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Assessment of cyclosporine A-induced ultrastructural changes in vascular wall using an experimental arterial autograft model

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Summary. The objective of this ultrastructural study was to assess the effects of cyclosporine A (CsA) in an experimental model of arterial autograft.

Fifty female Sprague-Dawley rats weighing 250-300 g were employed. Using a microsurgical technique, an arterial autograft measuring approximately 5 mm in length was placed in the right common iliac artery.

Two groups were established: group I (control), consisting of 25 animals subjected only to arterial autograft; and group II (pre- and postoperative CsA), also consisting of 25 animals which received a daily subcutaneous dose of 5 mg/kg CsA (Sandimmun, Sandoz®) on the four days preceding the surgery and thereafter, until sacrifice.

The animals were sacrificed on postoperative day 7, 14, 21, 30 and 50.

The specimens (autografts) obtained were studied under transmission and scanning electron microscopes.

In the control group, the process of endothelialization of the graft was completed by day 14. In the CsA-treated group, restoration of the endothelium took 50 days.

The development of intimal hyperplasia was delayed in the treated group. There were no morphological changes in its structure when compared to the control group.

The tunica media had thinned in the treated grafts, with loss of smooth muscle cells, fragmentation and lysis of the elastic lamina, presence of lipid-filled macrophages, and muscle cells with cytoplasmic lipid vacuoles.

In our opinion, these results suggest that the action of CsA mainly targets on the endothelium and smooth muscle cells, exerting a toxic effect in an *in vivo* arterial graft model.

Key words: Cyclosporine A, Intimal hyperplasia, Vascular endothelium, Arterial graft

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Introduction

Cyclosporine A (CsA) continues to be among the immunosuppressive drugs of choice employed in all the organ transplantation protocols. Its effects in vascular wall have been studied *in vitro* in both endothelial cells (EC) (Zoja et al., 1986; Lau et al., 1989) and in smooth muscle cells (SMC) (Ferns et al., 1990). *In vivo* studies from the ultrastructural point of view are practically nonexistent. We consider that this may be of unquestionable importance since the possible influence of the drug itself and its vehicle has been demonstrated in the onset of lesions similar to those found in the atherogenic process (Mennander et al., 1992).

Our objective was to perform an ultrastructural study of the effects of this drug using an experimental arterial autograft model that has been employed by our research group to assess the phenomenon involved in vascular wall healing (Bellón et al., 1990, 1994; Buján et al., 1992). We consider that the results obtained in rat autograft studies can even be compared with those produced by the drug in other animal species, including humans.

Materials and methods

Experimental animals

Female Sprague-Dawley rats weighing between 250 and 300 g were used.

Microsurgical technique

The microsurgical technique was assisted by operating microscopy (Wild M-650). The animals were anesthetized with a mixture of ketamine hydrochloride (0.5 mg/100 g body weight) and atropine (0.05 mg/100 g body weight), administered intraperitoneally. Median laparatomy was performed to allow access to the area of the right common iliac artery. This was isolated and separated from the iliac vein. A vascular segment of approximately 18 mm in length was obtained, after

which the artery was clamped with a Gilbert double microclamp and a 5 mm segment was resected for reimplantation (arterial autograft). The interpositions were linear, with re-construction of vascular continuity by means of end-to-end anastomosis with interrupted suture using 10/0 monofilament thread (Ethilon, Ethicon®). Once the anastomosis was completed, graft permeability was confirmed visually.

In no case was sterile surgical technique or anticoagulant medication employed.

Experimental design

Two study groups were established: group I (control), consisting of 25 animals subjected only to arterial autograft; group II (pre- and postoperative CsA), also consisting of 25 animals which, on the four days preceding the surgical procedure and postoperative, throughout the entire study period, received a daily subcutaneous dose of 5 mg/kg CsA (Sandimmun, Sandoz®). The injection site was rotated each day.

Study times

The animals were sacrificed on postoperative day 7, 14, 21, 30 and 50. A mean number of 5 grafts were employed for each study time.

Ultrastructural study

The segments were obtained from the animals by reoperation to achieve access to the graft and remove it, together with ample margins of iliac artery on either end.

The animals destined for electron microscopy were perfused at 100 mm Hg with a 3% glutaraldehyde and 1% paraformaldehyde solution. The samples destined for ultrastructural study were fragmented in small sections, placed in Milloning buffer (pH 7.3), postfixed in 2% osmium tetroxide, dehydrated in a gradual series of acetones and embedded in Araldite for thin sections. Afterwards, their contrast was enhanced with lead citrate and they were observed under a ZEISS 109 transmission electron microscope.

For scanning electron microscope (SEM) studies, after a short perfusion of the animals, the samples obtained were opened longitudinally under the operating microscope and submerged in 3% glutaraldehyde, placed in Milloning buffer (pH 7.3) for one hour, dehydrated in a graded series of acetones, reaching critical point in an E-3000 Polaron with CO_2, metallized with gold-palladium and observed under a ZEISS 950 DSM scanning electron microscope.

