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Inflammatory cells induce neointimal growth in a rat arterial autograft model

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Summary. Subendothelial invasion by leukocytes is a sign of intimal thickening in arteriosclerosis and in the response of a vessel to mechanical damage. Our study was designed to establish whether these cells are implicated in the formation of a neointima in an autologous arterial graft model in the rat and to evaluate the effects of cyclosporin A (CsA). Three study groups were established according to whether the animals were treated with CsA-Cp (Sandimmun)®, CsA-Et (ethanol vehicle) or received no treatment (control group). Both drug forms were administered (5 mg/kg/day, s.c.) from 4 days prior to surgery until the time of sacrifice. Antibodies specific for lymphocytes (CD4, CD8), monocytes/macrophages-ED1, smooth muscle α-actin and the von Willebrand factor (vWF) were used to identify the cells in the grafted arterial wall. In control grafts, the neointima had formed by 2 weeks post-implant. However, the cells comprising this layer generally presented no positivity whatsoever towards the antibodies employed. At 50 days, the new layer was observed to be formed by a vWF-positive endothelium and α-actin-positive cells. In all three groups, several polymorphonuclear (PMN) cells adhered to the denuded luminal surface from 7 days onwards. In the treated animals, neutrophils and monocytes were seen to infiltrate intimal and medial layers during the later post-implant stages. Around the third week post-implant, the neointima had reached the grafted segment from the distal portion of the recipient artery, and by 50 days it was similar to that seen in control specimens. Our findings suggest that: a) neutrophils play a role in neointimal thickening in this arterial autograft model; and b) CsA promotes the adhesion and infiltration of neutrophils in the injured arterial wall.

Key words: Neointimal thickening, Cyclosporin A, Arterial wall, Macrophages, Neutrophils

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Introduction

After a vascular lesion with loss of endothelium, white blood cells (PMN cells, monocytes and lymphocytes) adhere to the subendothelial surface and thereafter penetrate the vessel wall as far as the medial layer (Lucas et al., 1986; Bellón et al., 1995). Using different animal models of vascular injury, it has been established that the thickening of the intima is preceded by this infiltration of leukocytes (Tanaka et al., 1993; Rogers et al., 1996), which appears to trigger the onset and progression of arteriosclerosis following this type of injury (Walczak et al., 1999).

The inflammatory cells involved in this response (mainly T CD4+ cells) produce cytokines, matrix metalloproteinases and growth factors that stimulate the proliferation of smooth muscle cells and induce arterial occlusion after endothelial damage (Hancok et al., 1994; Weyand et al., 1998). Proliferating leukocytes contribute towards the phenotypic changes that occur in smooth muscle cells in coronary vasculopathy associated with transplant or accelerated atherogenesis (Shirasawa et al., 2000). However, the role of these cells in the reparatory response of autologous grafts has not yet been fully established. Our aim was to determine the extent of participation of the different inflammatory cells in the formation of the neointima using an arterial autograft model in the rat. A further aim was to evaluate the effect of both cyclosporin A (CsA) and its vehicle on these cells. This immunosuppressant selectively acts on T lymphocytes and also induces atherogenic changes in the grafted vessel (Bellón et al., 1995).

Materials and methods

Experimental animals

Female Sprague-Dawley rats weighing approximately 250-300 g at the time of surgery were employed. The animals were kept and handled according to European Committee guidelines for the care and use of laboratory animals (No. 28871-22A9).

Microsurgical technique

Animals were anaesthetised by the intraperitoneal administration of a mixture of ketamine chlorohydrate (0.5g/100g weight) and atropine (0.05mg/100g weight). A midline xypho-pubic laparotomy was performed to expose the right common iliac artery. The artery was separated from the iliac vein and a segment was dissected with the help of a Wild M-650 surgical microscope. The artery was clamped with a double Gilbert microclamp and a portion, 5 mm in length, was obtained. After preservation for 10 min in Ringer's lactate solution, the arterial segment was reimplanted by end-to-end anastomosis using 10/0 monofilament suture thread (Ethilon-Ethicon®), to achieve loose stitches. The surgical technique was non-sterile. No anticoagulant was used. Graft patency was visually confirmed.

Experimental design

Three study groups were established: control (n=25), animals undergoing arterial autograft; CsA-Cp (n=25), animals undergoing arterial autograft that were subcutaneously administered CsA (Sandimmun®, Sandoz, Spain) in an oil vehicle (Cremophor®, Sandoz, Spain) at a dose of 5 mg/kg/day, from 4 days before implant until sacrifice; and CsA-Et (n=25), animals undergoing arterial autograft treated with CsA in an ethanol excipient by the same route and at the same dose as in the previous group. This preparation of CsA was made according to a protocol described elsewhere (Bellón et al., 1995).

The animals were sacrificed at 7, 14, 21, 30 or 50 days post-implant. At each of these follow-up times, 5 grafts per group were subjected to evaluation.

Evaluation methods

Specimen preparation

Graft specimens were obtained by re-intervention. Prior to sacrifice, the animals were subjected to double perfusion with Ringer's lactate and 3% glutaraldehyde/1% paraformaldehyde (2:1) in Millonig buffer, via an intraventricular catheter at a pressure of 100 mm Hg. The grafted arterial segment was harvested along with a small portion of the recipient artery at each end. Samples were taken for light (LM) and transmission electron (TEM) microscopy.

Immunohistochemistry

For the identification of neointimal cells, specimens were fixed by immersion in 10% formaldehyde, embedded in paraffin and cut into 5 μm-thick longitudinal and transverse sections.

The monoclonal antibodies used were specific for rat monocytes/macrophages-ED1 (1:100) (MCA-341, Serotec, Oxford, UK), and CD4, W3/25 (1:50) (CTS 515G, Labgen, LabClinics, Barcelona) and CD8, MRC OX-8 rat lymphocytes (1:50) (CTS 418G, Labgen, LabClinics, Barcelona), smooth muscle α-actin (1:200) (1A4, A-2547, Sigma, St Louis, MO, USA), and the vWF of endothelial cells (1:1000) (F-3520, Sigma, St Louis, MO, USA). The activity of the antibodies specific for rat CD4 and CD8 lymphocytes employed has been tested on tissue fixed in formaldehyde/alcohol and embedded in paraffin (Whiteland et al., 1995; Buján et al., 2001). As a negative control of the technique used, tissue specimens were incubated with an isotype negative (IgM) of the corresponding primary antibody. Positive controls were transverse sections of rat heart tissue from an allogenic transplant that was positive for the antibodies used at the coronary artery level (Jurado et al., 1999; Buján et al., 2001). The antigen-antibody reaction was detected according to the peroxidase-antiperoxidase-diaminobenzidine and/or phosphatase alkaline-fast red methods. Cell nuclei were stained with haematoxylin or methyl green. The processed specimens were observed under a Zeiss Axiophot light microscope.

