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Cyclosporin A inhibits smooth muscle proliferation in the vascular response to injury

(atherosclerosis/arterial injury/T lymphocyte)

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ABSTRACT **The arterial response to injury is dominated by proliferation of smooth muscle cells and infiltration of blood-borne cells in the vascular intima. Arterial smooth muscle cell proliferation is under growth factor control, but how this regulation operates *in vivo* is unclear. We studied the effect on arterial response to mechanical injury of cyclosporin A, a drug that inhibits T-lymphocyte activation. Cyclosporin A treatment at surgery caused a persistent inhibition of the intimal proliferative lesion. Cyclosporin A also inhibited expression of Ia antigens on smooth muscle cells *in situ* but had no direct effects on smooth muscle cell proliferation in culture. Therefore, the inhibition of intimal cell proliferation appears to be mediated via the immune system.**

Studies in experimental animals have shown that arterial injury induces a response that resembles the intimal proliferation seen, for example, in atherosclerosis, postoperative intimal hyperplasia, and certain vasculitides (1). The arterial response to experimental injury is dominated by infiltration of blood-borne cells into the intima and by vascular cell migration and proliferation (1). Vascular smooth muscle cells migrate from the media into the intima, where they proliferate to form a lesion that is reminiscent of the fibrous cap of human atherosclerosis (1, 2). The mechanisms that control these phenomena are still not fully elucidated, although cell culture studies have shown that smooth muscle cells respond both chemotactically and by proliferation when exposed to the platelet-derived growth factor (3, 4). Animal experiments have demonstrated that platelets adhere to deendothelialized arteries, but the importance of platelet adhesion in other types of vascular injury has been questioned. Such studies have, however, pointed toward inflammatory cells as important components of the arterial response to injury and of the atherosclerotic lesion (1, 5–7). These inflammatory cells produce several types of growth factors, including the platelet-derived growth factor, the interleukins, and the transforming growth factors, all of which may be important for vascular growth control (8–11).

Most studies on the interactions between inflammatory cells and the vessel wall have focused on the role of the monocyte (1, 5–7). We have, however, recently shown that human atherosclerotic plaques contain a high proportion of T lymphocytes and have pointed out that they may modulate vascular response during the disease (12). This idea is supported by our observations on major histocompatibility complex (MHC) gene expression in vascular smooth muscle cells. In culture, these cells express the class II MHC antigen (Ia antigen) HLA-DR, when stimulated by the T-cell product, interferon γ (13). *In vivo*, smooth muscle cells express class II MHC antigens in human atherosclerotic plaques—but not in normal arteries (14, 15). In response to balloon catheter-induced arterial injury in rat, smooth muscle cells express class II antigens at the same time as T cells are infiltrating the experimental lesions (13). This indicates that T cells can control gene expression in smooth muscle cells and suggests that such a mechanism is operating in the atherosclerotic plaque. Consequently, it was postulated that suppression of T-cell activation might modulate the arterial response to injury.

The development of an intimal thickening after mechanical injury to the carotid artery was therefore analyzed in rats treated with cyclosporin A (CsA). This drug inhibits the interleukin 2-mediated autocrine stimulation of T-cell growth during activation of the T cell, as well as the secretion of other T-cell lymphokines (16–19); the net effect of CsA is therefore a suppression of the immune response.

MATERIALS AND METHODS

Experimental Model. Arterial injury was inflicted by the standard procedure of denuding the surface of the left carotid artery of Sprague–Dawley rats with a Fogarty balloon catheter (20, 21), which results in the formation of an intimal thickening that largely consists of proliferating intimal smooth muscle cells. Ten rats received i.p. injections of CsA [Sandoz, Basel; 30 mg/kg of body weight dissolved in Intralipid (Kabi Vitrum, Stockholm) (22)] at the day of surgery and the following day. Ten rats received Intralipid without CsA, and five rats received no injections at all. Blood was drawn from the tail vein at regular intervals for analysis of CsA concentration and platelet counts. The CsA concentration was analyzed in whole blood by a commercial RIA kit (Sandoz). The CsA level reached a maximum during the first 2 days after surgery, when it was 1483 ± 214 ng/ml. This value is in the same range as those in patients treated with CsA after organ transplantation (23, 24).