Results

We have systematized the ultrastructural findings,

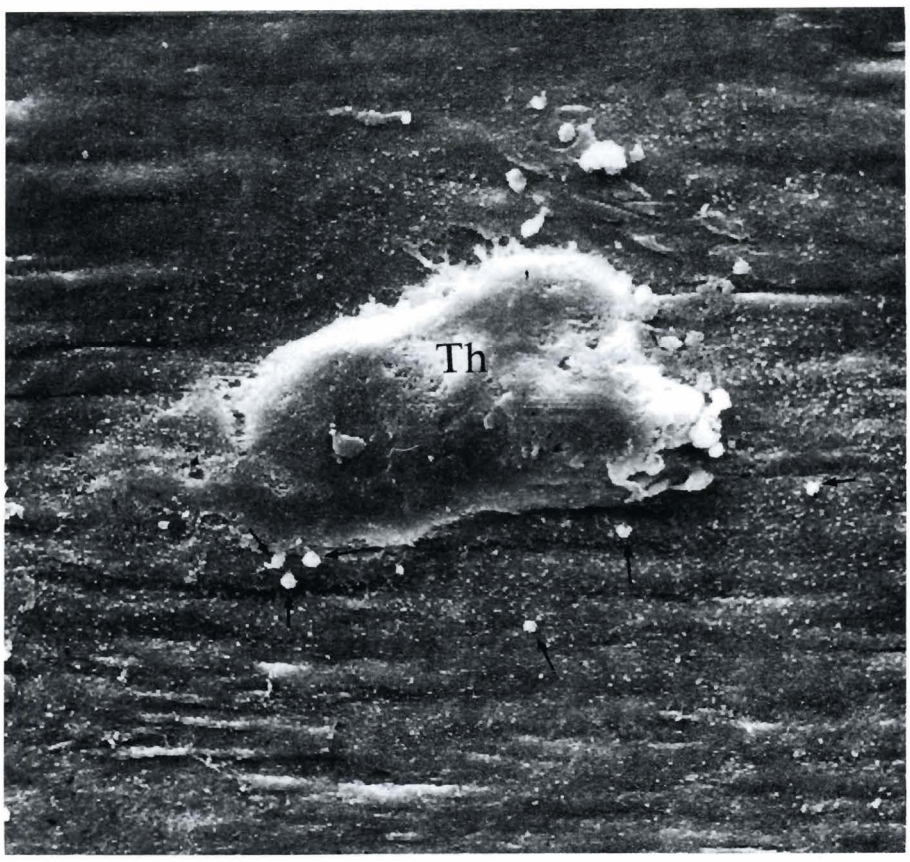


Fig. 1. Small thrombus (Th) attached to the internal arterial surface after 7 days of treatment (white cells: arrows). SEM, x 310

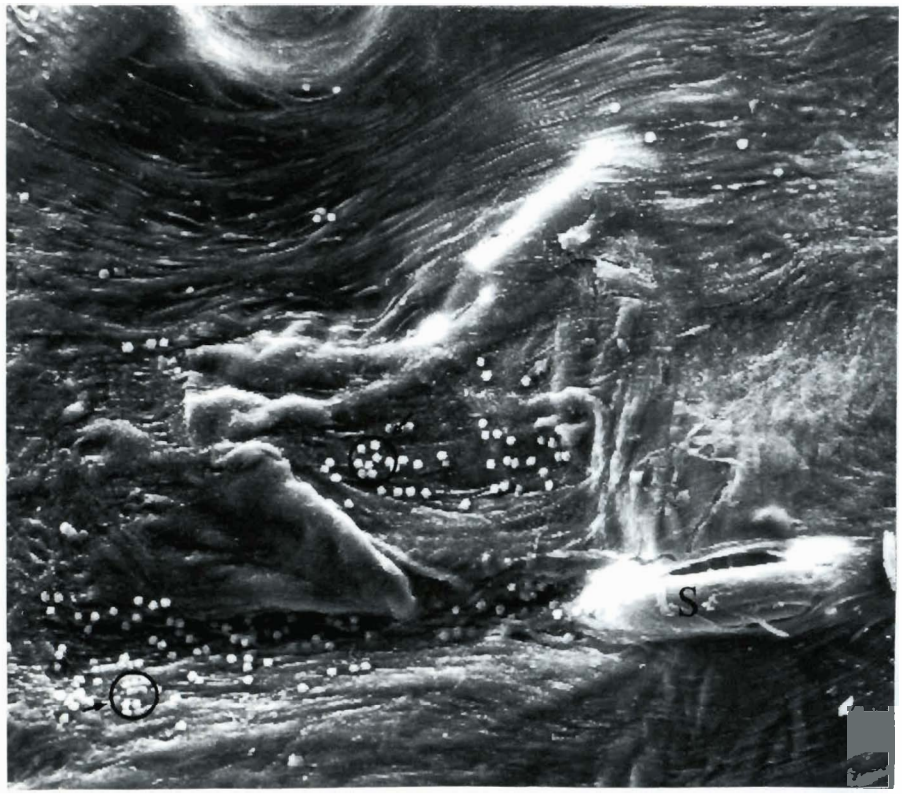


Fig. 2. White cells (arrows) adhering to the suture areas (suture: S) after 50 days of treatment. SEM, x 200

