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The Effect of Cyclosporine and Dexamethasone on Suppression of Medial Thickening after Arterial Injury in Rats

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= Abstract = **Vascular proliferative lesion is one of the most important causes of stenosis and late thrombosis in arterial reconstructions. We studied the effect of cyclosporine and dexamethasone on decreasing the hyperplastic response after experimental arterial injury in rats. Ninety female rats were assigned to one of four groups, each receiving daily subcutaneous injections of either (1) saline (control), or (2) cyclosporine(CYA) 5mg/Kg/D, or (3) dexamethasone (DEXA) 0. 1 mg/Kg/D, or (4) CYA 5mg/kg/D and DEXA 0. 1mg/kg/D. Injections were started 1 day before the intimal injury and continued daily for 6 weeks. Arterial injury was created in 90 rats by rotating a 1mm coronary dilator in the right common iliac artery. The vessels were harvested 1,4,6 weeks after injury and the thickness of the tunica media was measured. In the control group the injured iliac artery had significant medial thickening when compared to the noninjured ($P<0.0001$) 1 week after injury. Injured arteries treated with CYA or DEXA or CYA and DEXA showed significantly less medial thickening when compared to that of control ($P<0.001$) but no significant difference was noted among drug treated groups for 1 and 4 weeks of treatment. At 6 weeks, however, medial thickness in the CYA and DEXA group was significantly less than that of the CYA or DEXA group($P<0.05$).**

These data suggest that CYA and DEXA are effective in suppressing the hyperplatic myointimal reaction to an intimal injury. In addition, these data also provide the evidence that immunological mechanisms may modulate vascular proliferative lesions.

Key Words: *Myointimal hyperplasia, Immunosuppressive agents, Cyclosporine, Dexamethasone*

INTRODUCTION

Myointimal hyperplasia is described by abnormal proliferation of smooth muscle cells (SMCs) and connective tissue elements developed at arterial injury sites, and it has been implicated as one of the common causes of stenosis and possible late thrombosis after autologous vein grafting (Whittemore *et al.* 1981), endarterectomy (Callow 1982) and balloon catheter angioplasty (Holmes *et al.* 1984). Although the exact pathophysiologic mechanisms leading to the development of myointimal hyperplasia have not been characterized, the denuded intimal regions are immediately

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covered by a carpet of platelets. At the same time SMCs in the media begin to proliferate and migrate into intima according to mitogens by enzymatic activations(Fishman *et al.* 1975). The platelets have been investigated as the major factor in the development of hyperplastic response after vessel injury(Bowne-pope 1986; Fredman *et al.* 1977). Extensive work, however, on antiplatelet agents has yielded mixed results.

It has long been appreciated that the biologic responses of cellular proliferation and inflammation are closely associated. In the case of vascular injury the contribution of leukocytes has been largely thought to be limited to their role in augmented endothelial damage. Recently a more complex view of the role of inflammatory cells in the development of intimal hyperplasia has emerged.

Several reports suggest that inflammatory cells, in addition to their contribution to endothelial cell damage, may play an important role in stimulatory SMC recruitment and proliferation(Prescott *et al.* 1989; Lucas *et al.* 1986; Leibovich and Ross 1976).

We are interested in the role of immunosuppressive therapy in the suppression of myointimal hyperplasia as an inhibitor of such pathophysiologic processes. Recently limited effect in suppression of myointimal thickneing has been reported after steroid injections in rabbits(Colburn *et al.* 1992). Wengrovitz *et al.* (1990) reported that cyclosporine inhibited the development of medial thickening after experimental arterial injury. The purpose of this study is to know the effect of cyclosprorine(CYA) and dexamethasone(DEXA) on decreasing the hyperplastic response after experimental arterial injury in a rat model and to know whether the combined use of CYA and DEXA has any synergistic effect on decreasing the hyperplastic responses.

MATERIALS AND METHODS

Ninety female Sprague-Dawley rats weighing 200-250gm were used in this experiment. The animals were randomized into one of four groups. Each group received daily subcutaneous injections of (1) saline 0. 2cc/D (control), or (2) cyclosporine 5mg/kg/D, or (3) dexamethasone, 0.1mg/kg/D, or (4) cyclosporine, 5mg/kg/D and dexamethasone, 0. 1mg/kg/D. The animals were weighed and the dose of drug was adjusted weekly according to their weight. Injections were started from one day before intimal injury and continued until the day before they were killed.

Anesthesia was obtained by intramuscular injections of 50mg/kg ketamine. The animals were shaved and prepped with betadine, and through a midline laparotomy incision, a 1cm segment of the infrarenal aorta was isolated with a microbuldog clamp at the proximal site and 3-0 silk traction at the distal site. Through a longitudinal aortotomy, the injury was created by rotating a 1mm coronary dilator four times around the inside of the right iliac artery. After closing the aortotomy with interrupted 8-0 polypropylene suture under magnification, arterial flow was reestablished to the lower extremities. After adequate hemostasis, the abdominal wall was closed. Antibiotics and heparin were not used at any time during the procedure.

The animals were killed 1 week, 4 weeks and 6 weeks after intimal injury, respectively. After adequate anesthesia in the previously described manner, the infrarenal aorta was again controlled with a 3-0 silk ligature. Five millimeters of blood was withdrawn from the inferior vena cava for cyclosporine(CYA) whole blood level(approximately 22 hours since last CYA injection). These levels were determined by use of radioimmunoassay kits(Sandimun Kit, Switzerland). The aorta was perfused to fix the distal arterial tree, in situ, with 10% formaldehyde. Both right and left common iliac arteries were carefully dissected free and fixed in the perfusate. Arterial specimen were embedded in paraffin and 5μm sections cut at right angles to the longitudinal arterial axis. These sections cut from each arterial specimen were mounted on glass slides and stained with hematoxylin-eosin. Antifactor Ⅷ related antigen staining was performed on specimen fixed 1

hour after injury to document the extent and nature of the endothelial injury. This primary antibody (Vectastain avidin biotin peroxidase complex kit, Vector company) responses with Von Willebrand factor in the cytoplasm of endothelial cells. The thickness of the tunica media was measured by use of a micrometer scale. For measurement purposes the media was defined as the distance between the internal elastic lamina and outer smooth muscle cell. Measurements were made in two places in each specimen, 90 degrees apart, and averaged to arrive at a thickness used for statistical analysis.

