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THE APPEARANCE OF ENDOTHELIUM IN SMALL ARTERIES AFTER TREATMENT WITH 5-FLUOROURACIL. AN ELECTRON MICROSCOPIC STUDY OF LATE EFFECTS IN RABBITS

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Abstract

Cardiotoxicity is an unexplained toxic manifestation of 5-fluorouracil (5-FU). Its possible mechanism could be a direct cytotoxic effect on the vascular endothelium. We have tested this hypothesis in an experimental study in rabbits, using scanning and transmission electron microscopic evaluation of endothelium in small arteries (the central artery of the ear). The perfusion fixation method at physiological pressure and temperature was used. Both local and systemic effects of 5-FU on endothelium were studied 1, 3, 7, 14 and 30 days after *in vivo* treatment with 5-FU. Fifteen rabbits were used and five additional animals served as controls. The following parameters were evaluated: vessel wall and endothelial cell contraction, cell oedema, cytolysis, occurrence of denuded areas, platelet adhesion/aggregation and fibrin formation. For the description of each parameter, a scale of negative points (0.0-3.0) was used. We found severe cell damage with accompanying thrombus formation. The findings support the hypothesis that the thrombogenic effect of 5-FU, secondary to its direct cytotoxic effect on endothelium, is the pathophysiological mechanism behind 5-FU cardiotoxicity.

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

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Introduction

Vascular and heart endothelium is a site of considerable importance in the pathophysiology of cardiac and vascular diseases, due to its participation in vaso-regulatory mechanisms and to the role it plays in preventing thrombus formation (Mason *et al.*, 1977). Cardiotoxicity is a relatively unknown side effect of 5-fluorouracil (5-FU), one of the most widely used antineoplastic drugs in the treatment of human malignancies (Labianca *et al.*, 1982; Eskilsson *et al.*, 1988; Freeman and Constanza, 1988; Robben *et al.*, 1993). Various pathophysiological explanations for 5-FU induced cardiotoxicity have been suggested (Burger and Mannino, 1987; Gradishar and Vokes, 1990; Kuzel *et al.*, 1990). One possible mechanism might be a direct cytotoxic effect on the endothelial cells in cardiac vessels. Previously, we tested this hypothesis in an experimental study in rabbits (Cwikiel *et al.*, 1995). In these tests, the perfusion fixation method (Wieslander, 1987; Wieslander and Stjernquist, 1987) was used, followed by evaluation of the endothelium in small arteries 15-120 minutes after *in vivo* treatment with 5-FU using scanning (SEM) and transmission (TEM) electron microscopy. We found that 5-FU treatment resulted in cell damage consisting of disruption of the endothelial sheet and patchy exposure of the subendothelium, sometimes with adjacent thrombus formation. These findings of the early effect of 5-FU on vascular intima indicated that the pathophysiological mechanism behind 5-FU induced cardiotoxicity could be the thrombogenic effect of 5-FU, secondary to its direct cytotoxic effect on the endothelium. Morphological changes in the endothelium with accompanying disturbances of function could lead to a procoagulable state with thrombus formation, resulting in clinically apparent coronary ischemia. In the majority of patients, evidence of 5-FU cardiotoxicity is not immediately seen but appears usually 3-4 days after the start of treatment. The aim of the present study was to investigate if these clinical observations could correspond to more severe damage to the vascular intima, possibly with more frequent thrombus formation. Such an observation about 3 days after 5-FU treatment,

i.e., at the time for the clinically apparent coronary ischemia, could support the hypothesis that the thrombogenicity of 5-FU, secondary to its direct cytotoxic effect on endothelium, is the major pathophysiological mechanism underlying 5-FU induced cardiotoxicity. Therefore, in the present study, we evaluated the late effect of 5-FU on the intima and investigated morphological changes of the endothelium in small arteries 24 hours, 3 days, 7 days, 14 days and 30 days after 5-FU treatment.

Materials and Methods

Animals

Twenty male rabbits, weighing 2.5-3.0 kg, were used in the study. They were kept on a standard pellet diet and given water *ad libitum*. Anaesthesia was induced and maintained with repeated injections of pentobarbital (Mebumal 60 mg/ml, ACO Läkemedel, Helsingborg, Sweden) administrated in a marginal ear vein.

Small catheters (0.6 mm) were introduced into the central arteries of both ears. These catheters were used for the administration of 5-FU (Fluracedyl 50 mg/ml, Nycomed, Oslo, Norway) and saline, and subsequently, for perfusion fixation of the vessels.

Five rabbits received saline only at a volume of 0.5 ml/kg and served as controls. Fifteen rabbits were treated with 5-FU at a dose of 25 mg/kg, which corresponds well with human doses used in clinical situations, injected intra-arterially (i.a.) using the central artery of one ear. The animals were killed by an overdose of anaesthetic just before perfusion fixation, at 24 hours, 3 days, 7 days, 14 days or 30 days after 5-FU injections. Each time interval was represented by three animals.

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 (about 0.1-0.25 ml) of lidocaine (Xylocain 10 mg/ml, Astra, Södertälje, Sweden) were applied locally at 5 minutes and immediately prior to fixation.

The fixation solution was composed of 400 ml of 0.2 M phosphate buffer + 50 ml 50% glutaraldehyde + 90 ml 20% dextran T 70 + 455 ml distilled water. Perfusion fixation was performed by continuous infusion of perfusate at physiological pressure (120 mm Hg) and temperature (38.5°C) for 15 minutes. Thereafter, vascular specimens were taken from central arteries approximately 2 cm distal to the catheter tip and kept immersed in the fixative overnight.

Preparation for SEM

Specimens for SEM were fixed in 2.5% glutaraldehyde (in 0.15 M cacodylate buffer, pH 7.3) for 12 hours and then postfixed 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 (Eindhoven, Netherlands) operated at an accelerating voltage of 20 kV. Four standard magnifications were used: 500x, 1000x, 2500x and 5000x. Each vessel was photographed at three random locations in the proximal, central and distal part of the sample.

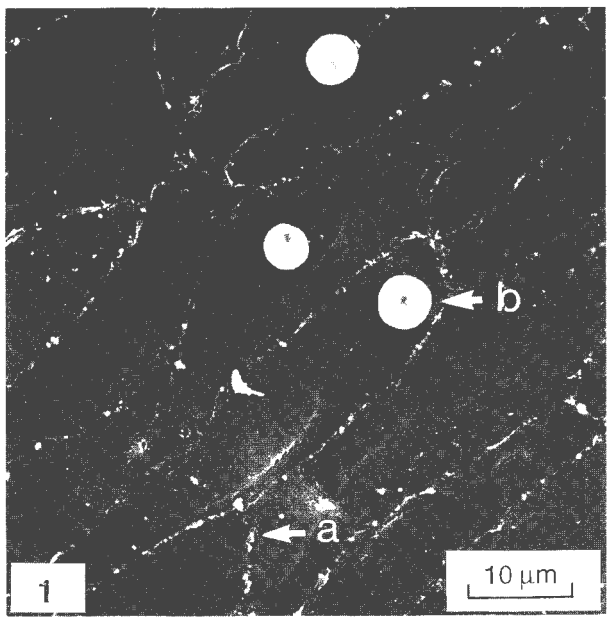


Figure 1. Control. Relaxed arterial vessel wall covered by a sheet of endothelium. A homogenous pattern of relaxed, flat endothelial cells arranged in the direction of the blood flow is seen. Cell nuclei protrude gently into the vessel lumen. At the cell edges, microvilli, so called "edgevilli" (arrow a), are present; erythrocyte (arrow b).

Preparation for TEM

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 TEM transmission electron microscope.

Evaluation of specimens

Two groups of vascular specimens were obtained from rabbits treated with 5-FU: one group was obtained from the central arteries of the untreated ears of the rabbits treated with 5-FU in the contralateral ear (group S), and the other group was from the central arteries of the ears used for i.a. 5-FU injections (group L). In group S, the systemic effect of 5-FU on the endothelium was studied, and in group L, the local effect of 5-FU on the

Figure 2. Control. A single-layered endothelial cell sheet rests on the internal elastic lamina (IEL). Neighboring cells overlap each other. The cell nuclei protrude into the vessel lumen.

vascular endothelium was evaluated. All specimens were evaluated by each of the authors independently, using the following parameters: vessel wall contraction, contraction of endothelial cells, occurrence of denuded areas, endothelial cell oedema, endothelial cell lysis, platelet adhesion and aggregation, fibrin formation, microvillus formation and occurrence of erythrocytes.