Fig. 3. Photomicrograph showing the restoration of the endothelium (E), as well as the partial detachment of some areas (arrows) of it after 50 days of treatment. SEM, x 500

analyzing the effects of CsA on the different components of the vascular wall.

a) Endothelium

In the control group, after the surgical aggression, deendothelialization was observed in the central area of the graft. In the adjacent areas, the endothelium advanced from the recipient artery, reaching the area of the suture, colonizing the middle portion of the graft in a centripetal direction. Under SEM, accumulations of fibrin, platelets and red blood cells appeared, together with the remains of EC. The endothelialization process was completed by day 14. Small clusters of white cells could be seen in the area of the suture.

CsA treatment increased the thrombogenesis following arterial interposition, sometimes provoking the occlusion of the vessel or the deposition of tiny thrombi visible under SEM (Fig. 1). Endothelial denudation was observed at day 7. Cell debris was dispersed over the internal elastic lamina, especially involving the central part of the micrograft. Abundant white cells were attached to the suture zones (Fig. 2). At three weeks, the process of endothelialization was not yet complete, there being denuded areas together with endothelialized regions. The restoration of the endothelium was reached by day 50 of the study (Fig. 3).

b) Myointima

The development of small patches of intimal hyperplasia was detected in the control group during the first week of repair, most notably in the distal portion. This response increased rapidly to involve the entire operative field, forming an unbroken layer after two weeks. Ultrastructurally, this layer consisted of myocytes of contractile phenotype and an abundant interstitial fibrillar matrix (Fig. 4).

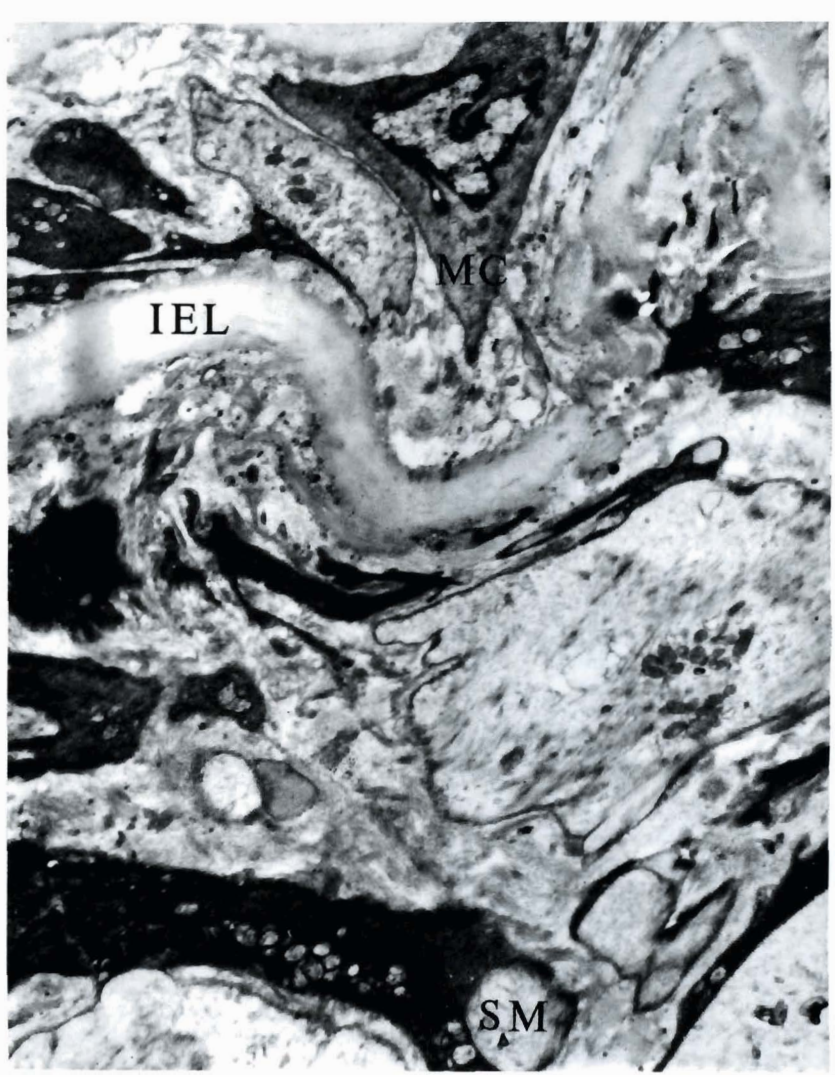
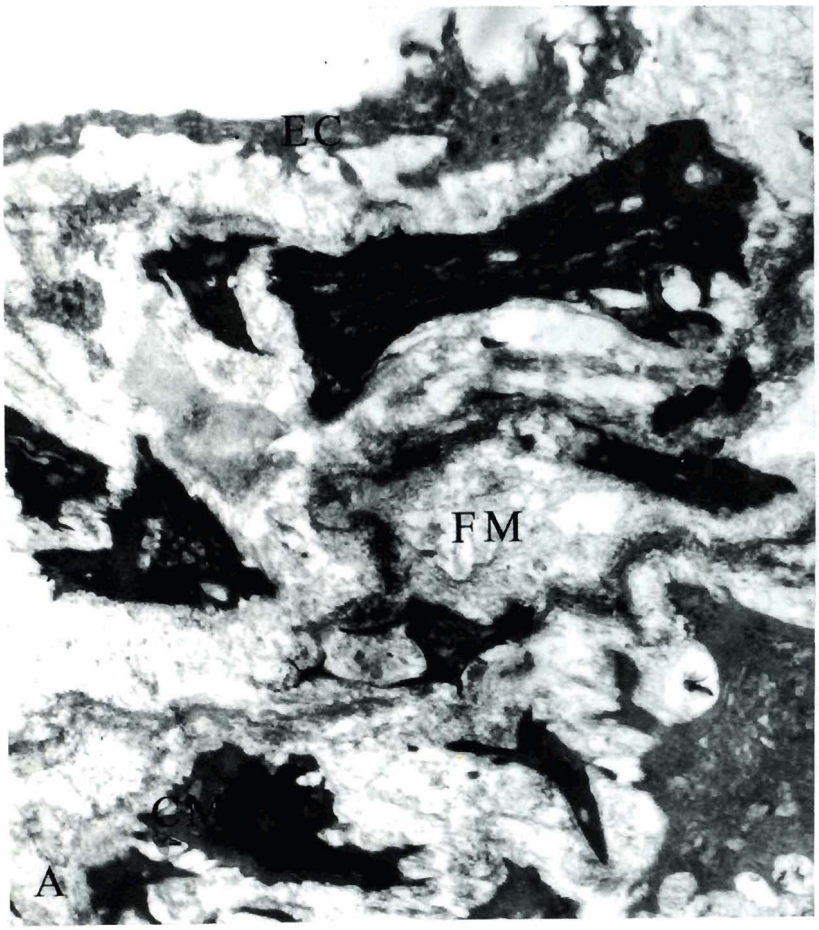