All rats were killed 2 weeks after surgery by perfusion with 1% phosphate-buffered paraformaldehyde under ether anesthesia. This time point was chosen because intimal smooth muscle proliferation is high and smooth muscle expression of Ia antigens is maximal by 2 weeks (13). The carotid arteries were sectioned at 100-μm intervals and analyzed by morphometry and immunofluorescence.

Rats (n = 4 per group) received CsA or vehicle during 2 consecutive days as described above. They were all injected with Evans blue i.v. (1.0 ml/kg of body weight) of a 0.25% solution in phosphate-buffered saline on day 3, and killed after 15 min during perfusion with paraformaldehyde. The aortas were pinned out, and a defined 28-mm segment of the thoracic part was analyzed for the size of the blue-stained area by the point-sampling method (25).

Cell Culture. Cells were isolated from the rat aorta by collagenase digestion (26) and grown in RPMI 1640 medium with 10% fetal bovine serum, penicillin G at 100 units/ml,

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Abbreviation: CsA, cyclosporin A.

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streptomycin at 100 μg/ml, and ascorbic acid at 50 μg/ml. The influence of CsA on the viability and growth of smooth muscle cells as well as any cytotoxic effects on these cells were investigated.

RESULTS

Effects of CsA on the Arterial Lesion. A prominent intimal thickening developed in all control rats (Fig. 1); the size of it was not affected by injection of the Intralipid vehicle alone (data not shown). To our surprise, we found only diminutive lesions in CsA-treated rats (Fig. 1). The difference in lesion volume between CsA-treated and control rats was highly significant (Fig. 2). Drug treatment affected all components of the lesion—i.e., smooth muscle cell number and extracellular tissue were both reduced (Fig. 1).

The drug also had a striking effect on the immunologic properties of the vessel wall. Expression of the Ia antigens, I-A and I-E, by intimal smooth muscle cells was reduced by a factor of four, and the frequency of leukocytes in the lesion was also significantly reduced (Fig. 3). In control rats, ≈1% of all cells expressed the leukocyte-specific antigen, leukocyte common antigen, and 0.3% of the cells could be identified as T cells using the W3/13 antibody (Fig. 4), whereas leukocytes were virtually absent from the lesions of CsA-treated rats. The *in vivo* effect of CsA on smooth muscle expression of I-A and I-E antigens is in accordance with similar observations on the endothelium (31) and suggests that expression of Ia antigens by vascular cells is controlled by activated T cells.

Effects of CsA on Platelets and Endothelium. The effect of CsA on the intima was not mediated via any quantitative platelet defect because the platelet counts were never diminished during treatment and were never significantly different between CsA-treated rats and controls (data not shown).

CsA has been reported to exert cytotoxic effects on cultured bovine aortic endothelial cells (32). We therefore tested whether the drug affected the arterial endothelium *in vivo*. Transendothelial albumin leakage was monitored in CsA-treated and control rats with the Evans blue technique, and the extent of blue areas did not differ significantly between the two groups (16.3 ± 3.0 mm^2 in CsA-treated and 15.3 ± 1.3 mm^2 in control rats, respectively). Thus, our data did not support the idea that the primary effect of CsA on the artery is an assault on the endothelium. In addition, it is difficult to see how a drug-mediated endothelial injury could affect a lesion that develops in a deendothelialized region of the vasculature.

