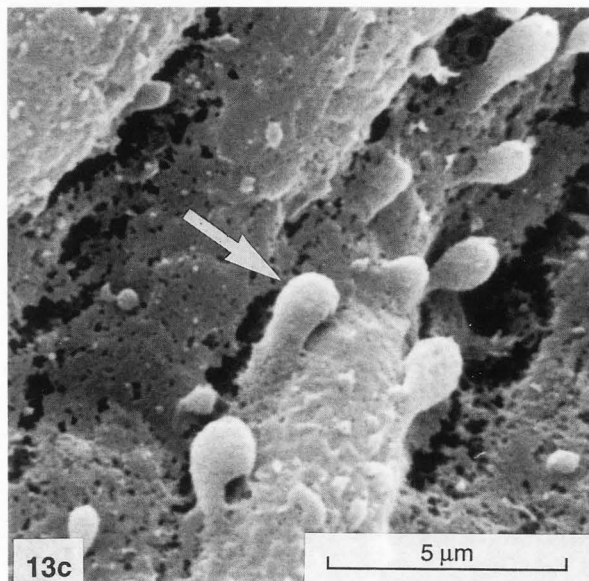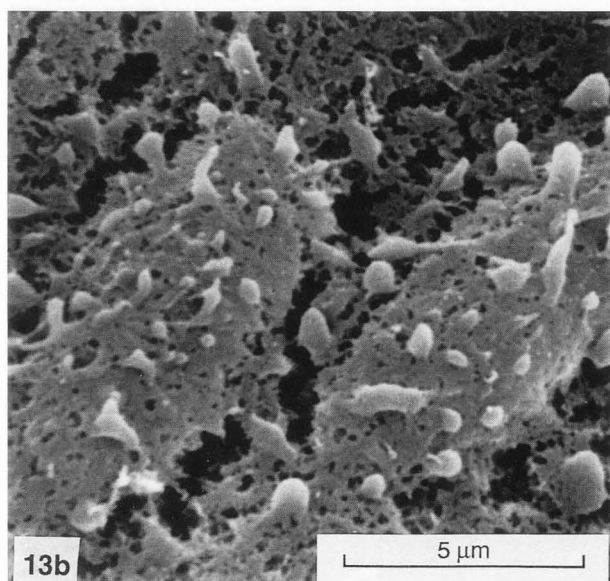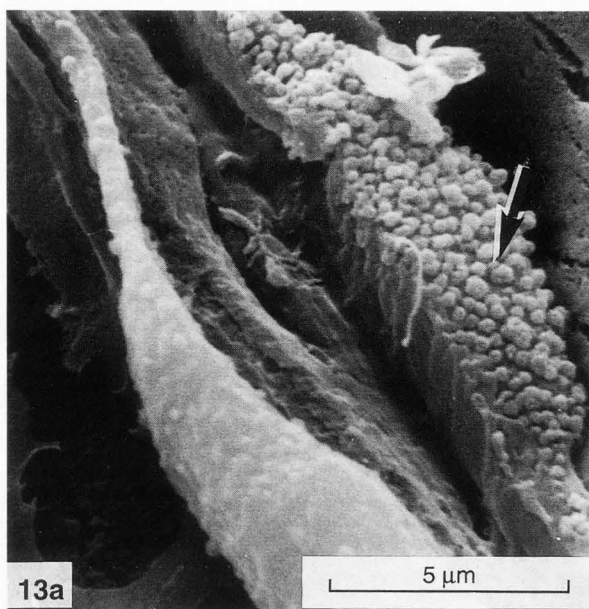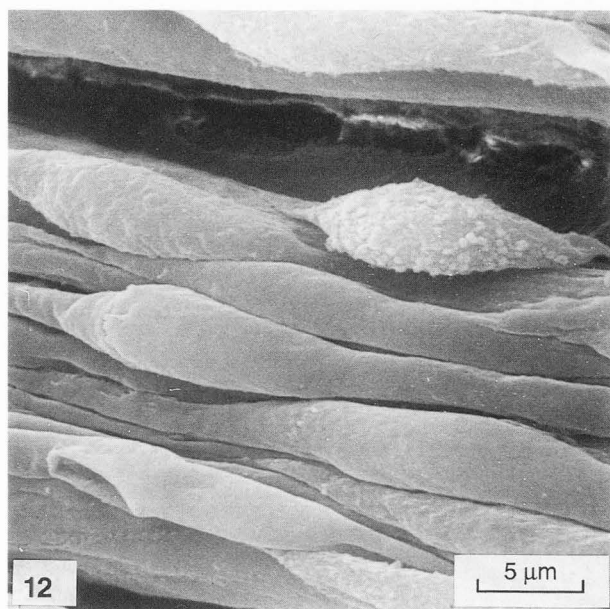
Figure 12. Contracted vessel wall with some edematous endothelial cells. Swollen cells with irregular cell surfaces. Thirty minutes after i.p. 5-FU injection.

Figure 13. Endothelial cells, demonstrating various forms of microvilli at their surface. (a) A group of microvilli, with the appearance of small, closely packed drops (arrow). 60 minutes after i.p. 5-FU injection. (b) Endothelial cells with isolated, large microvilli scattered over the whole surface of the cell. 15 minutes after i.a. 5-FU injection, local effect. (c) Endothelial cells with microvilli in the form of large, bizarre clubs, mainly located at the periphery of the cell (arrow). Cells are undergoing cytolysis. 15 minutes after i.a. 5-FU injection, local effect.