Fig. 4. Control group: ultrastructure of the arterial wall after 2 weeks (myointimal cell: MC; internal elastic lamina: IEL; secretory myocyte of the tunica media: SM). TEM, x 4,400



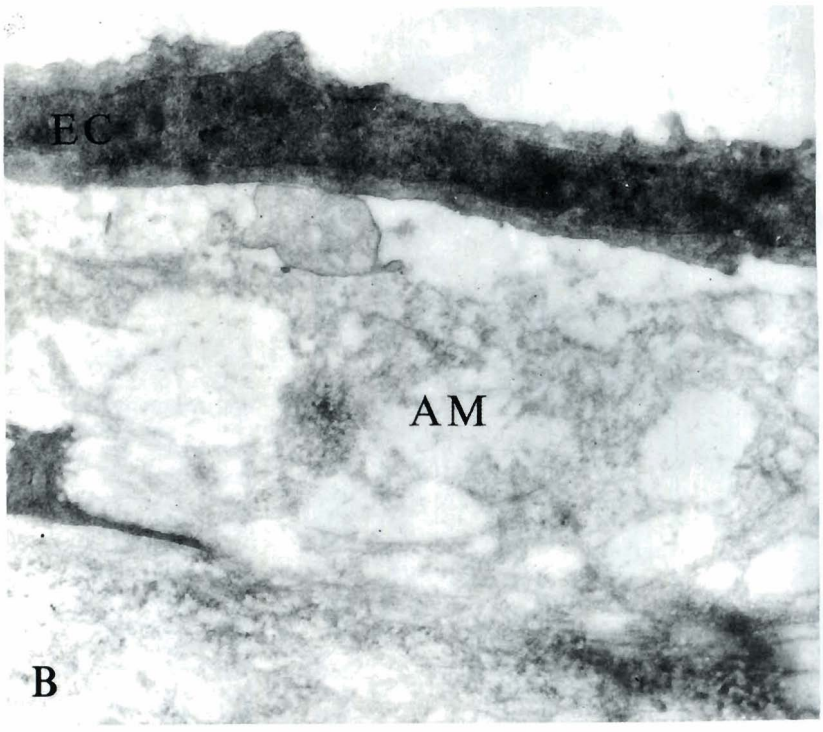


Fig. 5. Intimal hyperplasia (50 days of treatment). **a.** Ultrastructure of the myointimal layer, where contractile myocytes (CM) and abundant fibrillar matrix (FM) can be distinguished (endothelial cell: EC). TEM, x 3,000. **b.** Accumulations of amorphous material (AM) in the subendothelial matrix (endothelial cell: EC). TEM, x 7,000