Scores

For the description of each parameter, a scale of negative points (0.0-3.0) was used. The points expressed the ratio between the area covered with damaged and normal endothelium, respectively. Scores were linearly related to the percentage damaged area, and a score of 3.0 was equivalent to a 100% damaged area. In practice, scores were set in increments of 0.5 which is equivalent to an increase in damaged area of 17% percent. The scores for denuded areas, platelet accumulation and fibrin formation were multiplied by three, and those for endothelial cytolysis by two, because these latter reactions were judged to be more severe than, for example, endothelial cell contraction.

Each pathological phenomenon was estimated separately by mean of scores; the sum of negative points (given as its absolute number) was evaluated for each sample, and then the mean scores worked out for each group of vascular samples and each time, respectively.

Results

All animals survived the observation period without complications. All arteries were patent. Diameters of the fixed arteries varied between 0.6 and 1 mm.

Control groups

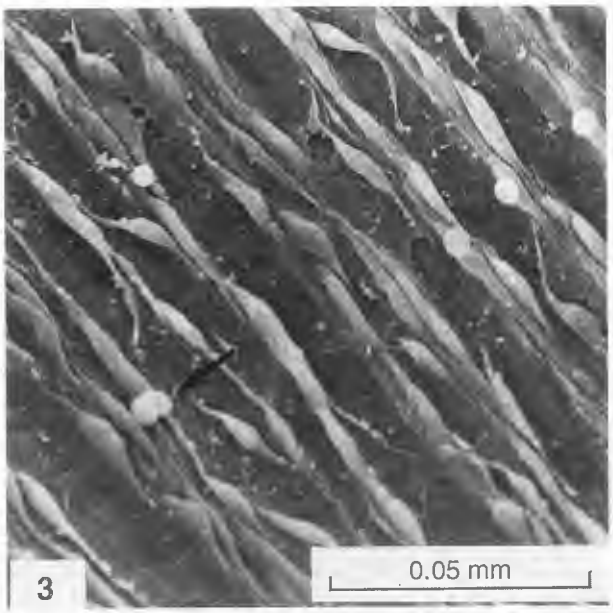
Scanning and transmission electron microscopy In all samples, relaxed vessel walls covered by a homogenous endothelial sheet were found on examinations with SEM (Fig. 1). TEM images showed a continuous single-cell layer, consisting of regular endothelial cells, slightly overlapping each other (Fig. 2).

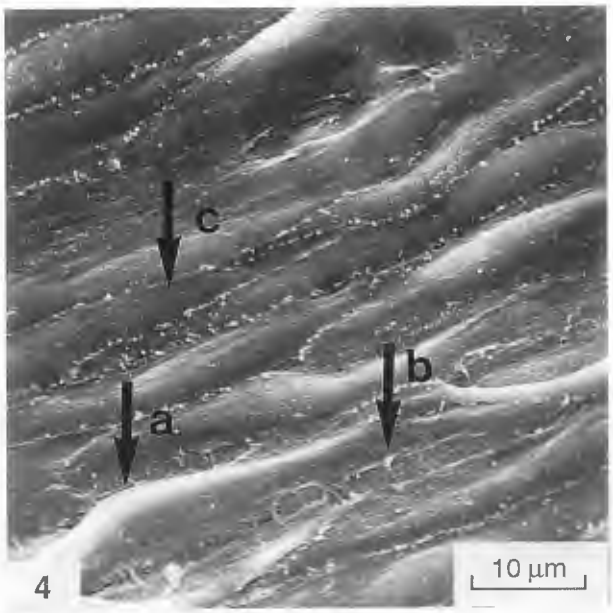
Treated groups

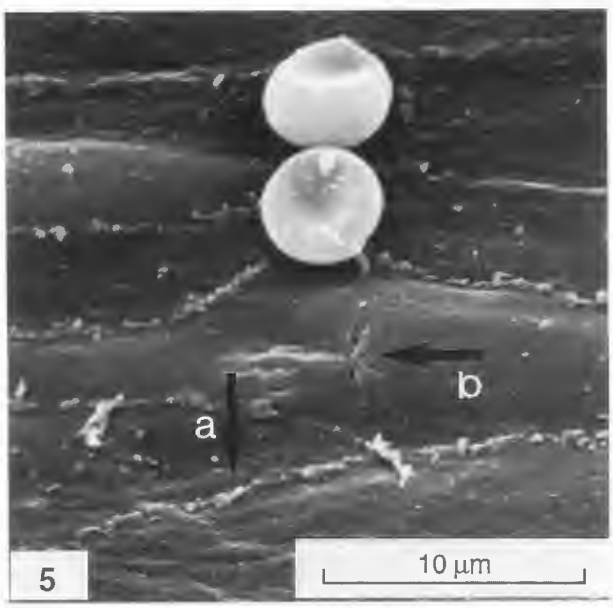
Scanning electron microscopy The majority of samples obtained from the treated rabbits presented a wide panorama of features synonymous with cell damage. Cell damage was evident through the whole material, even if its manifestations varied in appearance, extent and degree of severity from one sample to another.

The majority of samples obtained from the treated animals presented a more or less contracted vessel wall. The wall contraction which varied in degree, manifested itself as folds and ridges, occasionally covered by relaxed endothelial cells but usually by contracted ones (Fig. 3). Cell contraction could occur independently of vessel wall contraction, however, and was found even in the relatively relaxed areas of the vessel (Fig. 4).

The majority of samples from the two studied groups, S and L, and from all of the time aspects displayed a variety of phenomena attributable to cytolysis. Microvilli located at the cell edges, so called "edgevilli," common in controls, were here seen only in the well preserved areas of intima, with no disruption of cell connections or evidence of other severe damage (Fig. 5). Cell oedema was seen rather often, usually in contracted







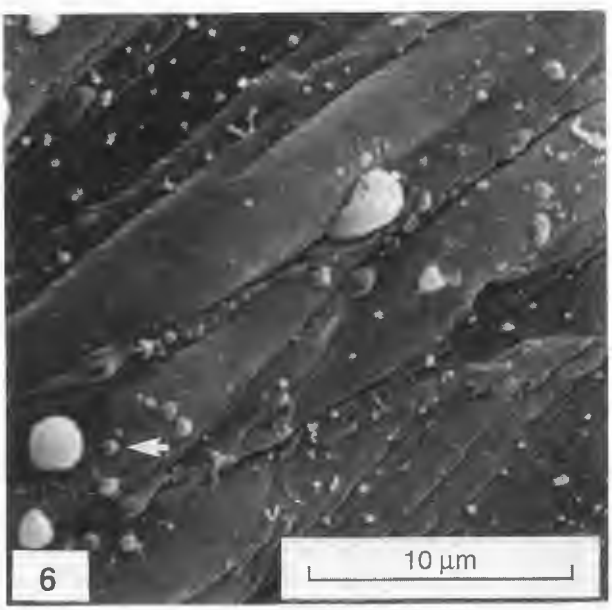
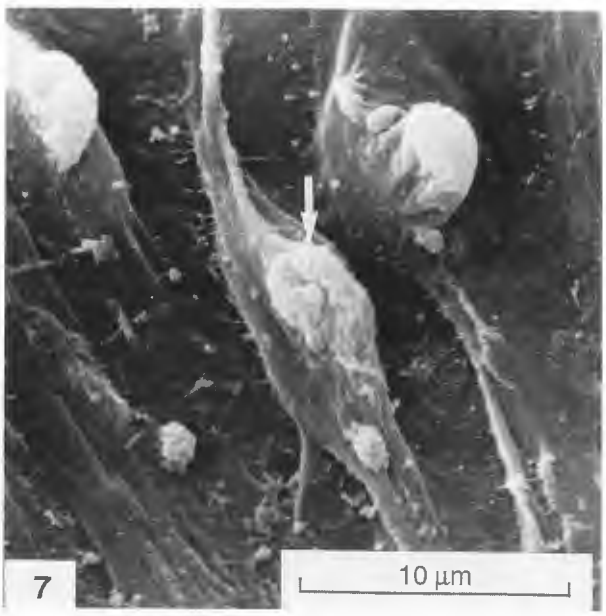


Figure 3. The contracted arterial vessel wall with a pattern of regular folds. Contracted endothelial cells lying on top of the folds. Erythrocytes close to ridges (arrow). Twenty four hours after i.a. 5-FU injection, systemic effect.

Figure 4. The relaxed vessel wall covered by the relaxed intima, in some areas presenting contracted endothelial cells (arrow a). Cell connections between contracted cells and their neighbors ones are not preserved. Gaps and denuded IEL can be observed (arrow b). The contracted cells seem detached from the underlying structures. The relaxed endothelial cells present "edgevilli" (arrow c). Twenty four hours after i.a. 5-FU injection, systemic effect.

Figure 5. Vessel intima with cells demonstrating "edgevilli" (arrow a). Cell connections are well preserved. "Rosette" in the central part of cell created by cell membrane contraction (arrow b). No other damage can be identified. Twenty four hours after i.a. 5-FU injection, systemic effect.