Treated groups

Scanning electron microscopy. Contrary to what was seen in normal, untreated vessels obtained by the perfusion fixation method and described above, the majority of samples from the rabbits treated with 5-FU presented contracted vessel walls. Folds and ridges of varying degrees were seen, always covered by contracted endothelial cells lying on top of the folds (Fig. 6). The contracted endothelial cells could occur independently of vessel wall contraction however, and were also seen in
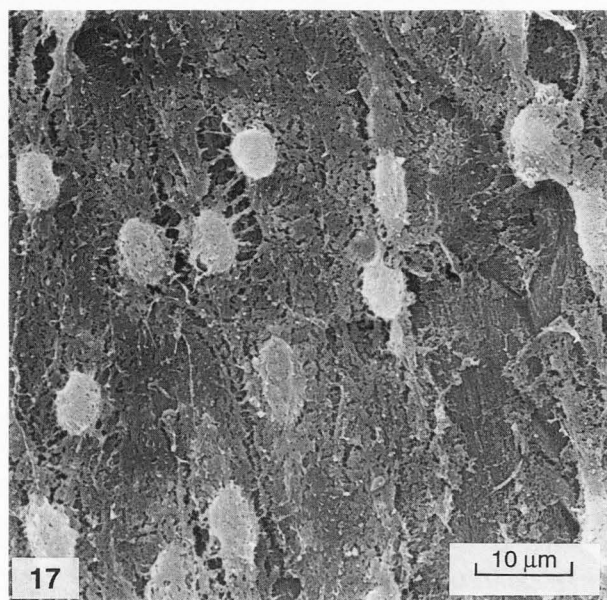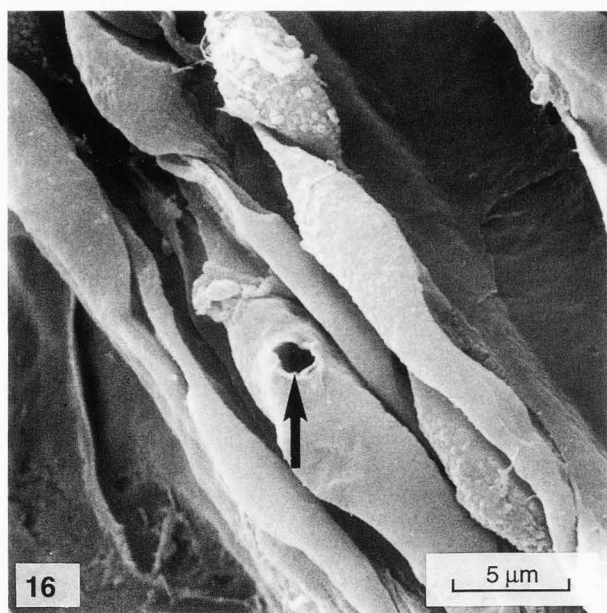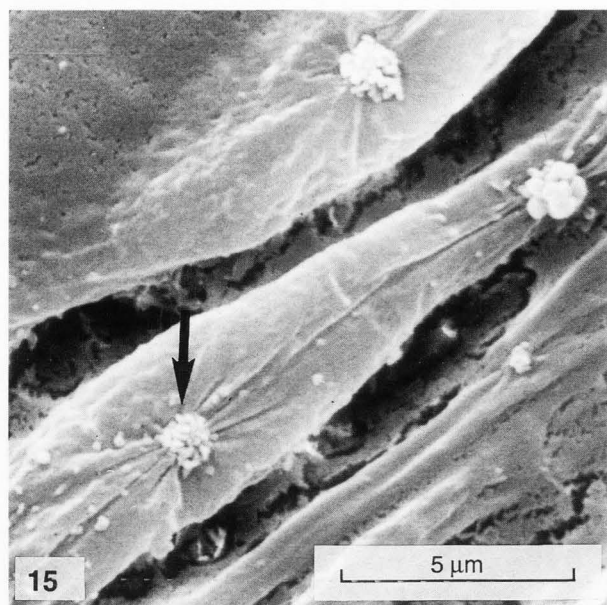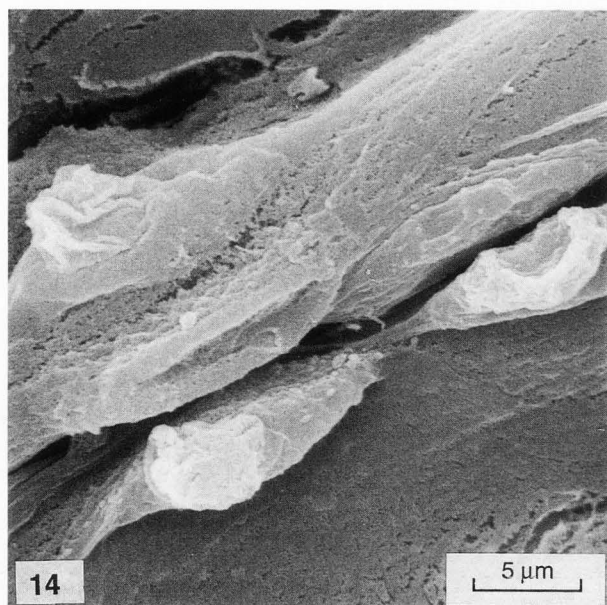
Figure 14. The ridge of a contracted arterial vessel wall covered by endothelial cells in a state of cytolysis, demonstrated by severe contraction and shrinkage of the cell membrane over a protruding nucleus. 60 minutes after i.p. 5-FU injection.

Figure 15. Severe cell membrane contraction in the central part of the cells forming a "rosette" (arrow). 30 minutes after i.a. 5-FU injection, systemic effect.

Figure 16. A ridge in a contracted vessel wall. One endothelial cell membrane shows perforation as a manifestation of cell lysis (arrow). Protrusion of nuclear material? 120 minutes after i.p. 5-FU treatment.