The maturity and stability of the smooth muscle cells (contractile phenotype) in the neointima were established using an immunohistochemical labelling technique for TEM. This analysis was based on the detection of α-actin filaments using the smooth muscle α-actin antibody (1A4, A-2547, Sigma). Negative and positive controls of the technique were the same as those described above.

Arterial segments were fixed by immersion in a mixture (2:1) of 0.5% glutaraldehyde/2% para-formaldehyde in Millonig which was replaced after 2 h with Millonig buffer (pH 7.3). Next, the specimens were dehydrated in ethanol and embedded in Lowicryl K4M. Polymerisation was by exposure to ultraviolet light at 4 ºC during the first 24 h, and at 25 ºC during the subsequent 48 h. The blocks were reworked in a Reichert TM-60 instrument and 70-80 nm tissue sections were obtained using a Reichert-Jung ultramicrotome. The sections were collected over Ni grids (100 Mesh Square, EMS, USA).

The grids were incubated in phosphate-buffered saline (PBS 1X) plus bovine serum albumin (BSA 1%) and glycine (20 mM) (30 min), and then incubated with the primary antibody (anti-α-actin, 1:200) (2 h) and secondary antibody labelled with colloidal gold (1:150) (immunogold EM conjugates goat anti-rabbit IgG, 30 nm Gold, Biocell) (2 h). All the steps were performed at room temperature in a humid atmosphere. Finally, the sections were contrasted with uranyl acetate (30 min) and examined using a Zeiss 109 transmission electron microscope.

Morphometry and cell quantification

The morphometric analysis was performed on 30 transverse 5-micron-thick histological sections (10 visual fields x40/section) using a computerised image analyser (MICROM). Neutrophils were counted by

subjecting 30 ultrathin sections to TEM examination (10 visual fields x2500/section). Estimation was made of: (1) the thickness of both the neointima (distance from the lumen to the internal elastic lamina) and media (distance from the internal to the and external elastic lamina) in the grafted segment; and (2) the number (%) of inflammatory (CD4, CD8, ED1 cells and neutrophils) and α-actin-positive cells comprising the intimal hyperplasia.

Statistical study

Morphometric measurements were analysed using a descriptive statistical method to calculate the arithmetic means and standard deviations. The Kruskal-Wallis test with the Dunn correction (inflammatory and α-actin-positive cells), and Mann-Whitney U test (intimal thickening) were applied to compare mean results corresponding to the different groups and follow-up stages.

Results

Microscopy and immunohistochemistry

A greater proportion of animals treated with CsA-Cp (9%) showed luminal thrombogenicity than those treated with CsA-Et (6%), although in no case did this lead to the death of the animal.

Seven days after surgery, the control group specimens showed endothelial denudation and a few platelets, monocytes and PMN cells adhered to the subendothelial matrix. Small groups of unlabelled cells were also observed in the internal elastic lamina. At two weeks post-implant, it was possible to see a neointima composed of an internal monolayer of flat cells, some of which showed positivity towards the endothelial antibody (vWF). Underlying these cells, several layers of cells, mostly showing a secretory phenotype (characterised by a lack of α-actin filaments), were noted. Only a few cells in the neointima reacted with the antibody against smooth muscle and ED1 macrophages, and there was a scarce presence of white blood cells (CD8- and CD4-positive). In areas close to the sites of anastomosis, some PMN cells (of neutrophilic appearance), ED1-positive macrophages and CD8-positive lymphocytes could be seen around the suture stitches. At 30 days, the neointima was composed of a vWF-positive continuous endothelium, α-actin-positive cells (Fig. 1a) and a few ED1-positive macrophages. By the 50th day post-implant, almost all the neointimal cells showed the morphology of a contractile smooth muscle cell. Gold granules (secondary antibody) outlined the α-actin filaments (Fig. 1b,c) and reflected the phenotypic change undertaken by these cells during the course of the reparatory hyperplasic response.

The medial layer of control group specimens was mainly composed of α-actin-positive cells. During the first week after surgery, some cells close to the adventitia showed secretory characteristics with loss of α-actin filaments. These cells increased in number from 14 to 21 days.

In the treatment groups, there was an increase in the number of PMN cells (of neutrophilic appearance) adhering to the luminal surface and infiltrating in the neointimal layer compared to control grafts (Fig. 2). Throughout the study period, these cells could be seen to infiltrate and penetrate the deepest zone of the media and areas adjacent to the vessels of the adventitia (Fig. 2b-d). These effects were most marked in the CsA-Cp group. In general, these cells showed negativity towards the antibodies tested and only a few were ED1-positive (Fig. 3). Most of the white blood cells showed the typical ultrastructure of PMN cells, mainly corresponding to neutrophils. In the CsA-Cp group, some neutrophils showed small lipid droplets in their cytoplasm. At 21 days post-implant, several cell layers were seen overlying the internal elastic lamina. These only affected the distal portion of the recipient artery and corresponding anastomosis. None of these cells were marked with the antibodies employed. At this stage, the medial layer of the graft showed substantial cell loss (Fig. 2), and basically, elastic laminae and other matrix proteins were all that remained. In the proximal portion of the recipient artery, cells of the media expressed α-actin filaments (at each stage), while at the distal end, cells of the deep media showed a secretory morphology. After a month of progression, a consolidated neointima could be observed on the grafted segment, which

Table 1. Proportion (%) of inflammatory and α-actin-positive cells comprising the intimal hyperplasia of the grafted segment in an arterial autograft model in the rat according to the study group (Control, CsA-Cp and CsA-Et) and time after implant (7, 14, 21, 30, 50 days).