Figure 6. Part of the contracted vessel wall presenting oedematous endothelial cells. Cell borders can not be distinctly discerned and edgevilli are not obvious. Cell membranes are irregular and sparsely covered by microvilli (arrow). Seven days after i.a. 5-FU injection, systemic effect.



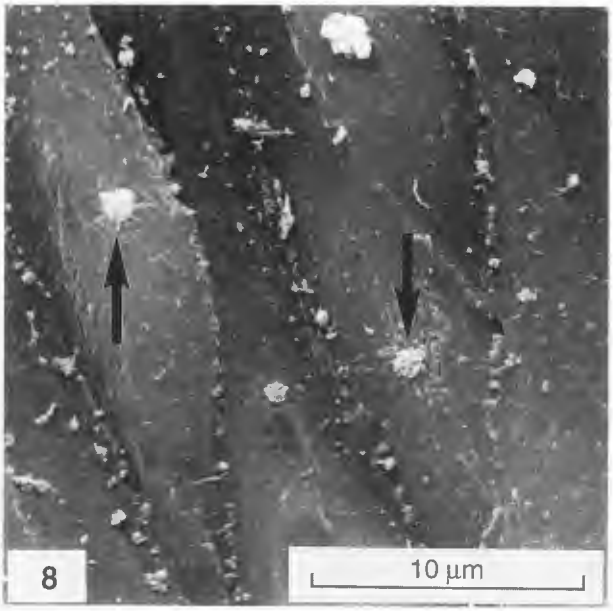


Figure 7. Relaxed vessel wall covered by contracted endothelial cells. Ongoing cytolysis manifested as shrinkage of cell membranes over the protruding nuclei (arrow). Intercellular gaps created by the withdrawal of cells from each other, revealing IEL. Thirty days after i.a. 5-FU injection, local effect.

Figure 8. Group of contracted endothelial cells presenting "rosettes" created by contraction of the cell membrane in the central part of cells (arrow). Seven days after i.a. 5-FU injection, systemic effect.

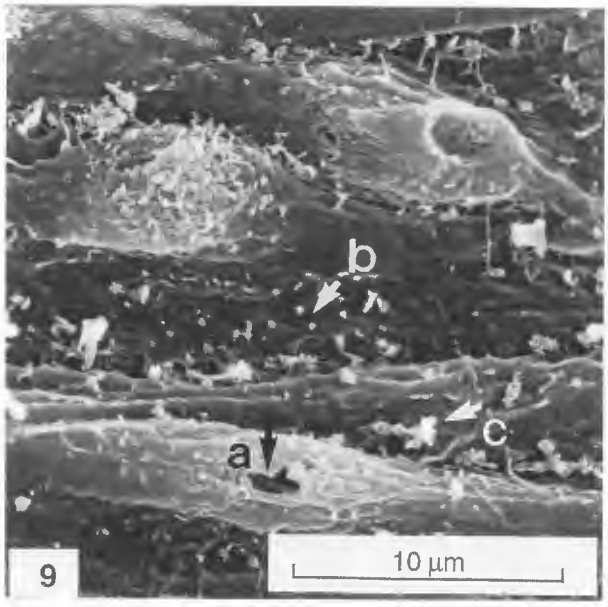


Figure 9. Area of relaxed vessel wall covered by contracted endothelial cells. Cytolysis appearing as severe irregularities of the cell membrane. Evidence of cell membrane shrinkage, contraction and perforation (arrow a). Cell borders can not be distinctly discerned, taking the form of intracellular cracks. Platelet adhesion (arrow b) and aggregation (arrow c). Seven days after i.a. 5-FU injection, local effect.

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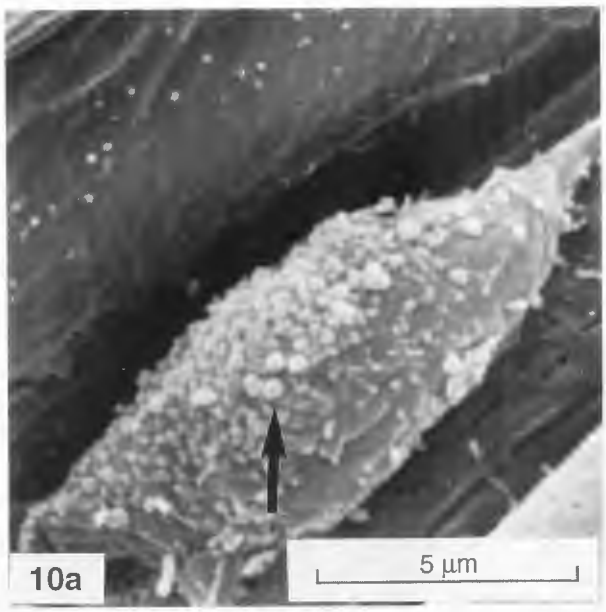
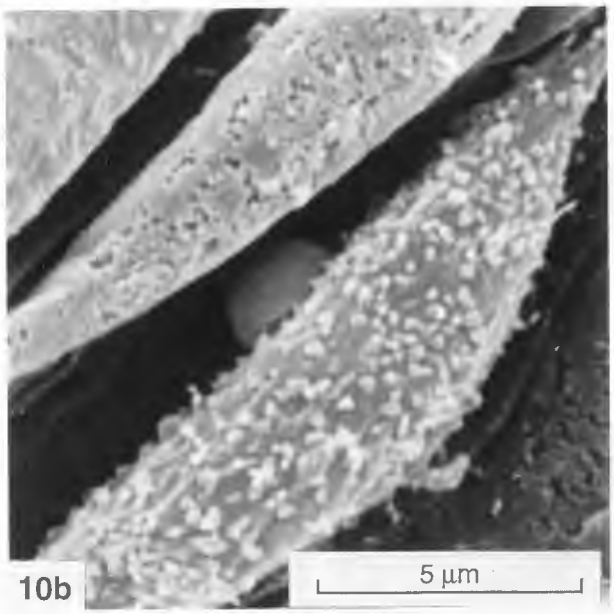
endothelial cells with no other features of severe damage (Fig. 6). Cell lysis appeared in a multitude of morphological manifestations. Membrane shrinkage, frequently located over protruding nuclei (Fig. 7), was common. A special presentation of cytolysis, the so-called "rosette" (Cwikiel *et al.*, 1995), created by severe membrane contraction in the central part of cell was also seen in this material (Fig. 8). The majority of samples studied presented cells with varying degrees of membrane irregularities (Fig. 9) and a magnitude of microvilli formations. Microvilli were usually located over the protruding nuclei as small drops (Fig. 10a), but they could also be bigger and scattered over the whole cell surface (Fig. 10b). Occasionally, microvilli were concentrated in the periphery of the cell surface and had the appearance of huge, bizarre clubs (Fig. 10c). Cytolysis manifested itself as cell membrane disruption, perforation (Fig. 11), cell shrinkage and partial or total cell detachment (Figs. 12 and 13). In some areas, there was almost total dissolution of the cell membrane, to the point where normal structures were difficult to identify (Fig. 14). Cracks and fissures in the intima, withdrawal of cells from each other, (Fig. 15) often leading to total disruption of the endothelial sheet (Fig. 16), total cell lysis and/or cell detachment resulting in denudation of the internal elastic lamina (IEL) (Fig. 17) were features frequently observed.





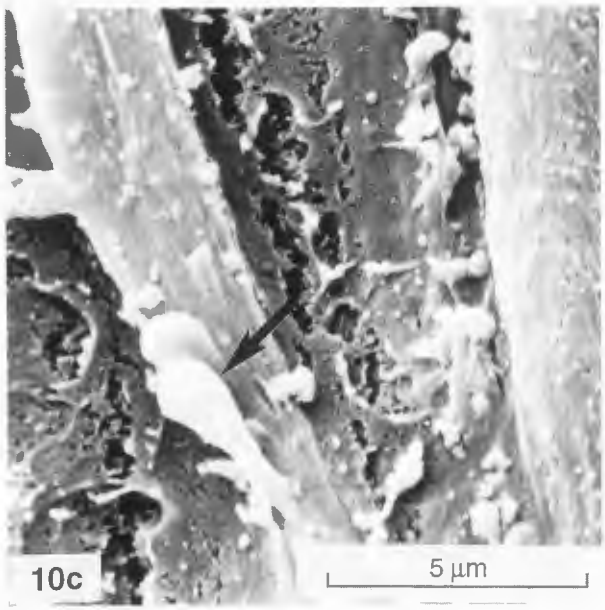


Figure 10. Endothelial cells, with manifestation of various forms of microvilli on their surface. **(a)** Small, drop-like microvilli located over the protruding nucleus (arrow). Three days after i.a. 5-FU injection, systemic effect. **(b)** Contracted endothelial cell with microvilli scattered over the whole cell surface. Three days after i.a. 5-FU injection, systemic effect. **(c)** Large, bizarre club-like microvilli located at the periphery of the cell (arrow). Fourteen days after i.a. 5-FU injection, systemic effect.