Figure 17. Diffuse cytolysis of endothelial cells in a relaxed vessel wall. No normal structure identifiable. The dense material seems to represent the remains of nuclei. 30 minutes after i. a. 5-FU injection, local effect.
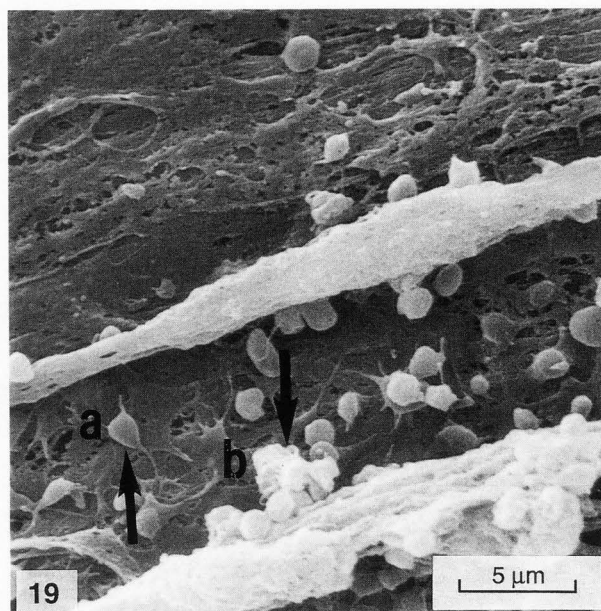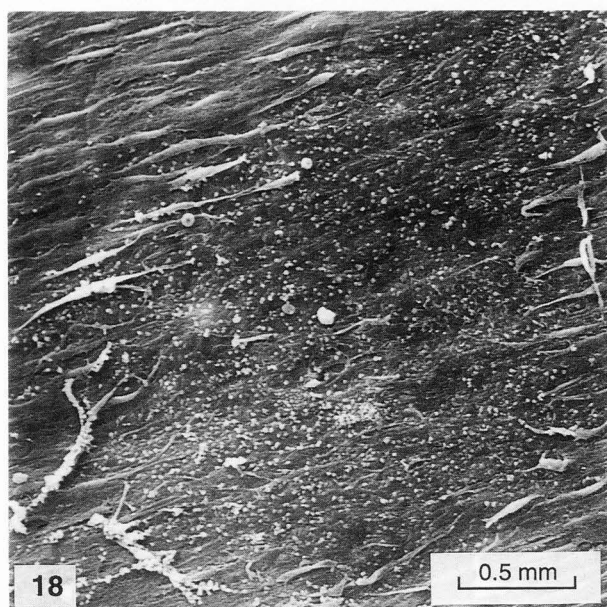
Figures 18 and 19. Relaxed arterial vessel wall at different magnifications. Intima with local endothelial sheet disruption and platelet adhesion (arrow a) and aggregation (arrow b). The endothelial sheet in other areas seems intact. 60 minutes after i.p. 5-FU treatment.

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the relatively relaxed areas of the vessels (Fig. 7). The relaxed, unfolded vessel walls with a homogeneous pattern of flat, regular endothelial cells with gently protruding nuclei, so characteristic of the normal intima, were not seen in this material. Most areas presented a wide panorama of different endothelial cell changes, frequently varying in appearance and grade of severity from one cell to another. Microvilli, present at the edges of cells, so called "edgevilli", were observed in most of the cells with no other evidence of severe damage (Fig. 8). Furthermore, edgevilli were not seen in cells with an ongoing disruption of cell connections. Where cells had contracted, creating denuded intercellular gaps, there were thin, filamentous processes extending between neighbor cells at the sites where edgevilli usually were seen. These seemed to be created by the withdrawal of the cells from each other (Fig. 9). Occasionally, connections between cells were still preserved by these processes, whereas other areas presented a complete disruption of the endothelial sheet (Fig. 10). Sometimes cell borders could not be distinctly discerned, the intercellular spaces taking the form of cracks and fissures (Fig. 11). Generally speaking, preserved cell connections, obvious in the normal intima, were less common here and complete disruption of the endothelial sheet, revealing underlying structures and forming denuded areas, was observed rather frequently.
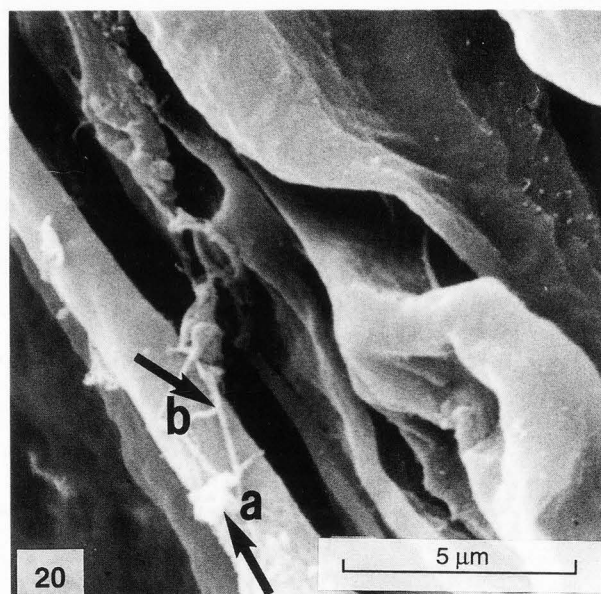
Figure 20. A ridge in a contracted vessel wall. Intima with platelet accumulation (arrow a) and fibrin formation (arrow b). One hundred and twenty minutes after i.p. 5-FU injection.

The contracted endothelial cells occasionally appeared to be swollen (Fig. 12), even if this feature was not as obvious as in the normal material. On the other hand, microvilli formations were much more widespread here and manifested a considerable morphological variation from one cell to another. They could have the form of small, closely packed drops (Fig. 13a) or be more isolated, bigger and scattered over the whole surface of

Mean value, score

Figure 21. The influence of 5-FU treatment on the appearance of endothelial cells in small arteries, described by mean of scores, in relation to time.

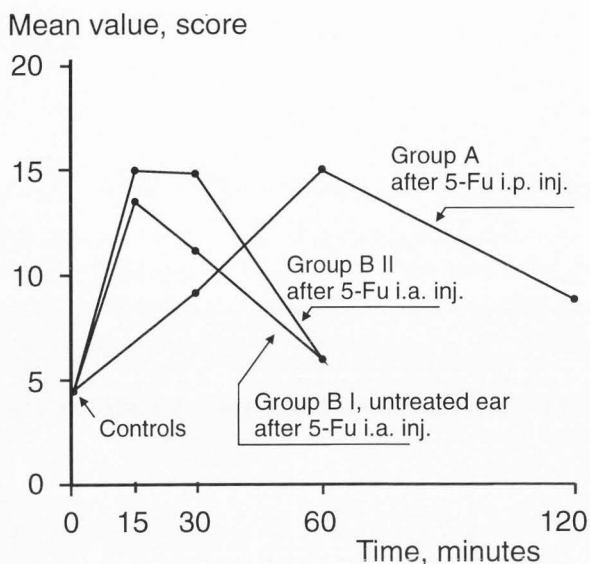the cell (Fig. 13b). Occasionally they appeared as large, bizarre clubs, usually located at the periphery of the cell surface (Fig. 13c). In some areas, microvilli formation was pronounced, with evidence of confluence, going over into cytolysis, no normal cell surface remaining. Furthermore, cytolysis could present a wide variety of morphological appearances. Irregularities of the cell membrane, protrusion of the cell nucleus and membrane shrinkage were seen (Fig. 14), sometimes followed by severe cell membrane contraction in the central part of the cell forming a "rosette" (Fig. 15). This peculiar presentation of cytolysis was frequently found in the present material. Cell membrane perforation (Fig. 16), disruption and, in the end, the total dissolution of cells, to the point where no normal structure could be identified (Fig. 17) was observed. This last mentioned extreme form of cytolysis was occasionally associated with platelet accumulation, varying in degree from one area to another and presenting adhesion or both adhesion and aggregation (Figs. 18 and 19). In addition, the thrombocyte accumulation was, in some areas, associated with fibrin formation (Fig. 20). These phenomena, i.e., the occurrence of platelet adhesion or aggregation and fibrin formation, were not seen in the normal material described before.