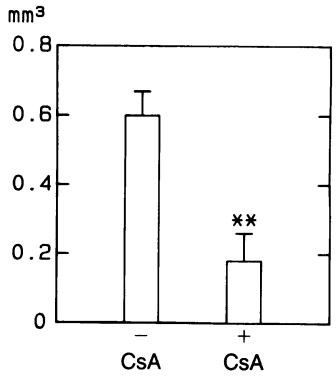

FIG. 2. Intimal volume (mean ± SEM) after balloon injury in rats with (+CsA) or without (−CsA) treatment. The carotid arteries were serially sectioned into 10-μm sections at 100-μm intervals, and hematoxylin/eosin-stained sections were analyzed for intimal areas by the point-sampling method (25). The length of the lesion was determined from the serial sections, and the volume of the lesion was calculated from length and area data. The difference between the two means is significant at $P < 0.005$ (**) by Student's t test. Both area and length of the lesions were significantly reduced in CsA-treated rats.

Effects of CsA on Smooth Muscle Cells in Culture. The drastic effect of CsA on smooth muscle proliferation in the intimal thickening *in vivo* led us to speculate that the drug might have direct effects on these cells, in addition to its effects on the immune system. This possibility was addressed in a series of cell culture experiments. CsA did not affect the continuous growth of rat aortic smooth muscle cells (data not shown). It did not affect DNA incorporation when quiescent cells were induced to proliferate by addition of serum (Table 1), and no striking effect was noticed on the growth of such cells (Fig. 5). A slight growth inhibition was seen after 9 days of continuous incubation with CsA, but a similar effect was also seen in vehicle-exposed controls (Fig. 5). Finally, CsA did not exert any cytotoxic effect on cultured smooth muscle cells (Table 1).

DISCUSSION

In conclusion, our *in vivo* data show that CsA effectively inhibits the development of an intimal thickening during the

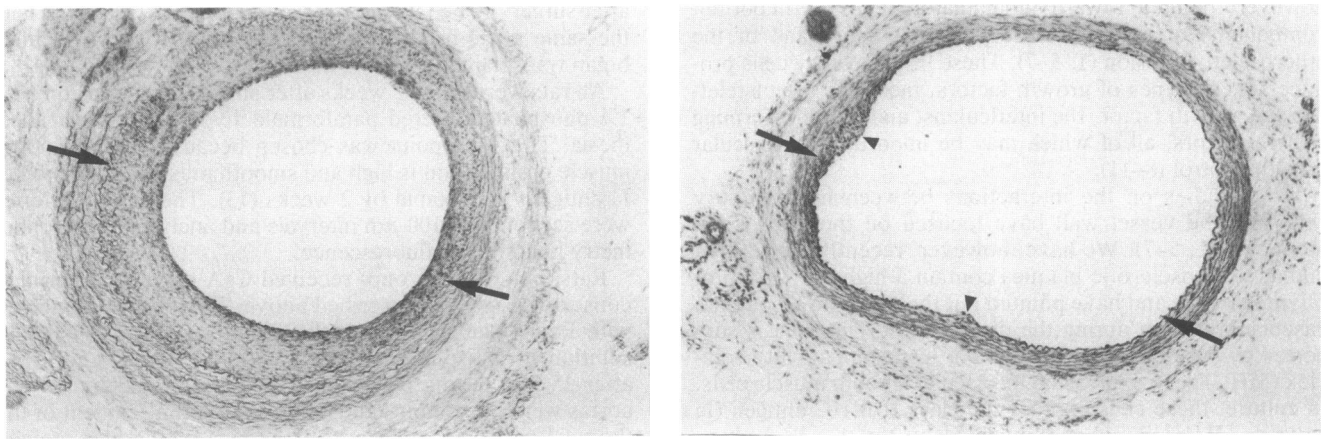

FIG. 1. Cross-sections of rat carotids 2 weeks after balloon catheter injury. (*Left*) Control rat. (*Right*) CsA-treated rat. Injury was inflicted by a Fogarty 2F balloon catheter, which was inserted through the right external carotid artery, inflated in the common carotid, and retracted in the inflated state. The procedure was repeated three times, the balloon was then withdrawn, and the external carotid and superficial wound were closed (for detail, see ref. 21). There was no obvious difference in wound healing or general condition between CsA-treated and control rats. The rats were killed 2 weeks after surgery by perfusion with 1% paraformaldehyde at physiologic pressure. The carotid arteries were removed and snap-frozen in liquid nitrogen as described (12). Frozen sections were hematoxylin/eosin-stained (×62.5 magnification). Arrows indicate the position of the internal elastic membrane, and arrowheads indicate the surface of the lesions.