Very severe damage of the intima, revealing a denuded IEL, varied in extent from one sample to another but was a feature represented through the whole material. In some samples, the investigated areas were denuded completely, while in the others, the IEL was only patchily exposed. This extreme form of cytolysis was associated with platelet accumulation in many areas. The phenomenon appeared occasionally as platelet adhesion (Fig. 18), occasionally, as both adhesion and aggregation (Fig. 19). Platelet accumulation could be found separately, but was usually associated with fibrin formation, which occasionally took the appearance of sporadic fibrin strands (Figs. 20 and 21), but very frequently appeared as an abundant fibrin net, covering large parts of the areas being studied (Figs. 22a and 22b, and 23a and 23b). However, platelet accumulation and fibrin formation was also observed in areas with a relatively well preserved intima, with no evidence of denudation of the IEL (Figs. 23a and 23b). Platelet accumulation and fibrin formation was the most conspicuous phenomenon in this material.

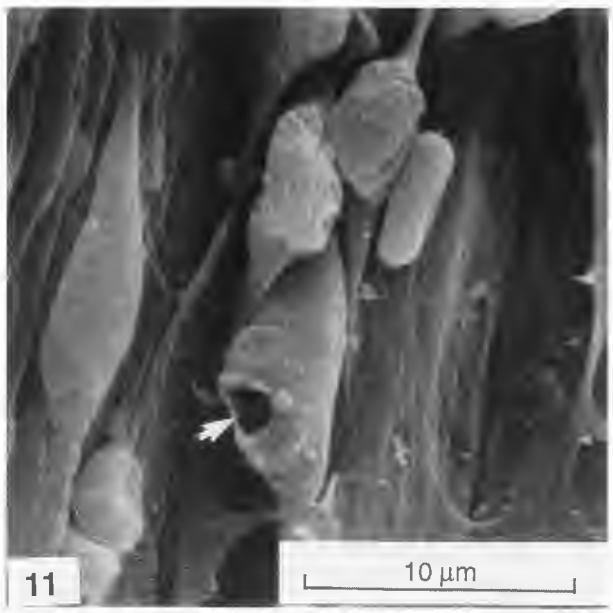
All of the above-mentioned manifestations of endothelial cell damage were observed in both groups of vessel specimens and at the all time aspects studied, varying in severity and extent from one group to another.

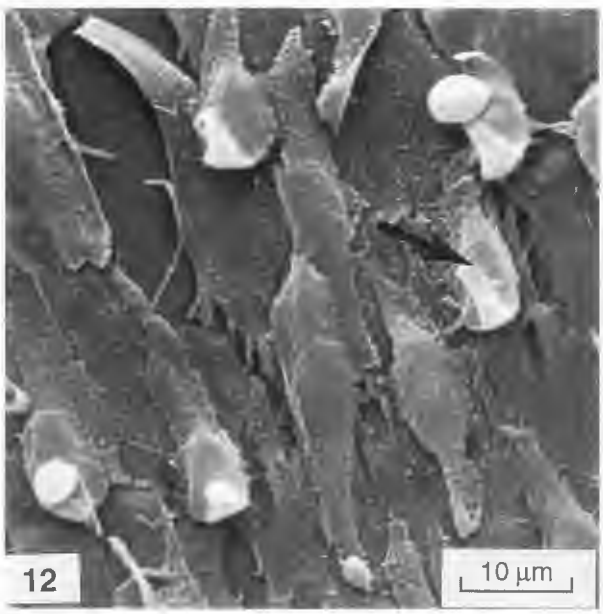
Scores

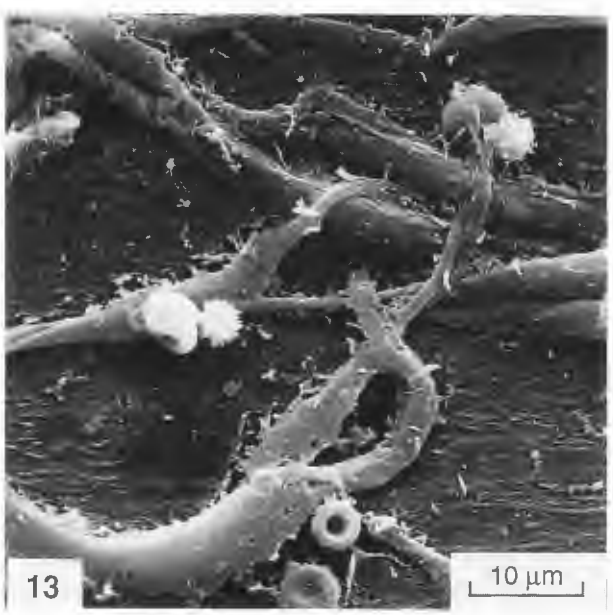
In the control group, the mean value of negative scores was 1.0; the score varied from 0.0 to 3.5 on a scale from 0.0 to 60, where 60 would indicate maximal damage for all parameters (Fig. 24). In treated groups, scores varied from 1.0 to 21.0 in group S and from 9.0 to 27.0 in group L (Fig. 24).

Transmission electron microscopy

The transmission electron micrographs (Figs. 25-30) show the images of arterial vessel walls in longitudinal







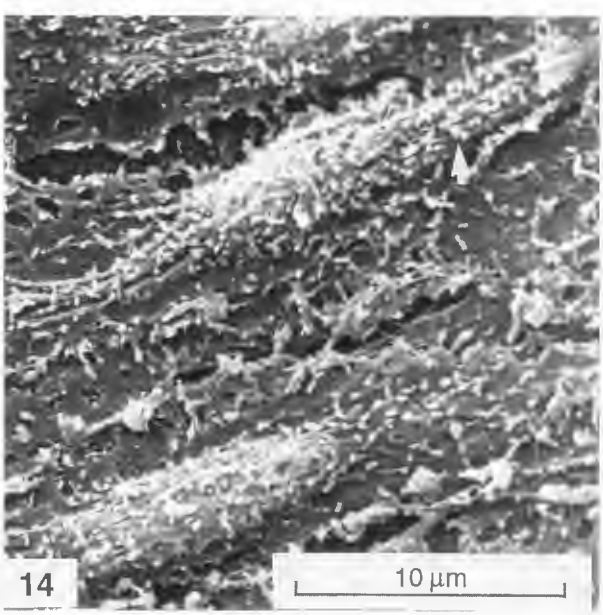
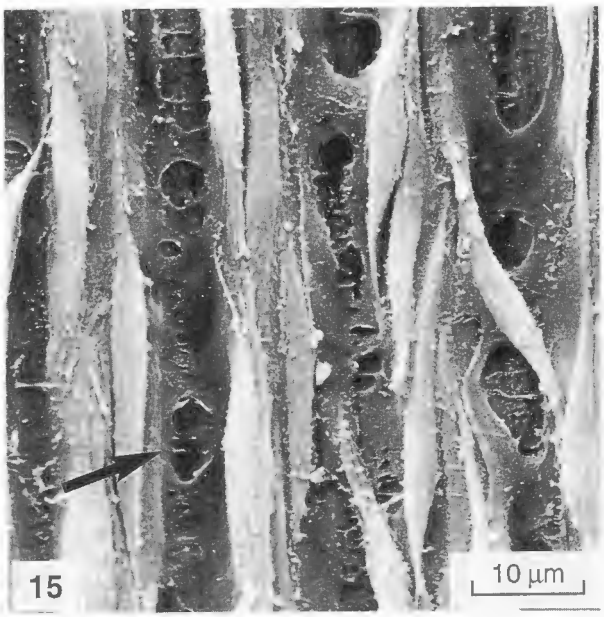


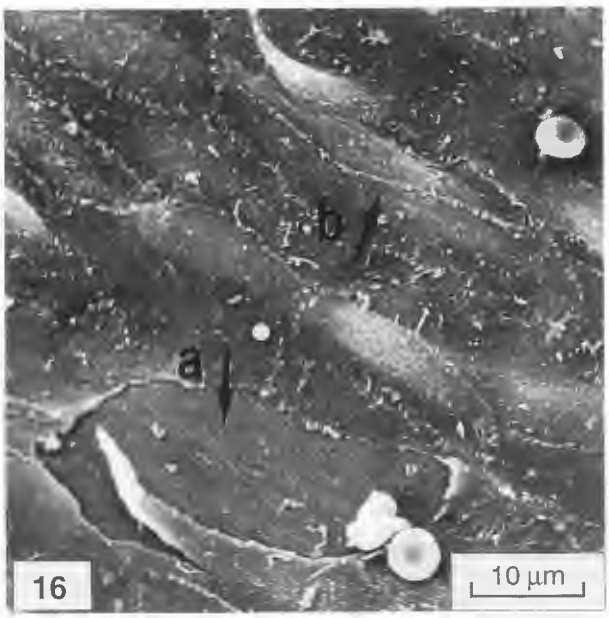
Figure 11. Group of contracted endothelial cells with ongoing cytolysis manifested here as cell membrane perforation (arrow). Thirty days after i.a. 5-FU injection, local effect.