All of the above-mentioned manifestations of endothelial cell damage were seen in all three groups of "treated" vessel specimens, varying in severity and extent from one group to another.

Each pathological phenomenon was estimated separately by mean of scores; the sum of negative points was evaluated for each sample and then the mean scores worked out for each group of vessel samples and each time respectively. The scores varied from 8.5 to 15.0 in group A, from 6.0 to 13.5 in group B I and from 6.0 to 15.0 in group B II (Fig. 21).

Transmission electron microscopy. The micrographs show the image of arterial walls in longitudinal section. Contrary to what is seen in normal material, but in agreement with the SEM findings, the majority of samples taken from the 5-FU treated animals, obtained by the perfusion fixation method and studied by TEM, showed more or less contracted vessel walls. These arterial walls had the appearance of distinct, wave-like folds involving the internal elastic lamina (IEL) and underlying connective tissue (Fig. 22). The relaxed, spindle-formed, homogeneous endothelial cells, typical for the normal material, overlapping at their edges and arranged in a single cell layer, were not observed here. Instead, almost all endothelial cells demonstrated varying degrees of disarrangement and a multitude of changes in cell membrane, cytoplasm, and nuclei. Endothelial cell contraction was most evident in the samples presenting a high degree of vessel wall contraction, even if this feature was also observed in fairly relaxed vessels. In the contracted samples, endothelial cells lay on top of the folded vessel wall (Fig. 22), occasionally still connected to each other, but usually with a total discontinuity of cell contacts. The edgevilli and endothelial cell edema, occasionally seen in SEM examination, were not observed here. Furthermore, in the TEM examination there was no evidence of the filamentous processes extending between neighbouring cells, which had been observed in SEM. The TEM examination revealed a multitude of microvilli manifestations, varying in form and size from one cell to another, featuring small, drop-like villi (Fig. 23), bigger, thinner and longer ones (Fig. 22) and huge, bizarre clubs, located at the periphery of the cell surface (Fig. 22). Apart from villi formation, cell membrane damage manifested itself as irregularity, blebbing, disruption, and finally, total dissolution of the membrane. Endothelial cell lysis showed itself as vacuolization of the cytoplasm and changes in cytoplasmic organelles, such as increased numbers and accumulation of mitochondria (Fig. 24) and swelling of the endoplasmic reticulum (Fig. 25). Fragmentation of the cytoplasm often resulting in the total breakdown of the endothelial cell (Fig. 26), with or without previous cell detachment, was seen. This phenomenon was common in this material. In almost all of the studied cells, there were multilobulated nuclei with peripheral chromatin accumulation and an increased number of distinctly marked nuclear pores (Fig. 23). Occasionally, condensation of
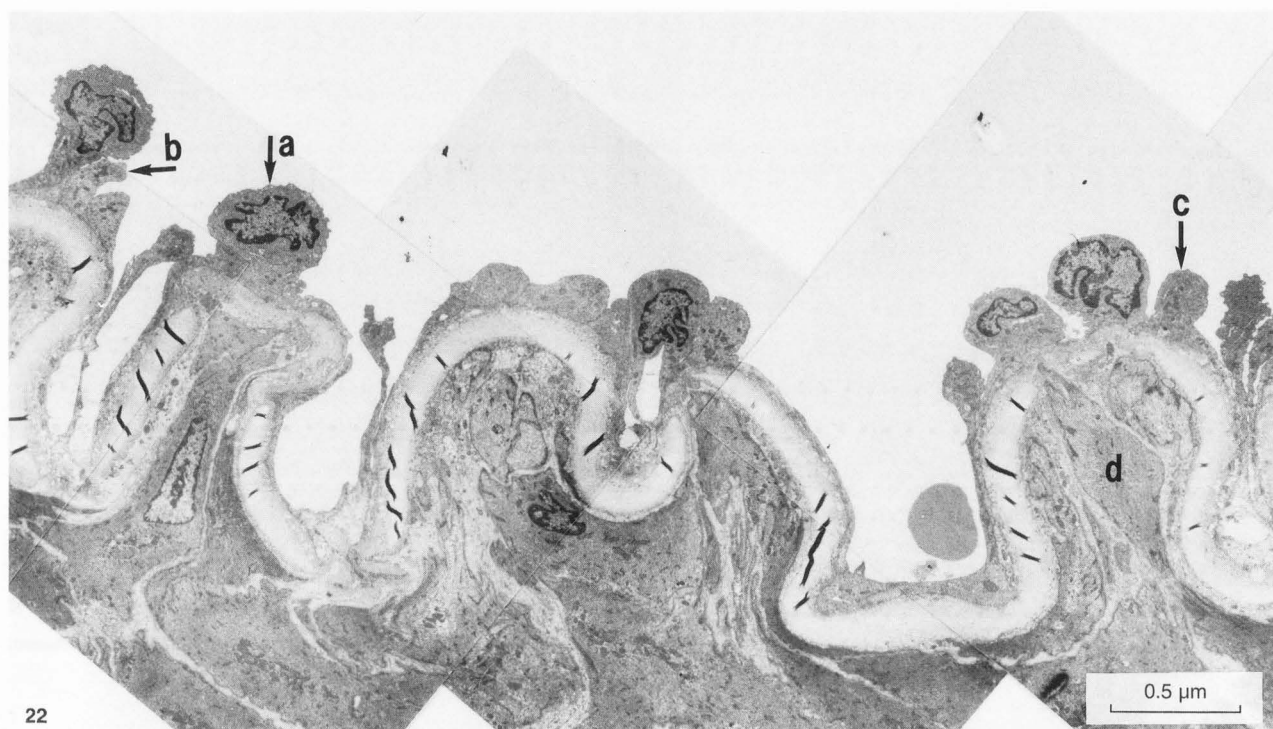
Figure 22. Contracted arterial vessel wall. Contracted endothelial cells lying on top of folds (arrow a), presenting pronounced, isolated microvilli (arrow b) and "club" villi (arrow c). Cell coming through an opening in the internal elastic lamina (d). 30 minutes after i.p. 5-FU injection.