	MONOCYTE/ MACROPHAGES (ED1-positive)	NEUTROPHILS	SMC (α-actin-positive)
Control			
7	---	1.5±0.70	---
14	2.1±0.99	12.4±0.69	1.2±0.42
21	10.5±1.26*	11.8±0.78	10.4±0.70
30	11.8±1.47*	5.2±0.78	58.6±0.69
50	4.8±1.03	3.2±0.42	80.6±0.84
CsA-Cp			
7	---	5.1±0.31	---
14	1.5±0.52	19.7±0.82	2.7±0.82
21	5.4±0.51	17.4±3.21	4.8±0.92
30	4.3±0.48	17.0±1.05*	46.7±0.82
50	0.9±0.31	15.5±0.70*	76.1±0.73
CsA-Et			
7	---	2.2±0.42	---
14	---	19.2±1.22	---
21	0.9±0.31	17.2±1.32	5.7±0.82
30	1.9±0.31	17.1±1.29*	51.3±1.76
50	1.3±0.48	12.6±1.77*	81.0±1.15

Results expressed as the mean±standard deviation. Asterisks indicate a significant difference according to the Kruscal Wallis test where $p<0.05$.

showed the same cellular characteristics as those noted in control group specimens (Fig. 1d-e). Lining the arterial lumen, we were able to observe a layer of vWF-positive endothelial cells (Fig. 4) and it was also possible to detect small vessels labelled with this antibody embedded in the new intimal layer.

Morphometry

Figure 5 shows the thickness values obtained for the medial and neointimal layers of grafts from the treatment and control groups.

A significant difference ($p>0.001$) in the thickness of

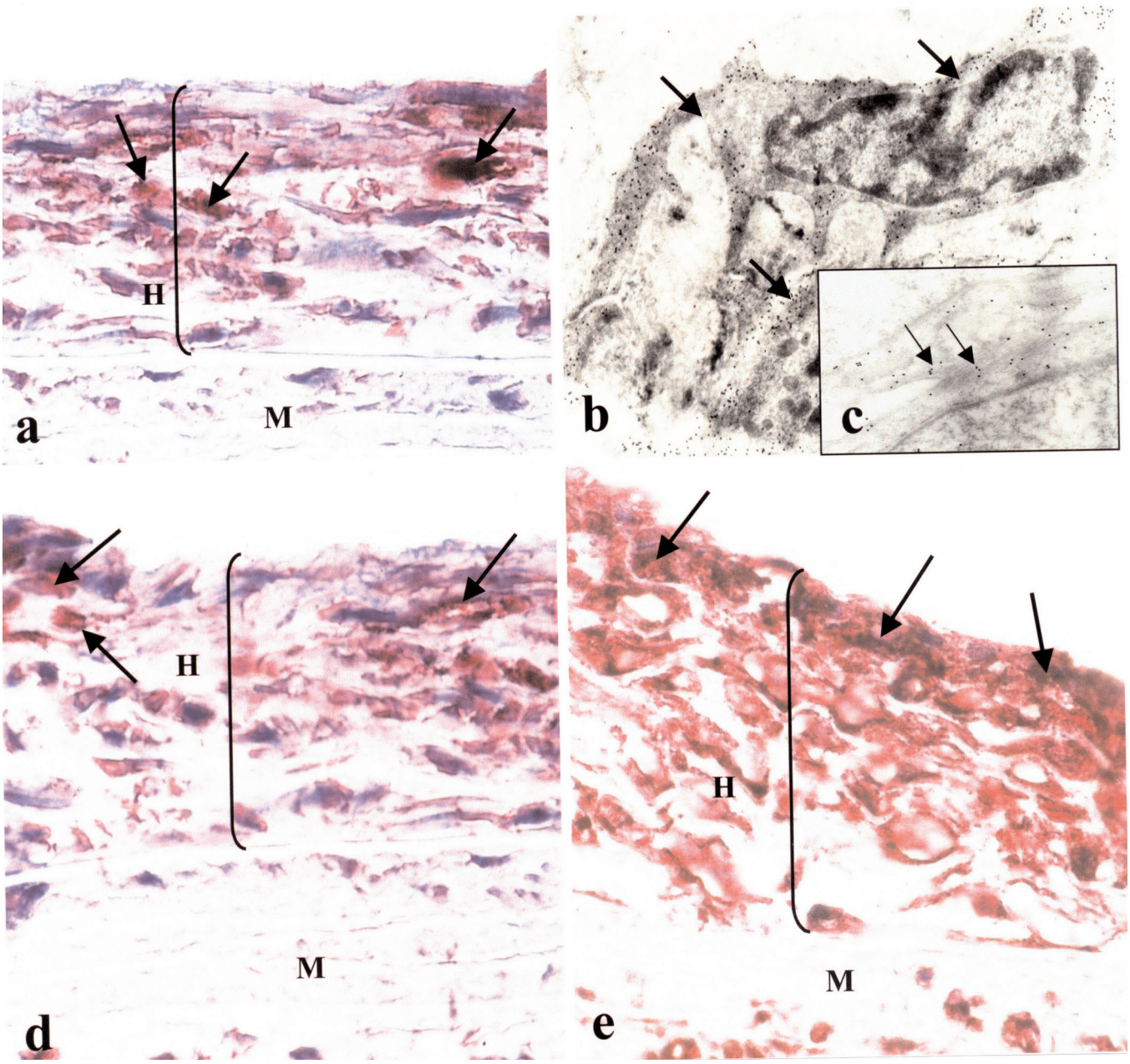

Fig. 1. Immunohistochemical labelling with the 1A4 (smooth muscle α-actin) antibody. **a.** Control autograft at 30 days showing a few α-actin-positive cells in the neointima (arrows). L.M, fast-red/haematoxylin, x 400. **b.** TEM image showing labelling (arrows) in cells of the myointima 50 days after arterial grafting in the control group. x 5000. **c.** Detailed image of gold granules (immunogold) outlining α-actin filaments in myointimal cells. x 20,000. **d.** α-actin-positive cells in the neointima (arrows) of a 30-day-old graft from an animal treated with CsA-Cp. L.M, fast-red/haematoxylin, x 630. **e.** Transverse section of a 50-day-old arterial graft from an animal treated with CsA-Et. All the cells of the myointima are labelled with the antibody. L.M, fast-red/haematoxylin, x 630. H: neointima or intimal hyperplasia; M: medial layer.

the media (Fig. 5a) was shown between control and treatment groups from 14 days onwards. In both treatment groups, this layer gradually became thinner from the initial follow-up stage. CsA led to medial cell loss with respect to the control group. Both CsA-Cp and CsA-Et delayed the onset of hyperplasia with respect to the control group (Fig. 5b). A discrete neointima was observed between 14 and 21 days after implant in animals treated with CsA-Cp, and hyperplasia became quantifiable for the first time at 21 days in those administered CsA-Et. From then on, hyperplasia increased to a maximum value at 50 days. Differences between the two treatment groups and the control group were statistically significant at 14, 21 and 30 days ($p<0.001$). However, at 50 days, hyperplasia was more intense in the CsA-Cp group than in the control group, but the difference was not significant. Neither were significant differences recorded between the CsA-Et and the control group at 50 days.