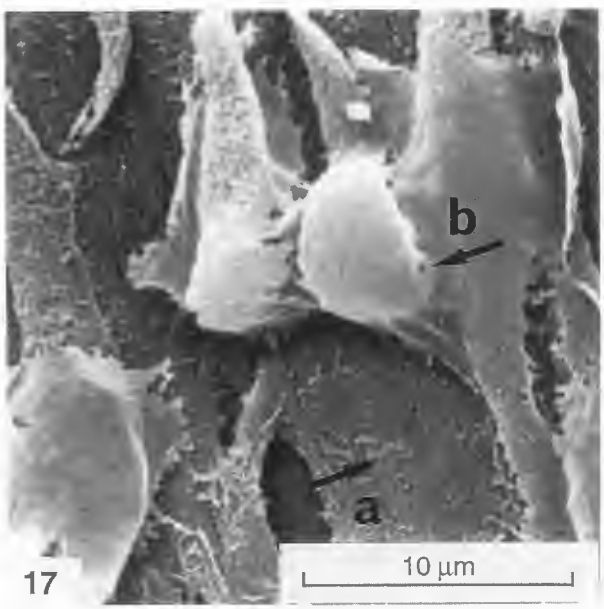
Figure 12. Relaxed arterial vessel wall covered by severely damaged intima, consisting of contracted endothelial cells partly detached from the subendothelium (arrow). Three days after i.a. 5-FU injection, systemic effect.

Figure 13. Relaxed vessel wall presenting very severe damage to the intima, resulting in almost total denudation of the IEL. The endothelial cells are partly or totally detached. Seven days after i.a. 5-FU injection, local effect.

Figure 14. Severe, diffuse cytolysis in the relaxed arterial wall. Massive dissolution of cell membranes. Cell borders not identifiable, taking the form of cracks and fissures. Internal elastic lamina partly denuded. Massive, diffuse adhesion of platelets (arrow). Twenty-four hours after i.a. 5-FU injection, local effect.







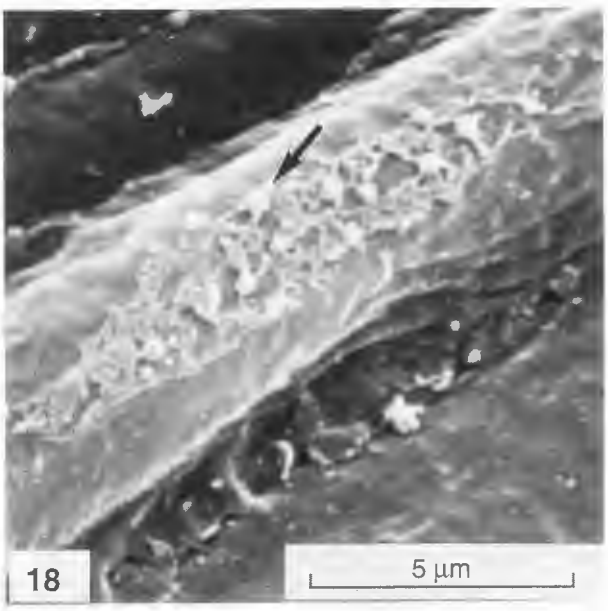
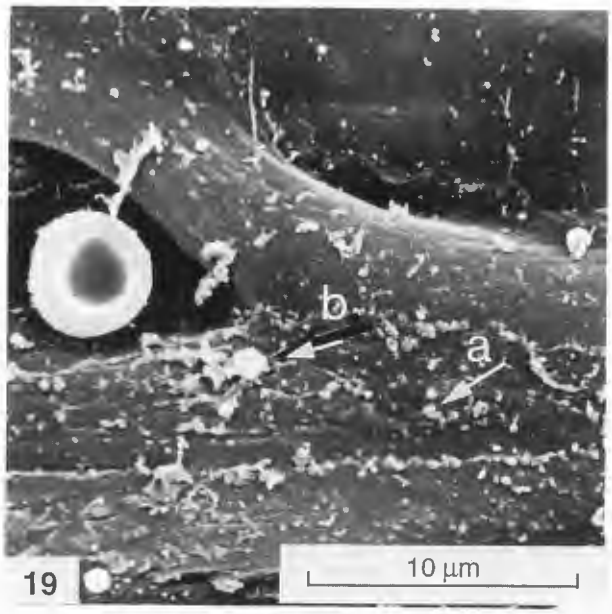


Figure 15. Contracted vessel wall with the folds covered by contracted endothelial cells. Occasionally, connections between the cells are still preserved. Some cells present ongoing disruptions of cell connections, creating denuded intercellular gaps (arrow). Three days after i.a. 5-FU injection, systemic effect.

Figure 16. Relaxed arterial vessel wall presenting local, total disruption of the intima (arrow a). The endothelial sheet in other areas shows still preserved cell connections with "edgevilli" (arrow b). Twenty-four hours after i.a. 5-FU injection, local effect.

Figure 17. Severe damage to the intima in a relaxed arterial vessel wall. Denuded IEL (arrow a) as the result of cell detachment and lysis. Fragment of the partly detached, damaged cell (arrow b). Three days after i.a. 5-FU injection, systemic effect.

Figure 18. Endothelial cell with a delicate, thin fibrin net on its surface. Platelet adhesion (arrow). Three days after i.a. 5-FU injection, local effect.



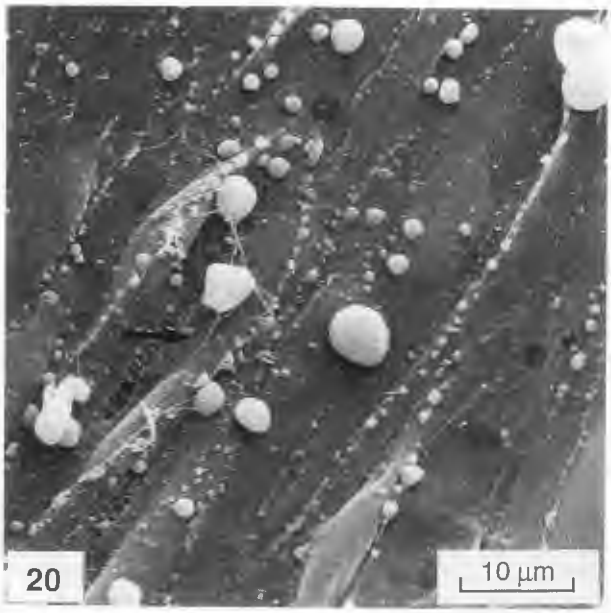


Figure 19. Area of relaxed intima presenting platelet accumulation, manifested as adhesion (arrow a) and aggregation (arrow b). Three days after i.a. 5-FU injection, local effect.

Figure 20. Relaxed arterial vessel wall. The endothelial sheet consists of relatively relaxed cells. Microvilli are scattered over the whole area, usually concentrated in the periphery of the cells. In the center, platelet accumulation and a thin fibrin strand (arrow). Seven days after i.a. 5-FU injection, local effect.

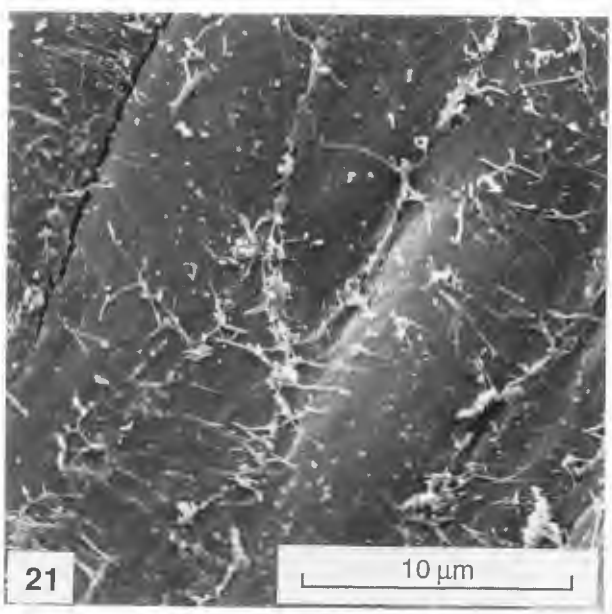


Figure 21. A vessel intima covered by a delicate net of thin fibrin strands. Twenty-four hours after i.a. 5-FU injection, local effect.