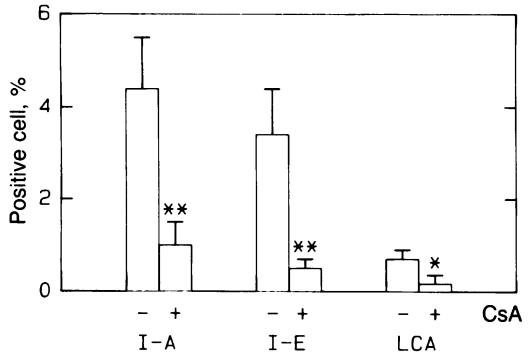

FIG. 3. Effect of CsA on Ia antigen expression by intimal cells *in vivo*. Frozen sections of lesions at 2 weeks after injury were subjected to immunocytochemical analysis as described (12). The percentage of intimal cells positive for I-A [monoclonal antibody (mAb) 0X6; ref. 27], I-E (mAb 0X17; ref. 28), and leukocyte common antigen (LCA) (mAb 0X1; ref. 29) is shown for CsA-treated (+) and control (−) rats. Differences between means are significantly different at $P < 0.01$ (**) and $P < 0.05$ (*), respectively. Serial sections stained for smooth muscle-specific actin antibodies (CGA7 mAb from D. Gordon, University of Washington) allowed identification of I-A and I-E positive cells as smooth muscle cells.

arterial response to injury. Our *in vitro* data do not show any direct effect of the drug on vascular smooth muscle cell proliferation. However, we should not exclude the possibility that CsA could exert certain effects on smooth muscle cells *in vivo*, which are not easily imitated in cell culture experiments. It can also not be excluded that CsA interferes with such functions of leukocytes as emigration or local activation in the arterial intima.

CsA exerts striking and specific effects on the immune system by blocking T lymphocyte activation (16–19). We have now demonstrated that the drug controls the immunologic properties of the vessel wall (Fig. 3). It seems reasonable, therefore, to suggest that CsA inhibits the arterial response to injury by blocking T cell-mediated control of vascular proliferation.

Activated T cells release both inhibitors of cell proliferation, such as interferon γ and lymphotoxin, and growth promoters such as interleukin 2 and transforming growth factor type β. The latter cytokine may have both stimulatory and inhibitory effects on the proliferation of mesenchymal cells (10). These factors are secreted locally in a tissue where T cells are activated, and they are also released into the circulation. It is therefore conceivable that T cells may

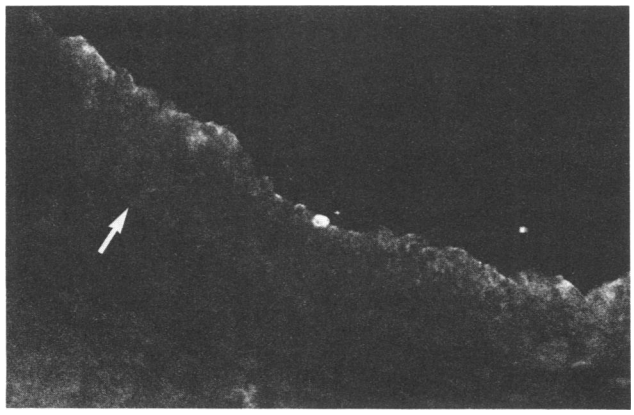

FIG. 4. Immunofluorescent detection of T lymphocyte in the intimal thickening. A frozen section of a 2-week lesion was treated with W3/13 (30) followed by biotinylated anti-mouse-IgG and fluorescein isothiocyanate–streptavidin. Arrow indicates the position of the internal elastic membrane. (×150).