The media was similar in thickness to the neointima from 30 days after implant.

Table 1 provides the counts of inflammatory and smooth muscle cells in the neointima. The number of CD4- and CD8-positive lymphocytes was low. Since these cells were restricted to areas close to the sutures it was not possible to perform random counts.

A larger proportion of labelled macrophages (anti-ED1) was detected in the control group than in the treatment groups at each of the follow-up times; these differences were significant (Kruscal Wallis, $p<0.001$) at 21 and 30 days. However, even in the control group, percentages never exceeded 14%.

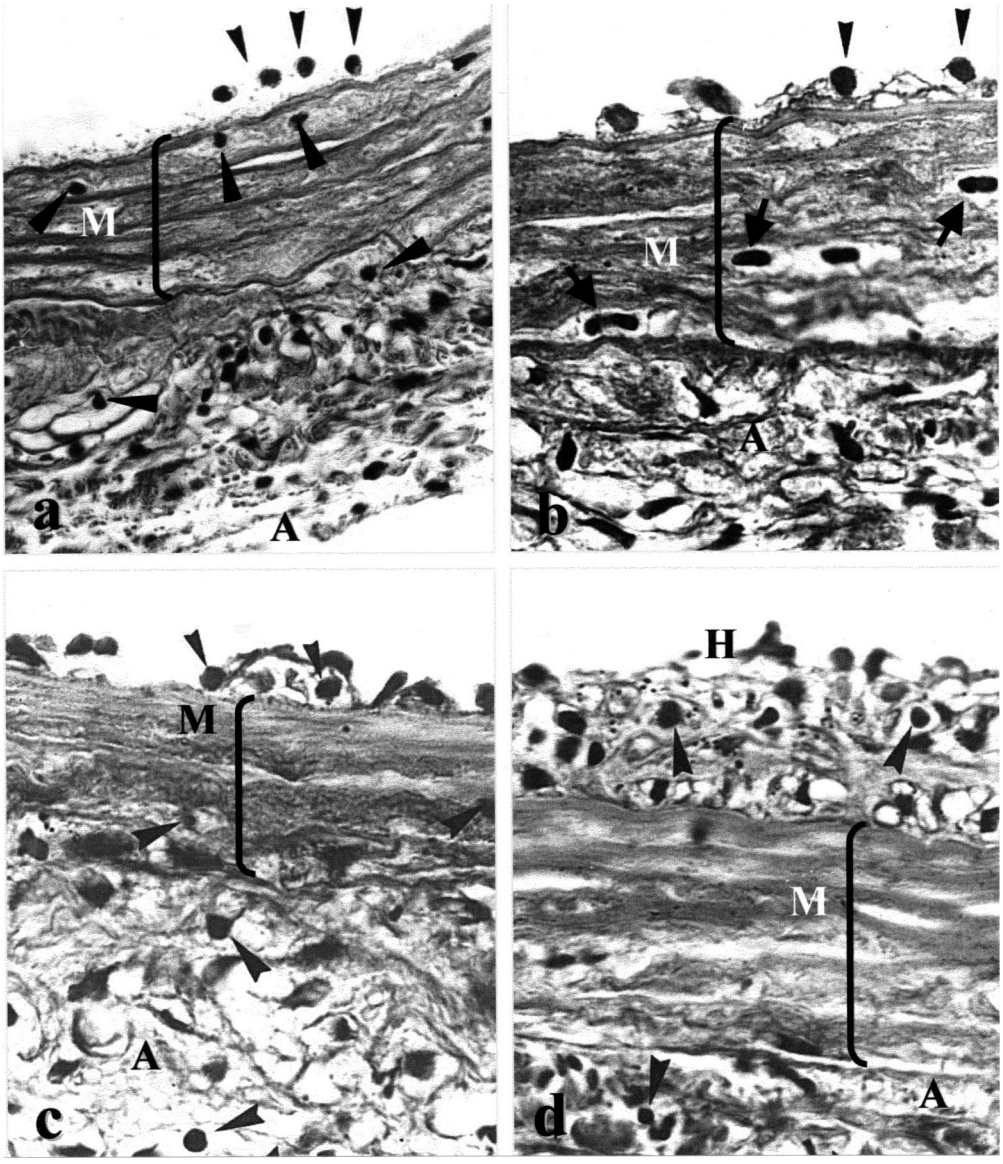

Fig. 2. Autograft from an animal treated with CsA-Cp. White blood cells (arrowheads) adhered to the denuded luminal surface and infiltrate the different arterial layers at 7 **(a),** 14 **(b)**, 21 **(c)** and 30 **(d)** days in the grafted arterial segment. PMN cells (arrows) colonising the medial layer of the graft at 14 days post-implant **(b)**. L.M. x 400. H: intimal hyperplasia; M: medial layer; A: adventitia.

Most outstanding differences were shown by the proportion of neutrophils in the intima of the grafts. However, as for the ED1-positive macrophages, percentages did not exceed 20% of the total number of cells of the intima. By 7 and 14 days after surgery, the neutrophil count was slightly higher in the treatment groups, and from the third week onwards, differences were more marked. The control group showed a drop in the number of neutrophils, while in grafts from animals treated with either CsA formulation, the percentage of neutrophils remained high (peaking at 14 days) until the end of the study. Thus, at 30 and 50 days the control and treatment groups showed significant differences in this variable (Kruscal Wallis, $p<0.001$).

Alpha-actin-positive cells showed an increase over time that was concurrent with the increase in thickness of the neointima. Differences noted between the control and treatment groups were only significant at 21 days ($p<0.05$).