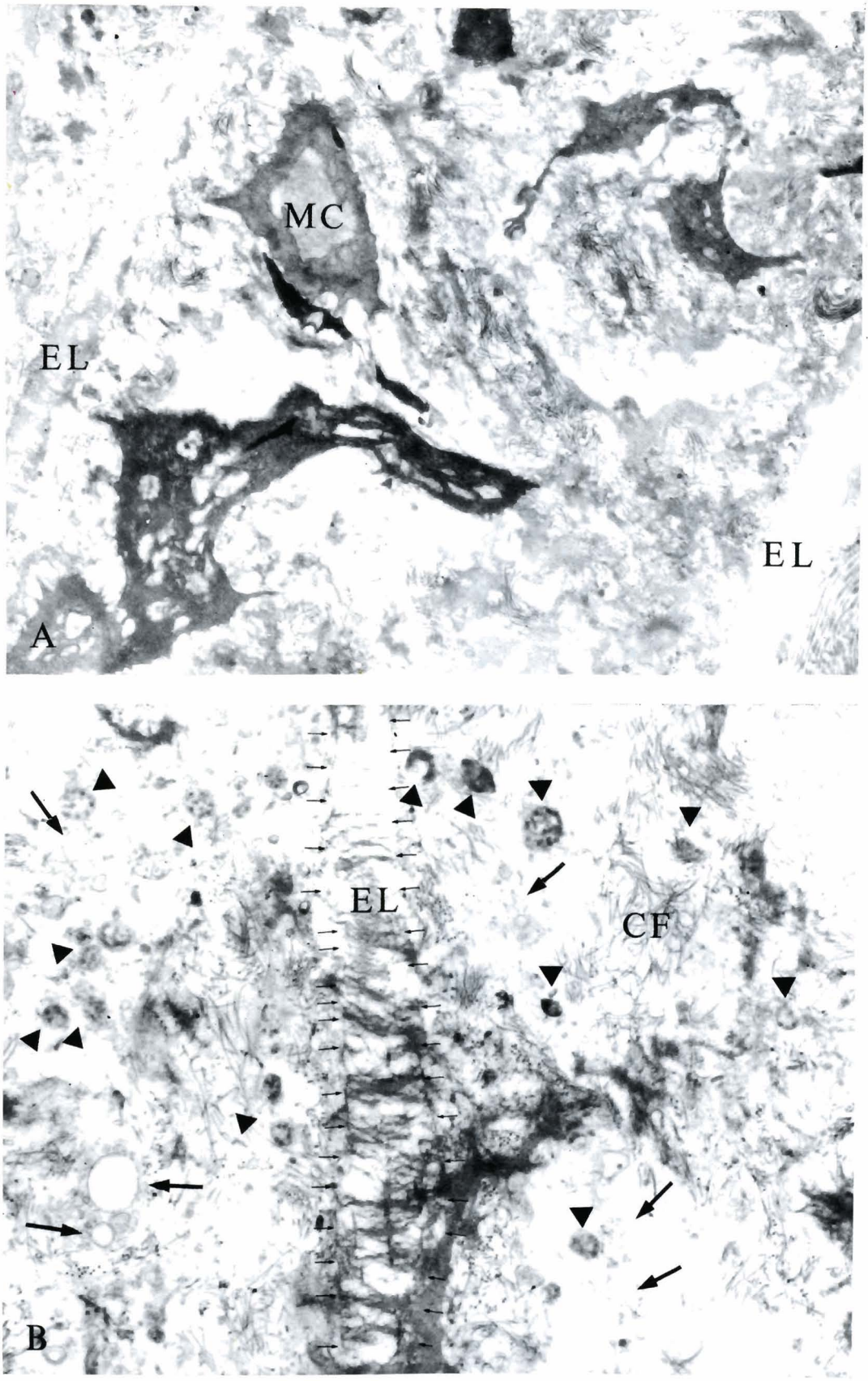


Fig. 6. Tunica media. **a.** Group of muscle cells (MC) remaining in the disorganized tunica media after 30 days of treatment (elastic lamina: EL). TEM, x 4,400. **b.** Extracellular matrix composed of degenerative microvesicles (arrowheads), membrane debris (large arrows) and abundant collagen fibres (CF) after 7 days of treatment. Fragmentation (small arrows) of the elastic lamina (EL). TEM, x 7,000

Following CsA treatment, there was a slight delay in the appearance of this hyperproliferative layer. After two weeks, clusters of myointimal cells were observed at the distal end of the artery, advancing toward the suture, while at the proximal end, this response had not yet been initiated. At day 21, the myointima formed a homogeneous cell layer covering the distal portion, while in the central area of the graft and the proximal end of the recipient artery, only small patches of hyperplasia were detected. The morphology of this layer was similar to that of the control group (Fig. 5a) with respect to cellular elements. However, the accumulation of amorphous material should be pointed out. This material endowed the subendothelial matrix with a stratum similar to that found in fat-rich diets, with a high proteoglycan composition (Fig. 5b), located in the stratum next to the endothelium.

c) Tunica media

In the control group, this layer presented secretory myocytes with abundant endoplasmic reticulum, preferentially located in the deepest area adjacent to the tunica adventitia (Fig. 4). The rest of this layer was formed predominantly by contractile myocytes. No changes were observed either in the collagen fibrillar matrix or in the elastic lamina.