Table 1. Effect of CsA on arterial smooth muscle viability and replication in culture

CsA added, ng/ml	Cell viability, % trypan blue-excluding cells	[^{3}H]Thymidine uptake, cpm × 10^{-3} per well
0	98.7 ± 0.3	42.4 ± 6.9
100	99.3 ± 0.3	ND
1000	99.0 ± 1.5	52.4 ± 2.7
2000	96.3 ± 0.7	ND

Cells in the fourth passage were growth-arrested in 0.5% fetal bovine serum (FBS), and CsA was added together with 10% FBS in full medium after 2 days. Viability analysis was done after 2 days in CsA by incubation with 0.25% trypan blue in serum-free phosphate-buffered saline for 5 min, followed by fixation in paraformaldehyde. The frequency of blue cells was determined in triplicate 2-cm^2 wells. Cell replication analysis was done in 10-cm^2 Petri dishes, and [^{3}H]thymidine (25 Ci/mmol, 5 μCi per well; 1 Ci = 37 GBq) was added together with CsA and FBS. After 2 days, cells were harvested by trypsinization and collected on Millipore filters. Trichloroacetic acid-insoluble radioactivity was determined by scintillation counting. All values are given as mean ± SEM, $n = 6$. ND, not determined.

stimulate smooth muscle growth, either systemically or in a paracrine fashion. Local concentrations of growth factors may be higher in the developing intimal thickening, both because the lack of endothelium enhances the influx of circulating factors and because of local release from infiltrating T cells. It is also possible that such a stimulation of smooth muscle growth is an indirect effect. It could, for example, be mediated by stimulating monocyte secretion of growth factors.

The observation that CsA inhibits intimal smooth muscle proliferation is interesting from a clinical point of view as well as for our understanding of the basic mechanisms of growth control in the arterial wall. The drug was effective in doses that are not acutely toxic and are of the same magnitude as those used clinically. The effect was similar to that obtained with heparin, which is the only other known pharmacologic inhibitor of the vascular response to injury (34). This merits further studies, both to identify the mechanism of drug action in this situation and to determine whether it could be clinically useful as an inhibitor of intimal proliferation and arterial stenosis.

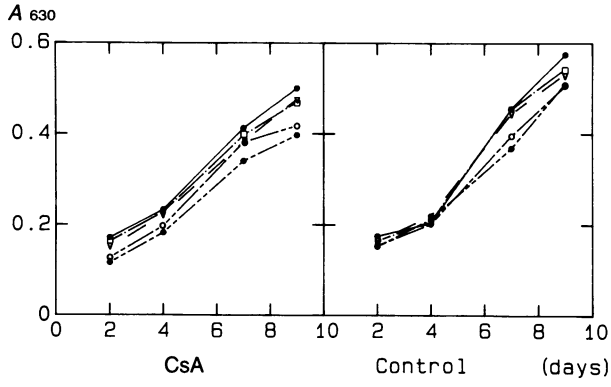

FIG. 5. Effect of CsA on the growth of arterial smooth muscle cells. Cells in the fourth passage were plated in 96-well microtiter plates, growth-inhibited by serum starvation (0.5% fetal bovine serum) for 48 hr, and then stimulated to enter the cell cycle by addition of 10% fetal bovine serum with or without CsA. The drug was added as a small volume of a stock solution dissolved in ethanol, and controls received equivalent amounts of ethanol. After various time intervals, the cell number was determined by a colorimetric method (33). (*Left*) Cells grown with 0 (●), 50 (□), 100 (▽), 1000 (○), and 2000 (●) ng of CsA per ml. (*Right*) Cells grown in a volume of ethanol vehicle equivalent to that added with the drug; vehicle was equivalent to 0 (●), 50 (□), 100 (▽), 1000 (○), and 2000 (●) ng of CsA per ml.

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