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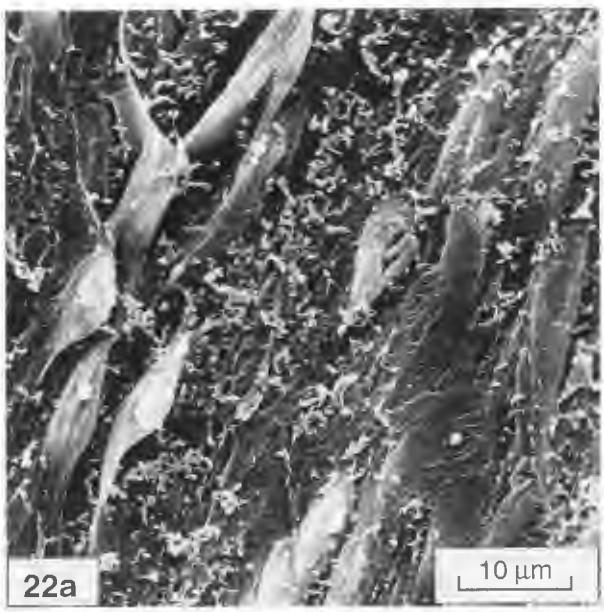
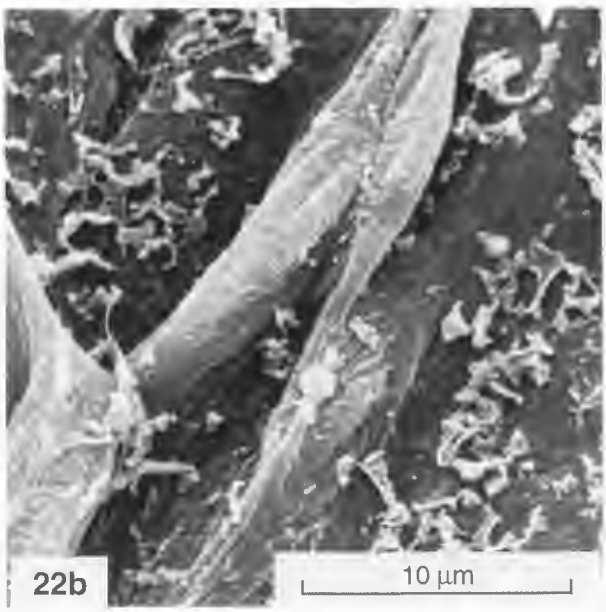
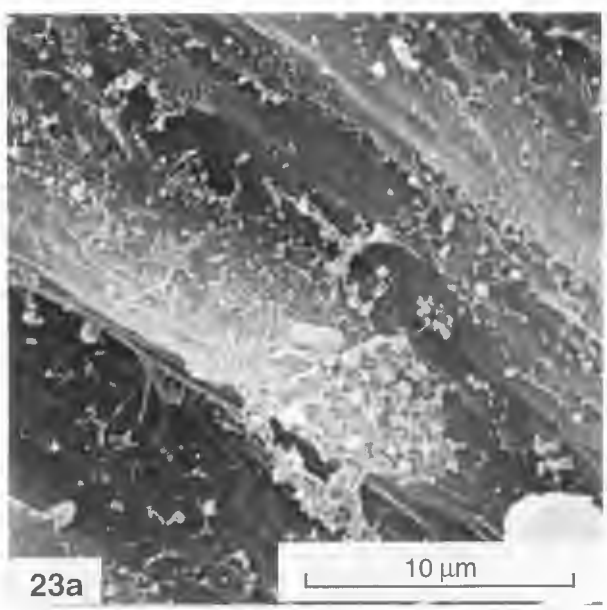
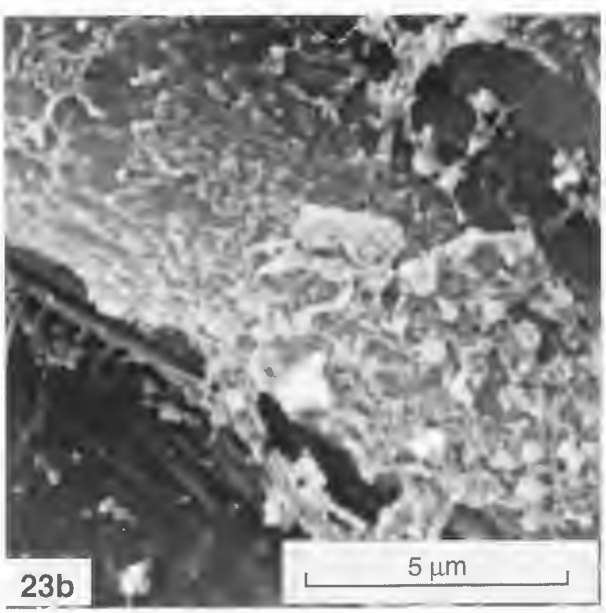
or cross section. As with SEM findings, the majority of samples taken from the 5-FU treated animals showed slightly contracted vessel walls. The vessel walls sometimes had wave-like folds involving the IEL and the underlying connective tissue (Fig. 25) and sometimes had almost no folds (Fig. 26). Irrespective of vessel wall contraction or relaxation, endothelial cells were usually contracted and manifested a variety of features attributable to cytolysis. Preserved cell connections, obvious in the normal material, were unusual here, and most of the endothelial cells demonstrated varying degree of disarrangement and discontinuity of cell contact (Figs. 25 and 26). Edgevilli and cell oedema, occasionally observed in SEM examinations, were not seen in TEM. As with SEM, cytolysis appeared in TEM with a great variation of morphological manifestations. Microvilli were frequently observed and presented in a multitude of different forms and sizes. They could appear as small drop-like villi scattered over the whole cell surface (Figs. 26 and 30) or present themselves as huge, bizarre clubs, located at the periphery of the cell surface (Fig. 25). Cytolysis could manifest itself as cell membrane alterations in the form of irregularity, blebbing and disruption (Fig. 27). Other appearances of cytolysis observed in this material were changes in cytoplasm and cytoplasmatic organelles, such as vacuolization of the cytoplasm (Fig. 27), accumulation of mitochondria (Fig. 28) and swelling of the endoplasmic reticulum. In some areas, damage to the cytoplasm was severe, resulting in fragmentation of the cell, occasionally, but not always, with previous cell detachment (Fig. 27). These phenomena, i.e., cell lysis and/or cell









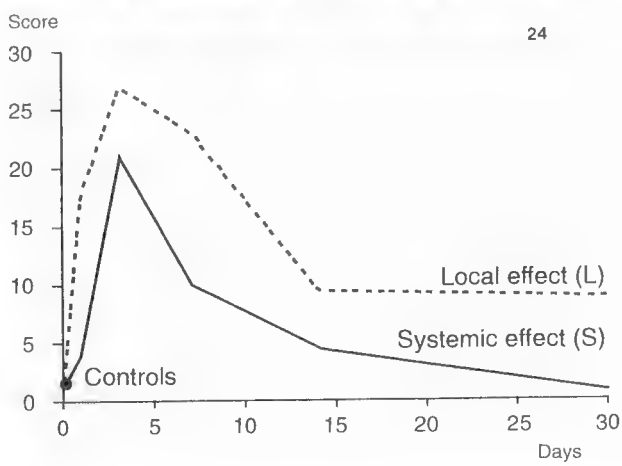


Figure 22a and 22b. Slightly contracted vessel wall at different magnifications. Massive fibrin net covering a large part of the endothelial sheet. Seven days after i.a. 5-FU injection, local effect.

Figure 23a and 23b. Slightly contracted vessel wall at different magnifications. Diffuse, massive platelet accumulation and fibrin formation. Three days after i.a. 5-FU injection, local effect.

Figure 24 (at left). The influence of 5-FU treatment on the appearance of endothelium in small arteries, described by mean of scores, in relation to time.