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chromatin was seen in cells undergoing lysis (Figs. 25 and 26). Another manifestation of nuclear damage was its total fragmentation in the damaged, vacuolized endothelial cell.

The TEM appearance of the 5-FU exposed arterial endothelium was characterized by the frequent occurrence of cell detachment and/or complete cell lysis, exposing the underlying structures. Many areas of the vessel walls, both contracted and relaxed, presented an exposed IEL (Fig. 27). Occasionally, wall damage was deeper, involving the IEL which seemed totally destroyed and discontinuous (Fig. 28). In this case, underlying tissues were edematous, manifested as increased intercellular spaces (Fig. 28). Another new phenomenon observed in this material was the presence of cells reminiscent of smooth muscle cells. They lay close to, or just underneath the damaged cells, and in close proximity to the openings in the IEL, possibly in the process of coming through these openings (Fig. 27). The features described above were all seen in samples representing all three groups and all of the time intervals after 5-FU treatment studied in our material. The frequency and severity of damage varied from one area to another and,

generally, seemed to correspond to the pattern of damage described in SEM when the means of scores were compared.

Discussion

The incidence of clinically apparent 5-FU cardiotoxicity varies in different materials and has so far not been clearly documented. In a retrospective review (Labianca et al., 1982) based on 1083 patients who received 5-FU, alone or in combination treatment with other agents, an incidence of adverse cardiac effects of 1.6% was reported. However, an incidence as high as 18% has been seen in other material (Eskilsson et al., 1988). A lot of effort has been put into trying to explain the underlying mechanism of 5-FU cardiotoxicity, but until now there have been few animal studies investigating the effects of 5-FU on the heart. Edema of myocardial fibres and loss of striation after 5-FU exposure have been described in a rat model by Levillain et al. (1972). In Japanese studies, the emphasis has been on biochemical mechanisms. For example, metabolic abnormalities in myocytes, resulting in the dysfunction of myocardium in guinea pigs
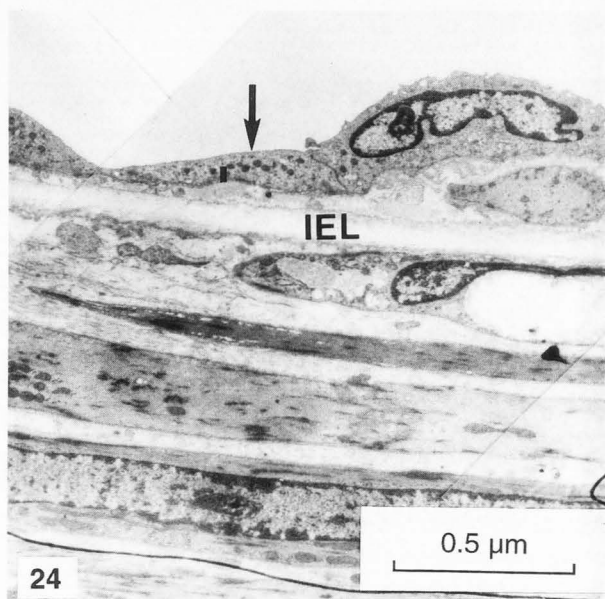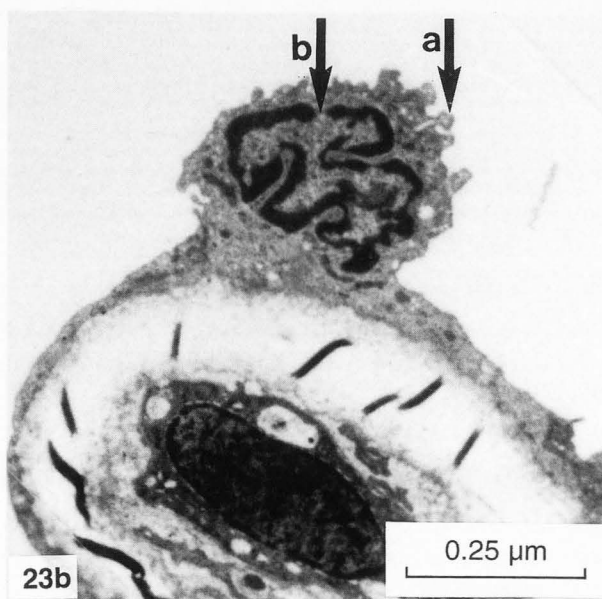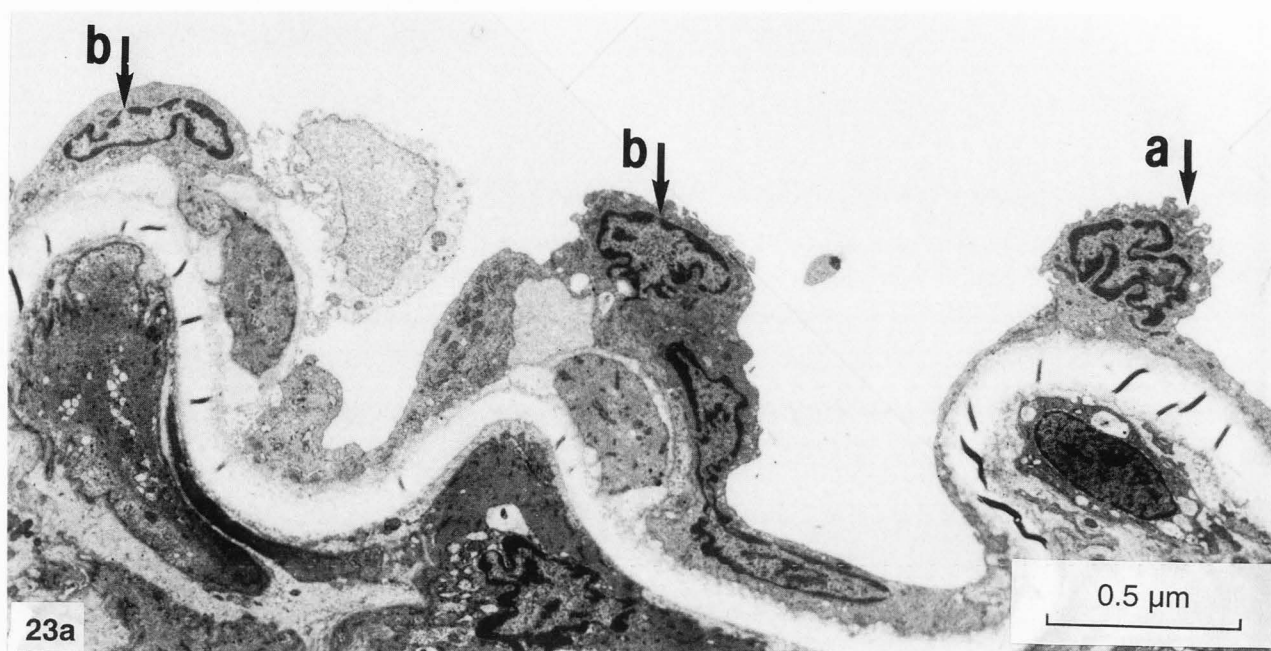
Figure 23. Contracted vessel wall; the area at the right of **Figure 23a** is presented enlarged in **Figure 23b.** Contracted endothelial cell lying on top of a fold, evidencing cell membrane shrinkage with small, drop-like microvilli on its surface (arrow a). Multilobulated nucleus with peripheral chromatin accumulation and an increased number of nuclear pores (arrow b). 30 minutes after i.p. 5-FU injection.