Discussion

Despite considerable differences in the cell and temporal characteristics of the inflammatory response in

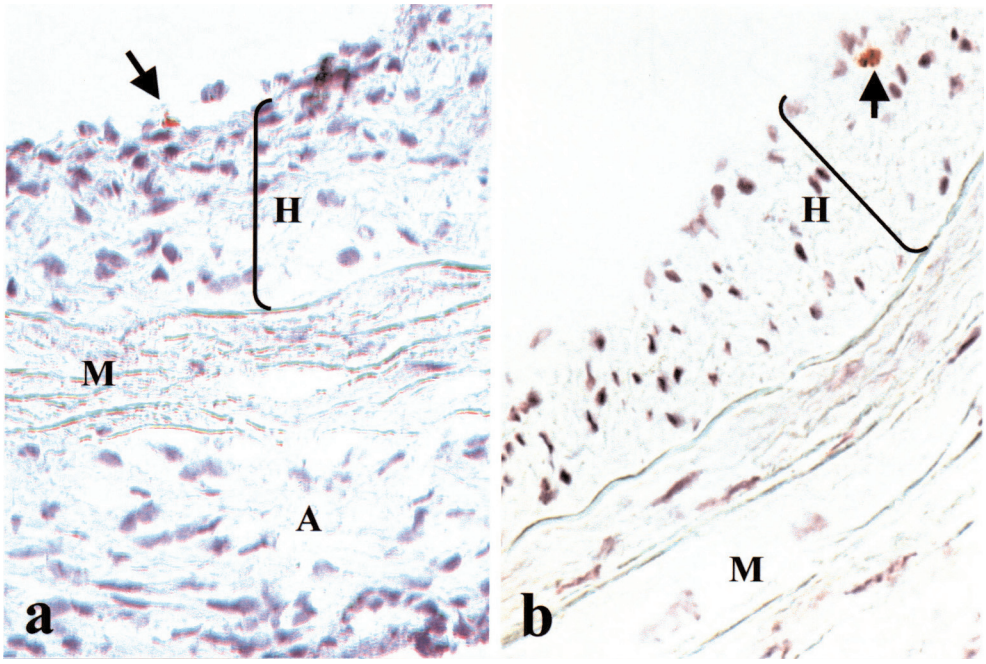

Fig. 3. ED1-positive monocytes/macrophage (arrows) in the neointima of 30-day-old grafts from animals treated with: **a.** CsA-Cp; **b.** CsA-Et. L.M, fast-red/haematoxylin, x 400. H = intimal hyperplasia; M: medial layer; A: adventitia.

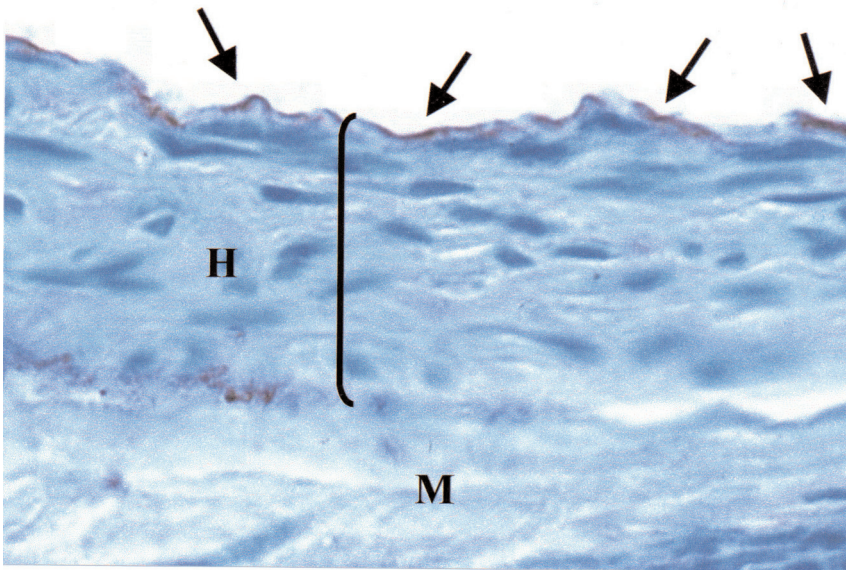

Fig. 4. vWF-positive endothelial cells (arrows) on the myointima of the grafted segment at 50 days post-implant (CsA-Et). L.M, diaminobenzidine-methyl green, x 400. H: intimal hyperplasia; M: medial layer.

different models of vascular injury (Welt et al., 2000), inflammatory cells are key mediators of restenosis following vascular damage.

Monocytes and macrophages control the proliferation and migration of smooth muscle cells, lipid metabolism and inflammation within the blood vessel wall. Circulating monocytes are among the first cells that infiltrate the vessel wall after experimentally-induced damage in different animal models (Gerrity, 1981; Rogers and Karnovsky, 1988; Rogers et al., 1996) and in spontaneous arteriosclerosis in human arteries (Jonasson et al., 1986).

Other inflammatory cells that infiltrate the arterial wall in the early stages of damage are neutrophils (Jorgensen et al., 1988; Richardson et al., 1990; Kockx et al., 1992, 1993; Roque et al., 2000; Welt et al., 2000). These cells play an important role in the development of intimal hyperplasia and in the progression of restenosis (Simon et al., 2000; Welt et al., 2000; Bienvenu et al., 2001). Moreover, smooth muscle cells proliferate when cocultured with neutrophils or in a neutrophil-conditioned medium (Cole et al., 1988).

CD4-positive cells or their secretion products have also been demonstrated to actively participate in the pathogenesis of the neointima following endothelial damage (Hancock et al., 1994).

In different models of mechanical aggression, luminal occlusion is associated with the accumulation of large quantities of CD4- and ED1-positive mononuclear cells, monocytes and neutrophils in the intima. Monocytes, macrophages and neutrophils adhere to the denuded luminal surface and infiltrate the media after endothelial denudation (Hanke et al., 1994; Rogers et al., 1996; Simon et al., 2000; Welt et al., 2000; Bienvenu et al., 2001). However, other studies have shown an absence of monocytes and macrophages in arteries denuded by a balloon-catheter (Yasukawa et al., 1997; Welt et al., 2000).

In human heart transplants, Hruban et al. (1990) suggested that heart vasculopathy is mediated by an endothelialitis directed by cytotoxic T lymphocytes (CD8- and CD2-positive) infiltrating the subendothelial space in affected vessels, together with the accumulation of macrophages and proliferation of smooth muscle cells in the intima. In contrast, Deng et al. (1995) found no significant relationship between inflammatory cells and the intimal thickening observed in coronary arteries following transplant.

Shi et al. (1996) observed that T CD4-positive cells (helper) and macrophages were essential for the development of concentric neointimal proliferation, which is characteristic of arteriosclerosis in the transplanted carotid artery in the mouse. However, T CD8-positive cells (cytotoxic) and "natural killer" cells do not participate in this process.