In the graft subjected to the action of CsA, there was considerable involvement of the tunica media. As in the first week, there was a visible disorganization and thinning of this layer, presenting areas of necrosis and few cellular elements alternating with others where muscle cells still remained (Fig. 6a). The extracellular matrix was full of a degenerative microvesicular component with membrane debris and abundant collagen fibres (Fig. 6b). Elastin digestion gave the elastic lamina a friable aspect, reducing them to discontinuous fibrillar material (Fig. 6a,b).

In later studies, fat-filled macrophage-like cells were observed, located in the deepest portion of this layer. Likewise, there were large lipid vacuoles in the cytoplasm of degenerated muscle cells and microvesicular clusters. It was also possible to observe the presence of cells with clear cytoplasm, dense granules and lobulated nuclei, which sometimes presented large vesicles in their interior (Fig. 7).

d) Adventitia

The response of this layer was similar in both study groups, consisting of a high rate of healing response. Fibroblasts, myocytic cells and large amounts of collagen were observed.

The increased white cell infiltration occurring after treatment with CsA should be pointed out, as well as the presence of cells that engulfed lipid vacuoles with their

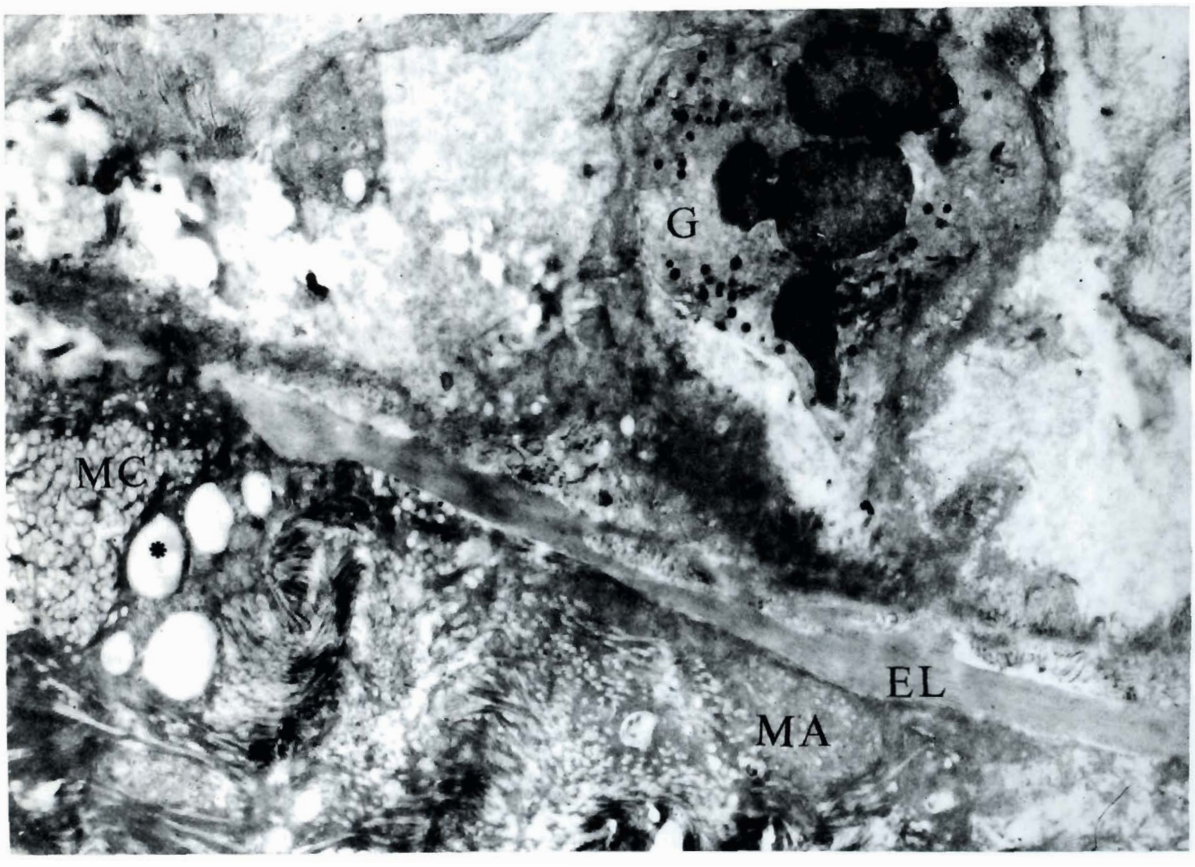


Fig. 7. Muscle cells (MC) in the process of degeneration (observe the vacuolization (*) of the cytoplasm), microvesicular accumulations (MA) and granulocytes (G) with lobulated nuclei after 21 days of cyclosporine treatment (elastic lamina: EL). TEM, x 3,000