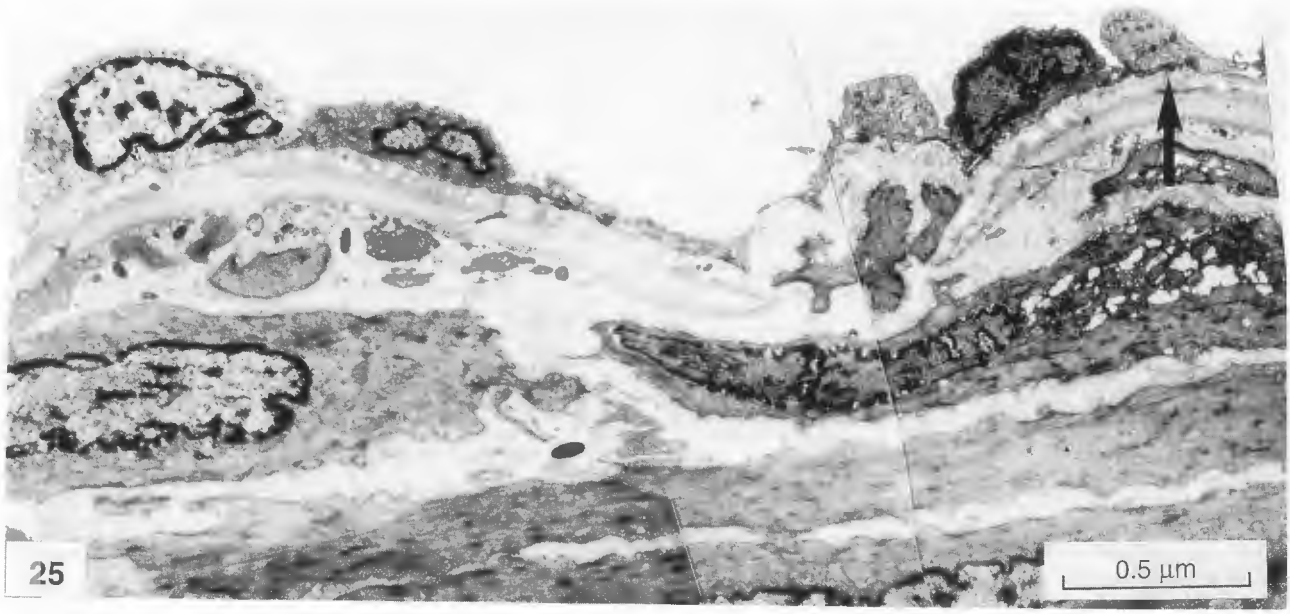


Figure 25. Slightly contracted arterial vessel wall. Contracted endothelial cells lying on top of the folds, presenting irregularities of the cell membrane, creating "club" villi (arrow). Cell borders can not be clearly discerned. Three days after i.a. 5-FU injection, systemic effect.

Figure 26. Relaxed vessel wall covered by contracted endothelial cells presenting microvilli (arrow a) and irregularities of the cell membrane. Multilobulated nuclei with peripheral chromatin accumulation and an increased number of distinctly marked nuclear pores (arrow b). Discontinuity of cell connections. Twenty-four hours after i.a. 5-FU injection, local effect.

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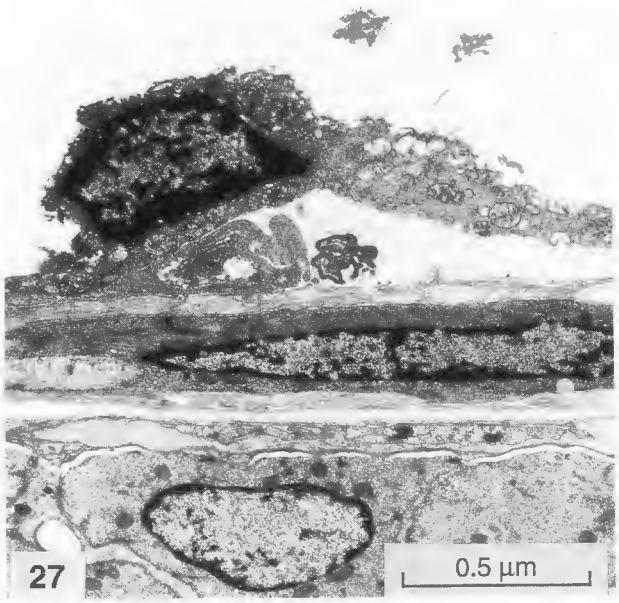
detachment frequently resulted in denudation of the underlying structures. The IEL, occasionally covered by cell remnants, occasionally totally bare, was seen in many areas of the vessel wall, both contracted and relaxed ones (Fig. 29). Damage to the vessel intima was occasionally followed by total destruction of the IEL, with accompanying oedema in the underlying tissues, indicated by increased intracellular spaces (Fig. 29). Most of the damaged endothelial cells contained multilobulated nuclei with peripheral chromatin accumulation



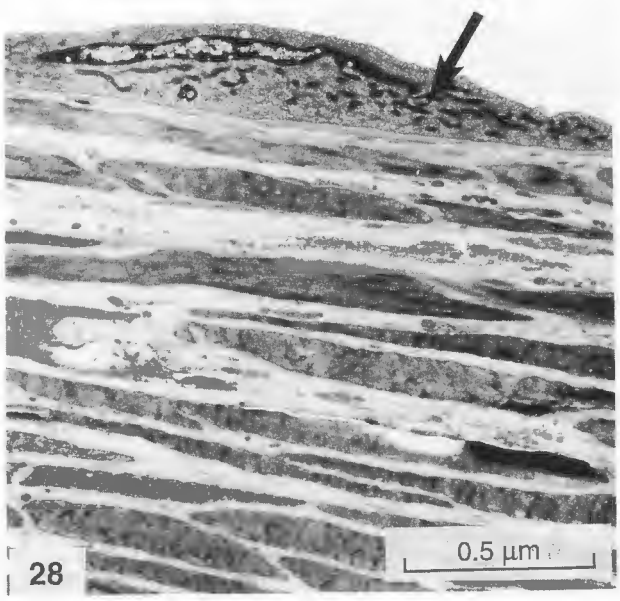


Figure 27. Relaxed vessel wall. Partly detached endothelial cell evidencing disruption of the cell membrane with vacuolization and dissolution of the cytoplasm. Seven days after i.a. 5-FU injection, systemic effect.

Figure 28. Relaxed vessel wall with an endothelial cell evidencing accumulation of mitochondria, which seem to have increased in number (arrow). Three days after i.a. 5-FU injection, local effect.

Figure 29. Relaxed arterial vessel wall presenting a denuded internal elastic lamina (IEL) which in places, is totally destroyed. Oedema of the underlying tissues manifested as increased intercellular spaces. Three days after i.a. 5-FU injection, systemic effect.

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and an increased number of distinctly marked nuclear pores (Fig. 26). Another phenomenon observed was the evidence of cells coming through openings in the IEL. These cells, reminiscent of smooth muscle cells, usually lay in close proximity to or just underneath the damaged endothelial cells (Fig. 30).

The evidence of platelet accumulation and fibrin formation, obvious in scanning electron micrographs, could not be distinguished in the material studied by TEM. All features described above, indicating damage to the endothelium, were observed in samples from both groups, S and L, representing all of the time intervals after treatment with 5-FU. The extent and degree of severity of the damage varied between groups and different time aspects but, generally, seemed to correspond to the pattern of damage described in SEM, when the means of scores were compared.

Discussion

The first reports on the relationship between cardiac complications and 5-FU treatment came in the seventies (Carpenter, 1972; Dent and McColl, 1975); and in the eighties, reports with larger materials and review articles about 5-FU induced cardiotoxicity were frequently seen (Labianca *et al.*, 1982). However, despite the fact that more then twenty years have gone since the first clinical reports on 5-FU induced cardiotoxicity, and a lot of effort has been expended to explain its mechanism, the pathophysiology of the syndrome still remains unclear.

The observations from our previous study (Cwikiel *et al.*, 1995), supported the hypothesis that 5-FU causes endothelial damage with following disturbances of function, leading to a procoagulable state, and thrombus formation. In the clinical situation, this mechanism could take the appearance of coronary ischaemia. In the present material, where longer time intervals were studied, the hypothesis was distinctly confirmed.

The appearance of the endothelium in the controls did not differ from that described previously (Cwikiel *et al.*, 1995). Scores were low, due to the absence of severe damage such as denudation, platelet accumulation and fibrin formation.

Contrary to what is seen in normal material but in accordance with the data presented previously describing the early, immediate effects of 5-FU on the endothelium (Cwikiel *et al.*, 1995), the majority of samples in the present study, representing all of the time aspects and both local and systemic effects of 5-FU treatment, show very severe endothelial damage. Such damage, frequently leading to disruption of the endothelial sheet and/or cell detachment, resulting in denudation of the IEL, was in most cases followed by platelet accumulation and fibrin formation. This phenomenon, was not observed in controls, and may, therefore, be interpreted as the effect of 5-FU. Thrombus formation, in the present material, was much more common than in the previous study, where the immediate effect of 5-FU on endothelium was assessed (Cwikiel *et al.*, 1995).