Figure 24. Endothelial cell lying on unfolded IEL. Irregularity of the cell membrane. Accumulation of mitochondria, which seem to have increased in number. Arrow. Multilobulated nucleus with peripheral chromatin accumulation. Sixty minutes after i.p. 5-FU injection.

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was reported by Matsubara *et al.* (1980) and Tamatsu *et al.* (1984). Satoh and Hashimoto (1983), demonstrated the positive inotropic and chronotropic effects of 5-FU on canine sinoatrial nodes and Mosseri *et al.* (1990) reported on 5-FU induced arterial vasospasm in isolated rings of rabbit aorta. So far, there have been no reports

Figure 25 (at right). Part of a detached endothelial cell. Evidence of chromatin condensation (a) and swelling of the endoplasmic reticulum (b). 15 minutes after i.a. 5-FU injection, systemic effect.
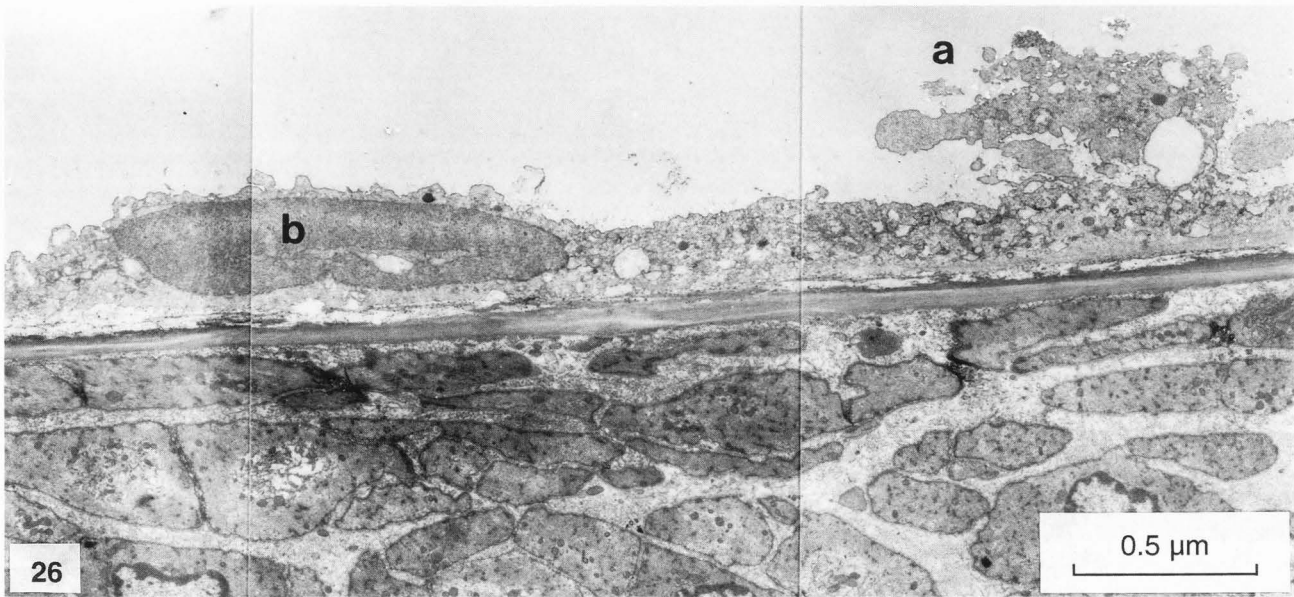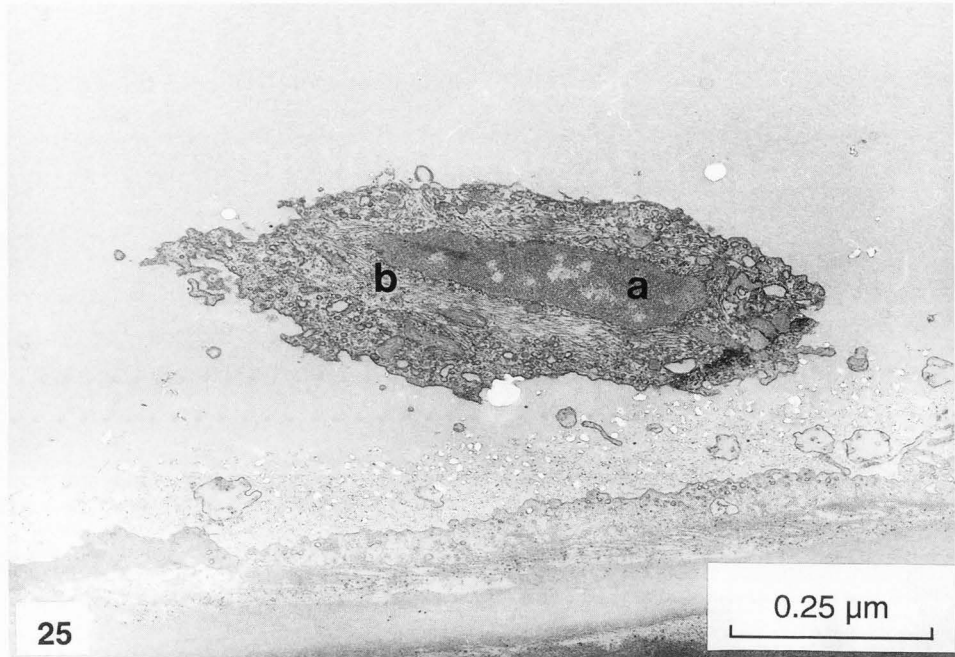

25

0.25 µm

26

0.5 µm

Figure 26. Total lysis of an endothelial cell with remnants of vacuolized, fragmented cytoplasm (a) and almost a nucleus exhibiting pronounced condensation of chromatin (b). 15 minutes after i.a. 5-FU injection, local effect.