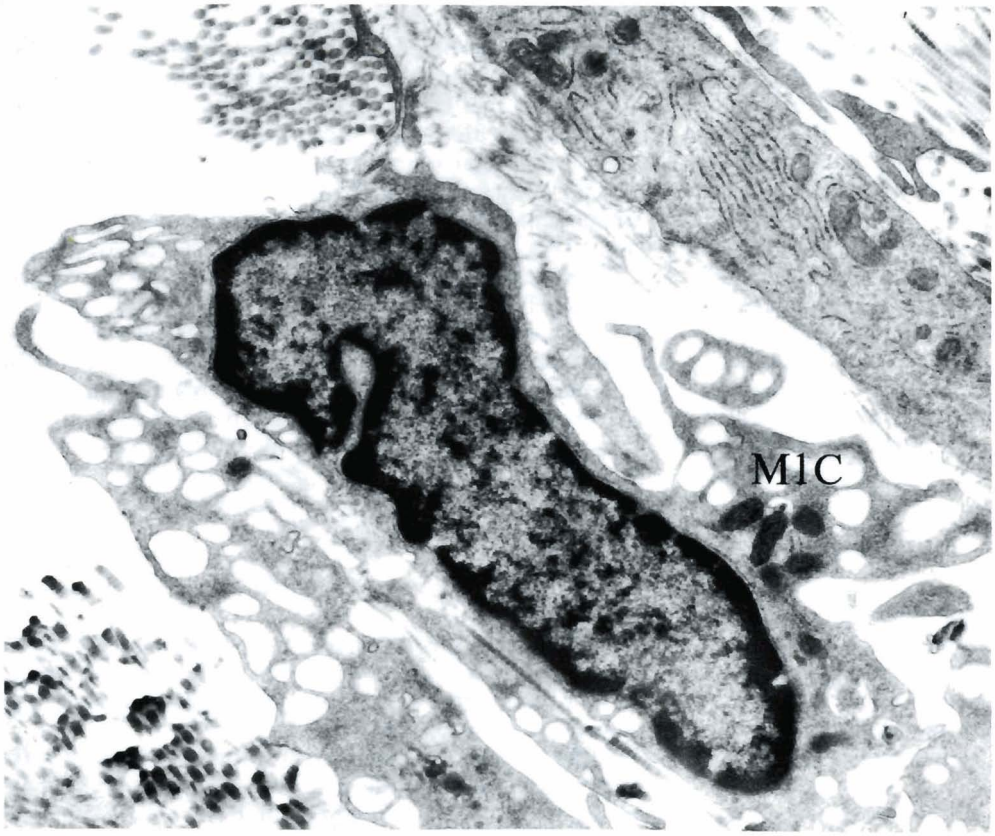


Fig. 8. Macrophage-like cell (MIC) with vesicular cytoplasm in the tunica adventitia after 14 days of treatment. TEM, x 6,300