Similarly, we observed that CsA induced white blood cell adherence to the luminal surface in both our treatment groups, and that adherence was greater when the CsA vehicle was oily. The white blood cells that infiltrate the vascular wall contribute to the development of an intimal hyperplasia of an atherogenic nature. Monocytes and macrophages release fibroblast growth factor and platelet-derived growth factor, both implicated in the development of arterial lesions. In our model, CD4- and CD8-positive cells were scarce in all the groups established and were confined to the areas of suture. This may be attributable to the fact that the grafts were autologous. However, we were previously able to detect cells with the ultrastructure of neutrophils (Buján et al., 1995) adhered to and infiltrating the different arterial layers, especially in grafts from animals treated with CsA (irrespective of the vehicle). Welt et al. (2000) reported an abundance of neutrophils in the tunica media

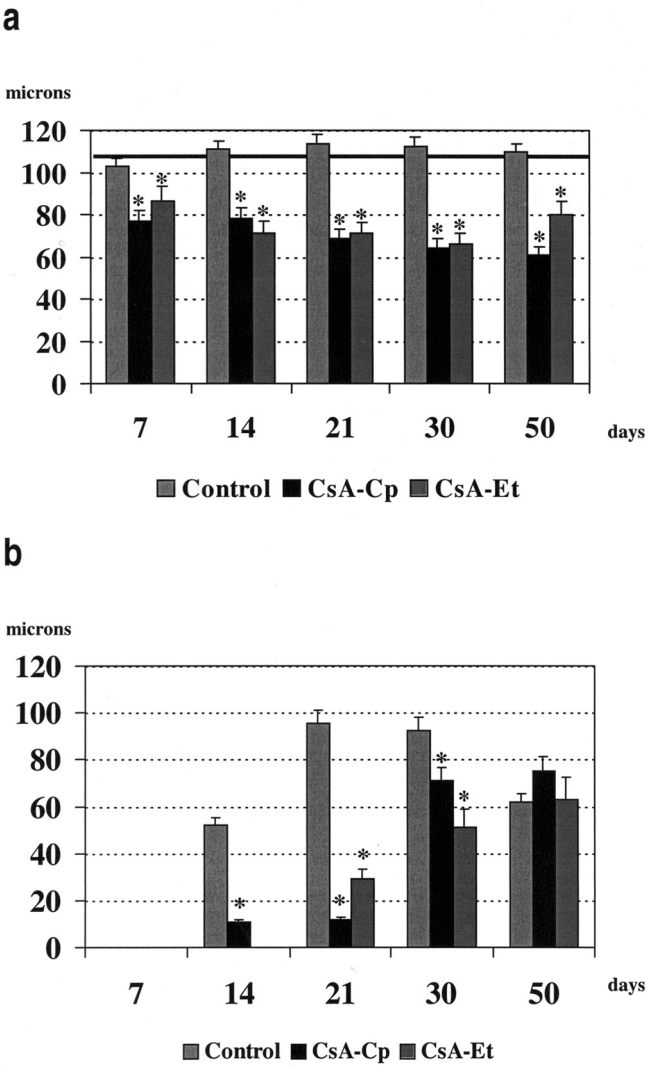

Fig. 5. Central portion of an arterial graft. **a.** Thickness of the medial layer in specimens from the untreated control and CsA-treated groups (changes in mean±SD). Black line represents mean thickness of the medial layer in a healthy rat iliac artery. *p<0.001. **b.** Progression of hyperplasia in grafts from untreated control and CsA-treated animals (changes in mean±SD): *p<0.001.

6 h (with a peak at 3 days) after endothelial denudation in an angioplasty model. These cells appear to perform the same functions as lymphocytes and macrophages in the neointima of allogenic grafts, attracting and promoting the proliferation of smooth muscle cells at this level. The deposition of neutrophils in the intima of damaged arteries has also been recently described in balloon catheter-induced models of angioplasty (Roque et al., 2000; Welt et al., 2000).

The greater presence of neutrophils in the present treatment groups may be explained by the fact that CsA interferes with the normal recovery of the endothelium (Buján et al., 1995). Endothelial cells play an important role in the modulation of the immune and inflammatory response (Colditz, 1985), producing substances that promote or inhibit the adhesion of neutrophils. The damaged endothelial barrier promotes the adhesion of these cells to the intima. However, this accumulation does not imply that the layer, which also shows slower development, is of greater thickness, but rather, that these cells replace other cell types in this model. Umemura et al. (1999) suggested that neutrophils may have a significant role in intimal thickening following endothelial damage.

On the other hand, CsA is highly toxic for the smooth muscle cells of the media; a notorious loss of this cell type being observed as early as one week post-implant. Potential mechanisms for the reduction of the media include the continuous migration of smooth muscle cells from the media to the intima, and sustained loss (via necrosis or apoptosis) in the absence of continued proliferation of medial smooth muscle cells (DeBlois et al., 1996; Hirsch et al., 1998). The resultant cell remnants might in turn act as chemotactic agents for neutrophils, provoking their infiltration in the graft wall. In this way, CsA may favour the arrival and persistence of neutrophils at the site of injury in the artery. It is these cells that would then be responsible for activating the proliferation of smooth muscle cells in the intima.

The inhibitory effects of CsA on T and B lymphocytes and macrophages are widely known. It was demonstrated that CsA inhibits T cell proliferation and blocks gamma-interferon production, a cytokine required for normal macrophage and monocyte functions (Kalman and Klimpel, 1983). However, little is known to date about its effects on neutrophil functions (Spisani et al., 2001). This drug has been described to show no inhibitory effects on PMN migration or phagocytic activity (Wish, 1986) or more specifically on the phagocytic role of neutrophils (Ormrod et al., 1990). A recent investigation by Scavuzzo et al. (2001), in which different CsA doses were employed (15, 100 and 500 ng/ml), has demonstrated that this drug fails to block in vitro neutrophil chemotaxis. These authors report that CsA only affects certain neutrophil functions, and in a dose- and time-dependent manner.

In the present model, the ischaemia-reperfusion process (Engler et al., 1986; Jurado et al., 1998) may also have contributed to the enhanced numbers of neutrophils. Some authors describe diminished infiltration and tissue accumulation of neutrophils in the damage caused by ischaemia-reperfusion after treatment with CsA in the liver (Suzuki et al., 1993; Matsuda et al., 1998; Mizuta et al., 1999) and in skin (Cetinkale et al., 1996). However, CsA fails to reduce the damage caused by accumulated neutrophils in the mucosa after heat injury (Gurbuz et al., 1997).