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on animal studies describing the effect of 5-FU on the cardiac or vascular endothelium, sites which seem to be involved in the phenomenon of 5-FU cardiotoxicity and which have been studied in this material.

This study examines two aspects of the effect of 5-FU on arterial endothelium, namely the local toxic effect and the systemic effect. When compared with normal material, obtained by the perfusion fixation method, this material demonstrated a much wider panorama of morphological changes in all of the studied groups. The appearance of the endothelium did not differ from group to group in terms of quality and quantity of observed features (Fig. 21). The only differences noted in this material were concerned with
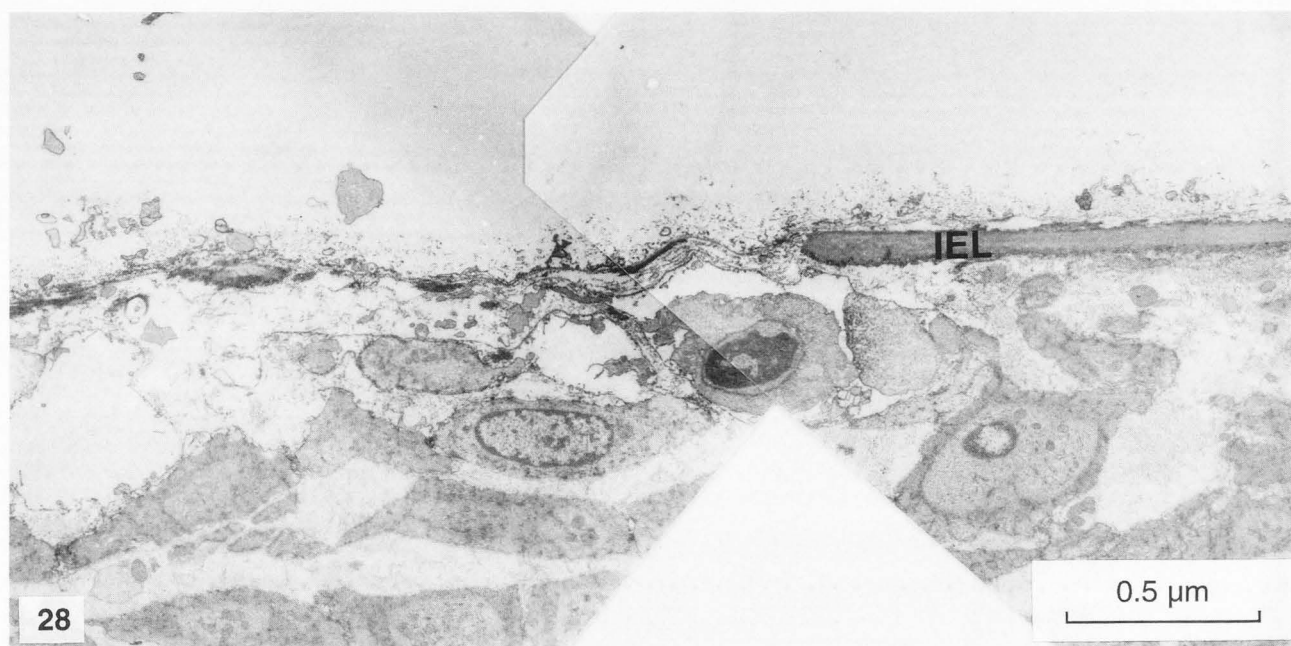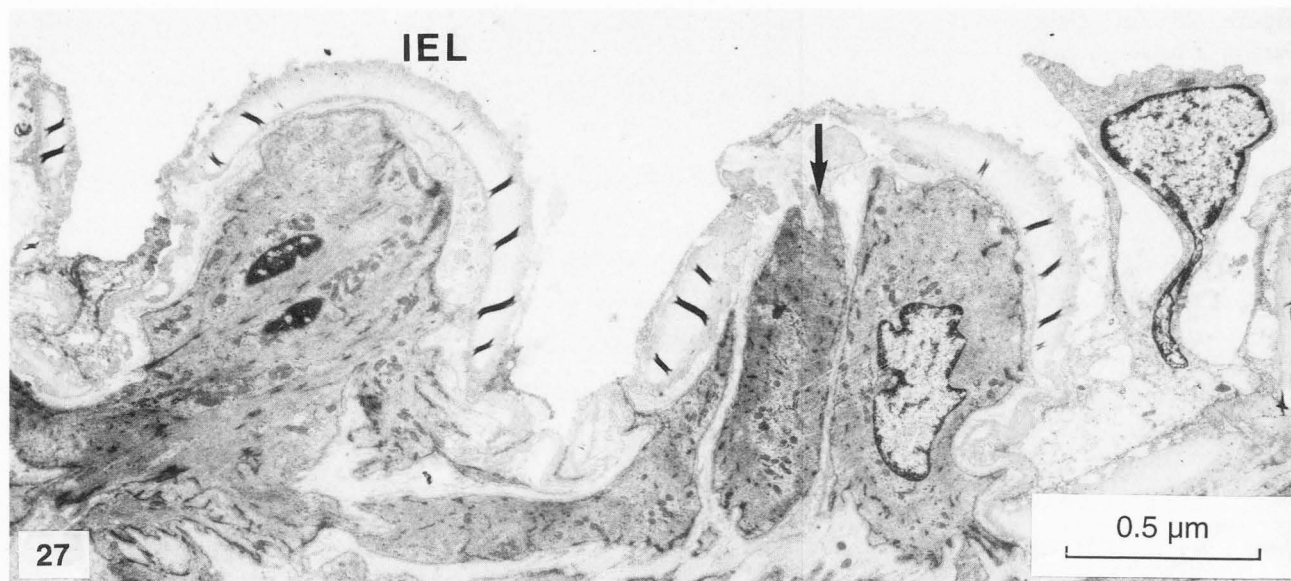
Figure 27. Part of a contracted vessel wall presenting a denuded internal elastic lamina (IEL). Cell coming through an opening in IEL. (Arrow). Thirty minutes after i.p. 5-FU injection.