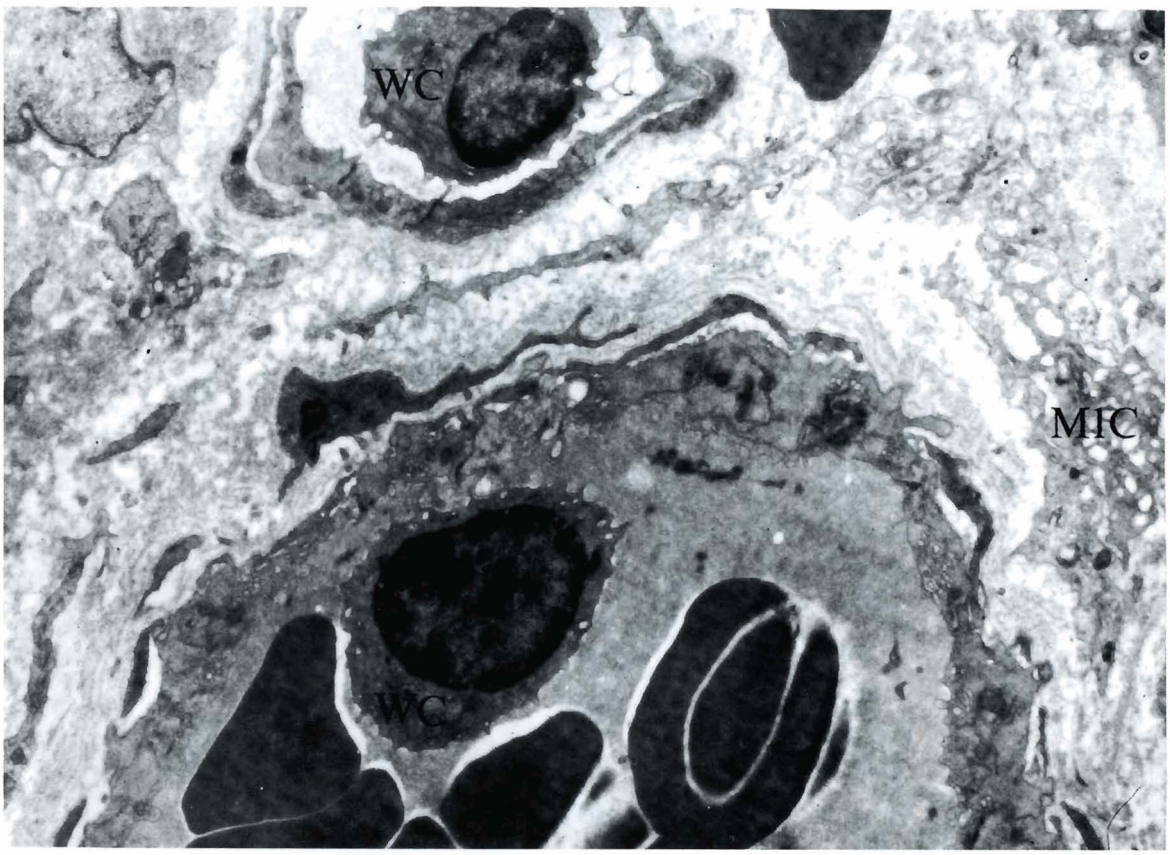


Fig. 9. Adventitia: white cells (WC) in the *vasa vasorum* lumen after 30 days of treatment (macrophage-like cells: MIC). TEM, x 3,000

cytoplasm (Fig. 8). White cells were also observed occupying the lumen of the *vasa vasorum* (Fig. 9).

Discussion

The vascular effects of CsA are numerous and involve all the components of the vascular wall.

In vitro studies of cultured EC performed by Zoja et al. (1986) demonstrated that CsA is toxic to these cells and that said toxicity is time and concentration-dependent. Ferns et al. (1990), again in cell cultures, observed a delay in the achievement of confluence in cultured EC. From the functional point of view, Rosenthal et al. (1989) and Brunkwall and Bergqvist (1993) observed that EC, both in culture and in experimental model set up in rabbit aorta, showed reduced prostacyclin release.

Extrapolating these results to our graft model, the increased thrombogenesis found in the animals subjected to continuous CsA treatment could be related to the delay in the endothelialization of the grafted portion, probably due to the toxic effects of CsA on the EC. The decrease in prostacyclin release on the part of the EC very probably exerts some influence as well. In any case, the delayed endothelialization of the grafts is highly significant in the treated group when compared with the control.

A large portion of the initial studies of the effects of CsA on the vascular wall focused on the inhibitory power of this drug over the intimal hyperplastic phenomenon (Jonasson et al., 1988; Wengrovitz et al., 1990). In agreement with Ferns et al. (1990), we do not consider CsA to inhibit intimal proliferation; rather it delays the onset of this process. On the other hand, we have found no morphological changes in the cellular components of this newly-formed coat.

It is at the level of the tunica media that significant changes take place. There is an evident thinning of the layer and loss of the myocytic population. Cells appear with clear cytoplasm containing small dense granules and lipid vacuoles. On occasion, the nuclei appear to be segmented. These cells may present the morphological features of neutrophils or, on the other hand, could constitute cells undergoing degeneration, or having monocytic-histiocytic features similar to those appearing in human arteriosclerotic processes (Haust, 1980). Lipid-filled macrophages were observed by Ferns et al. (1990). They can also be seen in our model, located in the deepest portion of this layer.

Finally, muscle cells with cytoplasmic lipid vacuoles are present, as well. Reidy (1991) considered CsA to be toxic to SMC. This toxic effect therefore, in our model could be the cause of the degeneration of the tunica media and the loss of SMC. This would explain the presence of cells with degenerative features that are especially observed in the early phases of our study.

All these CsA-induced modifications appear to agree with the elastase-activating factors, which would be responsible for the considerable fragmentation of the elastic lamina associated with this process.

The well-known theories of Hornebeck and Partridge (1975) and Robert and Robert (1980) to explain the degeneration of the elastic lamina in arteriosclerotic processes attribute an important role in the acceleration of the elastolytic enzymes to lipid and calcium.

Endothelial injury is suggested as a key element in triggering the arteriosclerotic process. However, there are several factors that appear to contribute to the degradation of the elastic lamina. Among these is mentioned the release of elastases from the arterial wall, such as the serine-protease produced by SMC (Robert et al., 1984) when they change from contractile to secretory phenotype. Another factor might be the correlation between LDL lipoproteins and the elastolytic activity of human and rat SMC (Bourdillon et al., 1984). Still another, the arrival at the site of the lesion of elastolytic enzymes of different origins, including platelet serine-protease, proteases such as apo A-I (Jacob et al., 1981) using serum lipoproteins as vehicles, and even metalloprotease enzymes bound to neutrophils and monocytes/macrophages (Davis et al., 1984).

On the basis of our results, we can summarize the findings provided by the ultrastructural study of the effect of CsA on the arterial wall of the autograft as follows: a) CsA modulates, but does not inhibit, the formation of the myointima; b) CsA delays the normal endothelialization process; c) CsA provokes damage to the tunica media that resembles the arteriosclerotic process with regard to both the cell substrate and the changes in the extracellular matrix.

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