Platelets deposited in the vessel lumen after endothelial denudation act as chemotactic agents for neutrophils (Diacovo et al., 1996), and this process may contribute to the intimal hyperplasia that follows endothelial damage (Umemura et al., 1999). Though in the present control group, platelet deposition did not give rise to thrombus formation, this complication was noted in the CsA treatment groups. Luminal thrombogenicity may be related to a toxic effect of the drug on the endothelium (which acts as an antithrombogenic barrier), since arterial reendothelialisation was delayed, exposing the highly thrombogenic subendothelial matrix to blood flow for a longer period. The increase in luminal thrombogenicity, observed in animals treated with CsA-Cp, may have been due to an additional, potentiated endothelial damage effect caused by the drug attributable to cremophor, its administration vehicle.

It has been established that treatment with CsA may reduce intimal thickness via a decrease in the infiltration of white blood cells in post-heart transplant arteriosclerosis (De Caterina et al., 1995; Koskinen et al., 1995). In our model, the appearance of intimal hyperplasia in animals treated with CsA was delayed by 1-2 weeks, regardless of the CsA administration vehicle, but once the neointima started to form, its progression was as rapid as in the control group. This delay was, nevertheless, probably more due to the toxic effect of the drug on the endothelium and the smooth muscle cells of the media than to its immunosuppressive action.

The present findings suggest that: a) neutrophils play an active role in intimal thickening during the repair process in this arterial autograft model; and b) CsA promotes the adhesion and infiltration of neutrophils in the damaged arterial wall.

References

Bellón J.M., Buján M.J., Jurado F., Hernando A., Gª-Honduvilla N., Domínguez B. and Contreras L. (1995). Atherogenic effects of cyclosporine in an experimental model of arterial autograft. Transplantation 60, 407-414.

Bienvenu J.G., Tanguay J.F., Chauvet P. and Merhi Y. (2001). Relationship between platelets and neutrophil adhesion and neointimal growth after repeated arterial wall injury induced by angioplasty in pigs. J. Vasc. Res. 38, 153-162.

Buján J., Bellón J.M., Jurado F., Hernando A. and Contreras L. (1995). Assessment of Cyclosporine-induced ultrastructural changes in the vascular wall using an experimental arterial autograft model. Histol. Histopathol. 10, 567-576.

Buján J., Jurado F., Gimeno M.J., Rodriguez M. and Bellón J.M. (2001).

Funtion of inflammatory cells and neoral cyclosporin-A in heart transplant-associated coronary vasculopathy. Histol. Histopathol. 16, 197-203.

Cetinkale O., Bilgic L., Ayan F. and Kandilci A. (1996). Neutrophil-mediated injury in ischemic skin flaps: amelioration of ischemic injury by cyclosporine in the rat. Ann. Plast. Surg. 37, 66-74.

Colditz I.G. (1985). Margination and emigration of leukocytes. Surv. Synth. Pathol. Res. 4, 44-68.

Cole C.W., Makhoul R.G., McCann R.L., O'Malley M.K. and Hagen P.O. (1988). A neutrophil derived factor(s) stimulates [3H]thymidine incorporation by vascular smooth muscle cells in vitro. Clin. Invest. Med. 11, 62-67.

DeBlois D., Viswanathan M., Su J.E., Clowes A.W., Saavedra J.M. and Schwartz S.M. (1996). Smooth muscle DNA replication in response to angiotensin II is regulated differently in the neointima and media at different times after balloon injury in the rat carotid artery. Role of AT1 receptor expression. Arterioscler. Thromb. Vasc. Biol. 16, 1130-1137.

De Caterina R., Tanaka H., Nakagawa T., Hamptman P. and Libby P. (1995). The direct effect of injectable cyclosporine and its vehicle, cremophor on endothelial vascular cell adhesion molecule-1 expression. Transplantation 60, 270-275.

Deng M.C., Bell S., Huie P., Pinto F., Hunt S.A., Stinson E.B., Sibley R., Hall B.M. and Valantine H.A. (1995). Cardiac allograft vascular disease. Relationship to microvascular cell surface markers and inflammatory cell phenotypes on endomyocardial biopsy. Circulation 91, 1647-1654.

Diacovo T.G., Roth S.J., Buccola J.M., Bainton D.F. and Springer T.A. (1996). Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. Blood 88, 146-157.

Engler R.L., Dahlgren M.D., Morris D.D., Peterson M.A. and Schmid-Schonbein G.W. (1986). Role of leukocytes in response to acute myocardial ischemia and reflow in dogs. Am. J. Physiol. 251, H314-323.

Gerrity R.G. (1981). The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am. J. Pathol. 103, 181-190.

Gurbuz V., Corak A., Yegen B.C., Kurtel H. and Alican I. (1997). Oxidative organ damage in a rat model of thermal injury: the effect of cyclosporin A. Burns 23, 37-42.

Hancock W.W., Adams D.H., Wyner L.R., Sayegh M.H. and Karnovsky M.J. (1994). CD4+ mononuclear cells induce cytokine expression, vascular smooth muscle cell proliferation, and arterial occlusion after endothelial injury. Am. J. Pathol. 145, 1008-1014.

Hanke H., Hassenstein S., Ulmer A., Kamenz J., Oberhoff M., Haase K.K., Baumbach A., Gown A.M. and Karsch K.R. (1994). Accumulation of macrophages in the arterial vessel wall following experimental balloon angioplasty. Eur. Heart J. 15, 691-698.

Hirsch G.M., Kearsey J., Burt T., Karnovsky M.J. and Lee T. (1998). Medial smooth muscle cell loss in arterial allografts occurs by cytolytic cell induced apoptosis. Eur. J. Cardiothorac. Surg. 14, 89-97.

Hruban R.H., Beschorner W.E., Baumgartner W.A., Augustine S.M., Ren H., Reitz B.A. and Hutchins G.M. (1990). Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endothelialitis. Am. J. Pathol. 137, 871-882.

Jonasson L., Holm J., Skalli O., Bondjers G. and Hansson G.K. (1986). Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. Arteriosclerosis 6, 131-138.

Jorgensen L., Grothe A.G., Groves H.M., Kinlough-Rathbone R.L., Richardson M. and Mustard J.F. (1988). Sequence of cellular responses in rabbit aortas following one and two injuries with a balloon catheter. Br. J. Exp. Pathol. 69, 473-486.

Jurado F., Bellón J.M., Pareja J.A., Golitsin A., Millán L., Pascual G. and Buján J. (1998). Effects of ischaemia-reperfusion and cyclosporin-A on cardiac muscle ultrastructure. Histol. Histopathol. 13, 761-774.

Jurado F., Bellón J.M., Golitsin A., Gimeno M.J., Pascual G. and Buján J. (1999). Role of macrophages in myocardial apoptosis following cardiac transplant. influence of immunosuppressive treatment. Histol. Histopathol. 14, 1033-1043.