Figure 28. Deep damage to the vessel wall. IEL partly totally destroyed. Edema of the underlying tissues manifested as increased intercellular space. Fifteen minutes after i.a. 5-FU injection, local effect.

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the time aspect, i.e., phenomena of the same severity were observed earlier after treatment in the group of animals injected with 5-FU intra-arterially, than in those injected intraperitoneally. This time difference can be explained by the slower drug absorption rate from the peritoneum into the vascular space. As seen in Figure 21, the severity of assessed features, described by mean

of scores, was lower after 60-120 minutes, which could be interpreted as a partial reversibility of the 5-FU injury. Some of the phenomena described in this material, such as vessel- and endothelial cell contraction, cell edema, villi formation and endothelial cytolysis were also seen in normal vessels; however, the score for these features was then fairly low. Other phenomena, such

as, denuded areas, platelet accumulation, and fibrin formation were not observed before in untreated (control) vessels, and therefore, judged to be the result of 5-FU treatment.

The pattern of folds and ridges observed when the vessel wall contracts is a part of normal vaso-regulation, but can also be induced by different traumata. Endothelial cells have an actin-myofilament system which enables them to adjust their shape and size. A common reaction to mild trauma is a reversible endothelial cell contraction, which becomes irreversible, however, if the trauma is more severe. This process can result in endothelial sheet disruption and exposure of the subendothelium, a potential focus for thrombus formation (Wieslander and Stjernquist, 1987). In this material, contrary to what was seen in the normal one, denuded areas were common, not only the result of endothelial sheet disruption, but, to a high degree, also caused by detachment of the damaged cells. These denuded areas with exposed internal elastic lamina (IEL) were frequently the sites of platelet accumulation and fibrin formation. This kind of injury leading to thrombus formation might seem irreversible. However, findings suggesting the possibility of repair processes were also found. The cell shown in Figure 27, migrating up through the opening in the bare IEL seems to be a smooth muscle cell undergoing transformation into an endothelial cell. This phenomenon was quite common in areas with severe injury and, therefore, we propose that it could be taken to represent the renewal of damaged endothelium. Observations from TEM show phenomena which can be related not only to cell damage but also to increased physiological activity and, therefore, may be attributed to repair processes. Signs of increased metabolic activity such as multilobulated nuclei, increased numbers of nuclear pores, rich amounts of endoplasmic reticulum and increased numbers and accumulation of mitochondria were seen. All these observations together indicate the cells to be very active and might therefore be interpreted to implicate an ongoing repair process.

The findings of our study show very severe damage to arterial endothelium following 5-FU treatment. In relation to the severity of this damage, features indicating thrombus formation are relatively poorly represented, although they do give support to the hypothesis of the thrombogenic effect of 5-FU being a major pathophysiological mechanism of its cardiotoxicity. Further studies evaluating the long term effect of 5-FU on endothelium are needed and the role of endothelium mediated, thrombus preventing substances must be kept in mind.

Acknowledgements

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Discussion with Reviewers

S. Aharinejad: Why did the authors use rabbits?
Authors: Rabbits have been used before in experimental studies using the perfusion fixation method (Wieslander, 1987). This model system has been shown to be reproducible, reliable and useful in practical applications.

M. Konerding: How much fixative was administered?
Authors: About 100-200 ml.

M. Konerding: How many vessels were prepared, examined and evaluated in each animal?
Authors: In the control group one vessel was prepared and in both groups of treated rabbits two vessels were prepared, examined and evaluated in each animal.

M. Konerding: How did you section the vessel longitudinally?
Authors: A longitudinal arteriotomy was performed on each vessel using microsurgical equipment.

M. Konerding: What was the quantity of pentobarbital? How long was the time between the intermittent injections? Was any heparin administrated?
Authors: Anaesthesia was induced by the injection of 40-50 mg of pentobarbital. Intermittent injections of 5-10 mg of pentobarbital were given about 1-2 times per hour during the preparation procedure. We did not use any heparin.

M. Konerding: Why did the authors choose a scale from minus 0.25-3.0? It remains unclear, whether a normal endothelium is scored with 0 or minus 0.25? What do the authors mean, e.g., by a score of 3.0 in the parameter "denuded areas"? Does it mean, that 100% or 50% or 10% of the endothelium is denuded?
Authors: We used this scale since it has been shown before (Wieslander, 1987) to be very useful in the evaluation of endothelial damage. A normal endothelium is given score 0. The score 3.0 in the parameter "denuded areas" means that 100% of the endothelium is denuded.

M. Konerding: Can lidocain minimize the vascular contraction induced by glutaraldehyde?
Authors: Yes.

V. Yang: Are there any ultrastructural changes indicating that the endothelial cells are in the process of repair? Are there any endothelial cells or macrophages migrating through the damaged area?
Authors: The score system showed rather unanimous results in all the groups of treated vessels in the later phases of observation with scores increasing towards values observed in the controls. This phenomenon might possibly be interpreted to be a sign of repair process. Our hypothesis seems to be supported by the observation of a smooth muscle cell migrating through an opening in the IEL. In our interpretation this observation could possibly represent a repair phenomenon.

M. Konerding: How do you discriminate microvilli on the cell surface from blebs and spikes?
Authors: We based our interpretation of features observed in endothelium as microvilli, upon the definition of these phenomena previously described by Wieslander (1987).

G. Pasquinelli: Although the TEM image (Fig. 26) shows necrosis of an endothelial cell, no interruption in the antithrombogenic lining is present, nor features suggesting possible repair from sliding of adjacent endothelium or from the cell below. How do you explain this?
Authors: Occasionally, the damage to the endothelial sheet observed in our material was very widespread and severe, with no normal cells remaining. However, such damage was not always connected with observations of phenomena suggesting repair processes, as e.g., in Fig 26, which shows the SEM examination of the local effect of 5-FU on the endothelium, 15 minutes after the i.a. injection. The lack of the features attributable to the repair processes might be related to the short observation time.