The endothelial injury, described by mean of scores, was more severe in the days after 5-FU treatment (Fig. 24) than in the minutes and hours after treatment (Cwikiel *et al.*, 1995). The scores were highest on the third day after 5-FU treatment, because features attributed to severe intima damage, such as denudation of the subendothelium with accompanying platelet accumulation and fibrin formation were most strongly manifested at that time. These experimental observations correspond very well with the timing of clinical signs of cardiotoxicity, such as angina, electrocardiogram alterations, myocardial infarction or arrhythmias, which appear usually about 3-4 days after the start of 5-FU treatment.

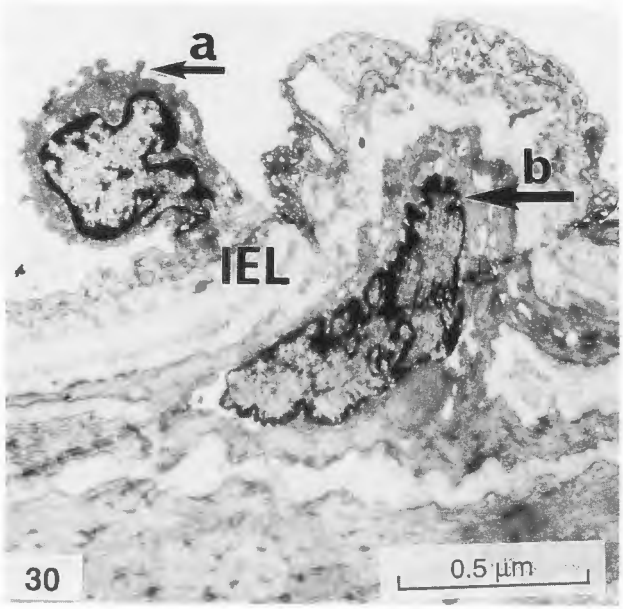

Figure 30. Slightly contracted arterial vessel wall with a contracted, damaged endothelial cell presenting microvilli (arrow a). Cell coming through an opening in the internal elastic lamina (IEL) (arrow b). Three days after i.a. 5-FU injection, systemic effect.

The scores at 7 days after treatment were still high, but at the longer time intervals, they declined towards values seen in controls. This phenomenon can be interpreted as a partial reversibility of 5-FU injury. There were findings in the TEM examinations suggesting renewal of the damaged endothelium, such as migration of smooth muscle cells through openings in the IEL, possibly with further transformation into endothelial cells. As seen in Figure 24, the severity of endothelial injury, described by means of scores, was higher and more persistent in group L, i.e., vessel specimens in which the local 5-FU effect on endothelium was studied. This finding corresponds well with the clinically apparent thrombophlebitis, usually of rather long duration, which is seen after intravenous injections of 5-FU.

Vascular endothelium synthesizes and secretes a large number of vasoactive substances which diffuse to the underlying smooth muscle cells causing vasoconstriction or vasodilatation.

The vasodilatory factors include endothelium-derived nitric oxide (EDNO), prostacycline, acetylcholine, substance P and endothelium-derived hyperpolarizing factor. Vasoconstrictor substances produced by endothelial cells include endothelin, angiotensin II, vasoconstrictor prostaglandins and the superoxide anions which inactivate EDNO (Vane *et al.*, 1990; Harisson, 1992).

Another function of endothelium is the regulation of

intravascular thrombosis through the expression of different pro- and anticoagulant substances. Good preservation of the endothelial sheet is of the utmost importance for its anticoagulant function. The electronegative charge of the endothelium prevents platelet deposition on its surface. An intact endothelium produces adenosine phosphatase which rapidly degrades the platelet product adenosine diphosphate, preventing platelet aggregation. Other antithrombotic substances synthesized by the endothelium are tissue plasminogen activator, which converts plasminogen to active plasmin, prostacycline and EDNO, both inhibiting platelet aggregation, antithrombin III, which inhibits thrombin activity, and thrombomodulin, which binds thrombin and thus inhibits all its procoagulant effects. To the procoagulant products of the endothelium belong: tissue factor which activates factor VII, leading to the coagulation cascade, von Willebrand factor, a key component of the coagulation cascade, and plasminogen activator inhibitor (Mason *et al.*, 1977; Nimmrich *et al.*, 1981; Davies and Hagen, 1993).

Because of the key role which the well preserved endothelium plays in anticoagulant mechanisms, it seems rather obvious that the severe injury caused by 5-FU would result in expression of the endothelium's procoagulant effects with resulting thrombus formation. Another experiment performed by the authors (Cwikiel *et al.*, in manuscript), where the effects of 5-FU on vascular endothelium *in vitro* in a cell-culture model were studied, showed a significant increase in the release of prostacyclin by endothelial cells exposed to 5-FU. The release of prostacyclin increased with increasing concentrations of 5-FU and was highest after about 24-48 hours incubation. It would appear that the primary reaction to endothelial injury is the increased release and leakage of vasodilatory, anticoagulant substances, and only when this mechanism becomes insufficient do the procoagulant effects take over and find expression in thrombus formation. The process is later followed by repair and thrombolytic mechanisms. The findings of our present study and the course of events shown in Figure 24 seem to correspond with the mechanisms mentioned above.

The more detailed pathophysiological mechanism of thrombogenesis caused by 5-FU deserves more attention and will be investigated in further studies. However, our study seems to confirm the hypothesis that the thrombogenic effect of 5-FU, secondary to its cytotoxic effect on endothelium, is the major pathophysiological mechanism behind 5-FU induced cardiotoxicity.

Acknowledgements

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Discussion with Reviewers

R.H. Christofferson: What happened with the arterial catheters from injection to, e.g., day 30? Did you remove them or leave them in place? Does not removal cause thrombotization of the artery?
Authors: We removed the arterial catheters after injection of 5-FU and saline, respectively. In order to avoid the thrombotization of the artery, which we previously experienced in similar situations, we have, in the present study, placed catheters for perfusion fixation about 0.5 cm from the 5-FU injection site. We have not seen any thrombotization.

R.H. Christofferson: Were both ears (L and S) perfusion fixated simultaneously?
Authors: Yes.

R.H. Christofferson: How were the SEM specimens opened for inspection?
Authors: All the specimens, both SEM and TEM, were opened longitudinally.

R.H. Christofferson: Which parameters are the two most important in the score system? Can they be quantified? I would rather see that you omitted the damage score.
Authors: The score system was used in order to, as objectively as possible, quantify the damage to the endothelium. The system used in this study was introduced by Wieslander (1987) and has been shown to be useful, reliable and reproducible. The scale of negative points from 0.0 to 3.0 was used for description of each parameter; in practice, scoring was done in increments of 0.5 points. The score for denuded areas, platelet accumulation and fibrin formation were judged to represent the most severe damage to the endothelium, and therefore, were multiplied by three.

R.H. Christofferson: The "rosette" to me is a heating artefact that often is seen after focusing on the specimen at higher magnification. Were they observed in the controls?
Authors: Yes, "rosettes" were also seen in control material. Since they were, however, more frequently observed in the treated material, we interpret them to represent real damage to the endothelium.

R.H. Christofferson: Endothelial cells are known to have a slow turn-over, and only rarely incorporate tritiated thymidine in autoradiographic studies. Why would they take up 5-FU? Could part of the toxic effect observed be explained by the high pH (8.5-9.2) and sodium hydroxide in the injection solution?
Authors: Sodium hydroxide is a known component of injection solutions of a variety of drugs used in clinical praxis. That holds also true for high pH. For instance, the injection solution of Methotrexate contains sodium hydroxide and has a high pH, but is not cardiotoxic.

R.H. Christofferson: Why did you not chose to investigate the endothelial morphology of the coronary arteries?
Authors: Investigation of the coronary arteries in animals is rather complicated, especially when direct infusion of the drugs and perfusion fixation is required. Because of these practical reasons we have chosen to investigate arteries in the ears, the model which is well tested and established. Findings of the systemic effect of 5-FU in our material (group S), which reflect the general drug effect, could, in our mind, be applicable also for the coronary arteries.

R.H. Christofferson: Is not a substantial part of the cardiotoxicity of 5-FU due to its late effects on the myocardial cells? After all, acute angina pectoris is rather unusual side-effect at therapy.
Authors: Cardiotoxicity of 5-FU, mainly angina pectoris, is a known side-effect of this drug. An incidence as high as 18% has been reported (Eskilsson *et al.*, 1988). 5-FU induced cardiotoxicity is in contrast to, e.g., antracycline-induced cardiotoxicity, not cumulative, and usually disappears after discontinuing the 5-FU infusion. This does not support the assumption that myocardial cells are involved in the pathophysiology of the syndrome.

Z. Somosy: Did you find apoptosis of endothelial cells upon 5-FU treatment?
Authors: No, we have not seen any feature that could be clearly attributed to apoptosis.

Z. Somosy: Do you have any data in your material about changes of cytoskeletal elements?
Authors: Unfortunately, we did not study the cytoskeletal elements in this material.