Kalman V.K. and Klimpel G.R. (1983). Cyclosporin A inhibits the production of gamma interferon (IFN-g) but does not inhibit production of virus-induced IFN a/b. Cell. Immunol. 78, 122-129.

Kockx M.M., De Meyer G.R., Jacob W.A., Bult H. and Herman A.G. (1992). Triphasic sequence of neointimal formation in the cuffed carotid artery of the rabbit. Arterioscler. Thromb. 12, 1447-1457.

Kockx M.M., De Meyer G.R., Andries L.J., Bult H., Jacob W.A. and Herman A.G. (1993). The endothelium during cuff-induced neointima formation in the rabbit carotid artery. Arterioscler. Thromb. 13, 1874-1884.

Koskinen P.K., Lemstrom K.B. and Hayry P.J. (1995). How cyclosporine modifies histological and molecular events in the vascular wall during chronic rejection of the rat cardiac allograft. Am. J. Pathol. 146, 972-980.

Lucas J.F. 3d, Makhoul R.G., Cole C.W., Mikat E., McCann R.L. and Hagen P.O. (1986). Mononuclear cells adhere to sites of vascular balloon catheter injury. Curr. Surg. 43, 112-115.

Matsuda T., Yamaguchi Y., Matsumura F., Akizuki E., Okabe K., Liang J., Ohshiro H., Ichiguchi O., Yamada S., Mori K. and Ogawa M. (1998). Immunosuppressants decrease neutrophil chemoattractant and attenuate ischemia/reperfusion injury of the liver in rats. J. Trauma. 44, 475-484.

Mizuta K., Ohmori M., Miyashita F., Kitoh Y., Fujimura A., Mori M., Kanno T., Hashizume K. and Kobayashi E. (1999). Effect of pretreatment with FTY720 and cyclosporin on ischaemia-reperfusion injury of the liver in rats. J. Pharm. Pharmacol. 51, 1423-1428.

Ormrod D.J., Cawley S. and Miller T.E. (1990). Cyclosporin a modulation of the acute inflammatory response: an explanation for the effect of CsA on host defences in infection. J. Exp. Pathol. 71, 69-82.

Richardson M., Hatton M.W., Buchanan M.R. and Moore S. (1990). Wound healing in the media of the normolipemic rabbit carotid artery injured by air drying or by balloon catheter de-endothelialization. Am. J. Pathol. 137, 1453-1465.

Rogers K.A. and Karnovsky M.J. (1988). A rapid method for the detection of early stages of atherosclerotic lesion formation. Am. J. Pathol. 133, 451-455.

Rogers C., Welt F.G., Karnovsky M.J. and Edelman E.R. (1996). Monocyte recruitment and neointimal hyperplasia in rabbits. Coupled inhibitory effects of heparin. Arterioscler. Thromb. Vasc. Biol. 16, 1312-1318.

Roque M., Fallon J.T., Badimon J.J., Zhang W.X., Taubman M.B. and Reis E.D. (2000). Mouse model of femoral artery denudation injury associated with the rapid accumulation of adhesion molecules on the luminal surface and recruitment of neutrophils. Arterioscler.

Thromb. Vasc. Biol. 20, 335-342.

Scavuzzo M., Sagripanti A., Mosca F. and Ambrogi F. (2001). Modulation of beta2 integrin phenotype, adhesion, chemotaxis, and oxidative burst of neutrophils by cyclosporine. Biomed. Pharmacother. 55, 61-69.

Shi C., Lee W.S., He Q., Zhang D., Fletcher D.L. Jr., Newell J.B. and Haber E. (1996). Immunologic basis of transplant-associated arteriosclerosis. Proc. Natl. Acad. Sci. USA 93, 4051-4056.

Shirasawa B., Hamano K., Ueda M., Ito H., Kobayashi T., Fujimura Y., Kojima A. and Esato K. (2000). Contribution of proliferating leukocytes to phenotypic change in smooth muscle cells during the development of coronary arteriosclerosis in transplanted hearts. Eur. Surg. Res. 32, 30-38.

Simon D.I., Dhen Z., Seifert P., Edelman E.R., Ballantyne C.M. and Rogers C. (2000). Decreased neointimal formation in Mac-1-/- mice reveals a role for inflammation in vascular repair after angioplasty. J. Clin. Invest. 105, 293-300.

Spisani S., Fabbri E., Muccinelli M., Cariani A., Barbin L., Trotta F. and Dovigo L. (2001). Inhibition of neutrophil responses by cyclosporin A. An insight into molecular mechanisms. Rheumatology 40, 794-800.

Suzuki S., Toledo-Pereyra L.H., Rodriguez F.J. and Cejalvo D. (1993). Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. Transplantation 55, 1265-1272.

Tanaka H., Sukhova G.K., Swanson S.J., Clinton S.K., Ganz P., Cybulsky M.I. and Libby P. (1993). Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury. Circulation 88, 1788-1803.

Umemura K. (1999). Roles of platelets and neutrophils in intimal thickening following endothelial injury. Nippon Rinsho 57, 1480-1485.

Walczak E., Krus S., Fiejka E., Walski M., Celary-Walska R., Dabrowski M., Witkowski A., Chmielak Z. and Ruzyllo W. (1999). Light and electron microscopic picture of atherosclerotic plaque in stable and unstable angina. Pol. J. Pathol. 50, 87-92.

Welt F.G., Edelman E.R., Simon D.I. and Rogers C. (2000). Neutrophil, not macrophage, infiltration precedes neointimal thickening in balloon-injured arteries. Arterioscler. Thromb. Vasc. Biol. 20, 2553-2558.

Weyand C.M., Brandes J.C., Schmidt D., Fulbright J.W. and Goronzy J.J. (1998). Functional properties of CD4+ CD28- T cells in the aging immune system. Mech. Ageing Dev. 102, 131-147.

Whiteland J.L., Nicholls S.M., Shimeld C., Easty D.L. Williams N.A. and Hill T.J. (1995). Immunohistochemical detection of T-cell subsets and other leukocytes in paraffin-embedded rat and mouse tissues with monoclonal antibodies. J. Histochem. Cytochem. 43, 313-320.

Wish J.B. (1986). Immunologic effects of cyclosporine. Transplant. Proc. 18, 15-18.

Yasukawa H., Imaizumi T., Matsuoka H., Nakashima A. and Morimatsu M. (1997). Inhibition of intimal hyperplasia after balloon injury by antibodies to intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1. Circulation 95, 1515-1522.

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