Data were expressed as mean ± standard error of mean. The significance of difference was determined by use of two way analysis of variance (ANOVA), and p values less than 0.05 were considered significant.

RESULTS

There were 3 deaths in the control group, 2 deaths in the CYA group, 4 deaths in the CYA and DEXA group during the experimental period; causes of death were operative bleeding in 2, evisceration in 1 and unknown in 6. Also 9 rats were excluded from analytical data due to intraabdominal abscess in 4, severe weight loss in 1, and other reasons in 4. Light microscopy of specimen injured 1 hour after injury revealed endothelial denudation that became circumferentially complete by a negative antifactor Ⅷ antigen staining. The uninjured (left) iliac artery had a positive stain with antifactor Ⅷ antigen (Fig 1). The internal elastic lamina remained essentially intact in all specimen for each time interval, which estab-

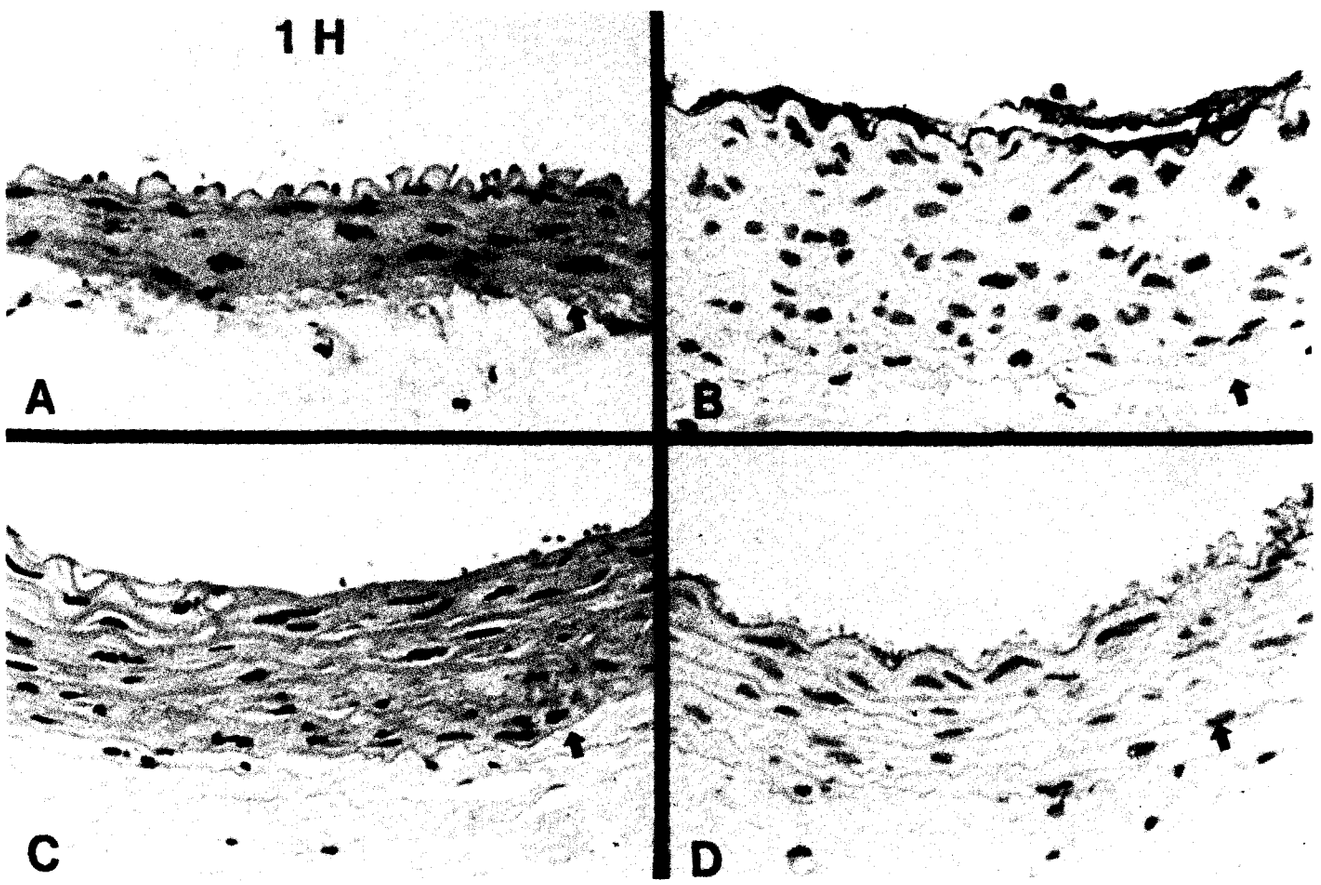

Fig. 1. Microscopic finding of the experimental groups 1 hour after injury. A,B: Noninjured artery shows well preserved endothelium and strong reactivity against factor Ⅷ-related antigen. C, D: Injured artery shows total denudation of endothelial cell and non-reactivity against factor Ⅷ-related antigen. Arrow indicates outer smooth muscle layer (A. C : Hematoxylin-Eosin stain, x 200, B. D. : Avidin-Biotin Complex peroxidase stain, x 400).

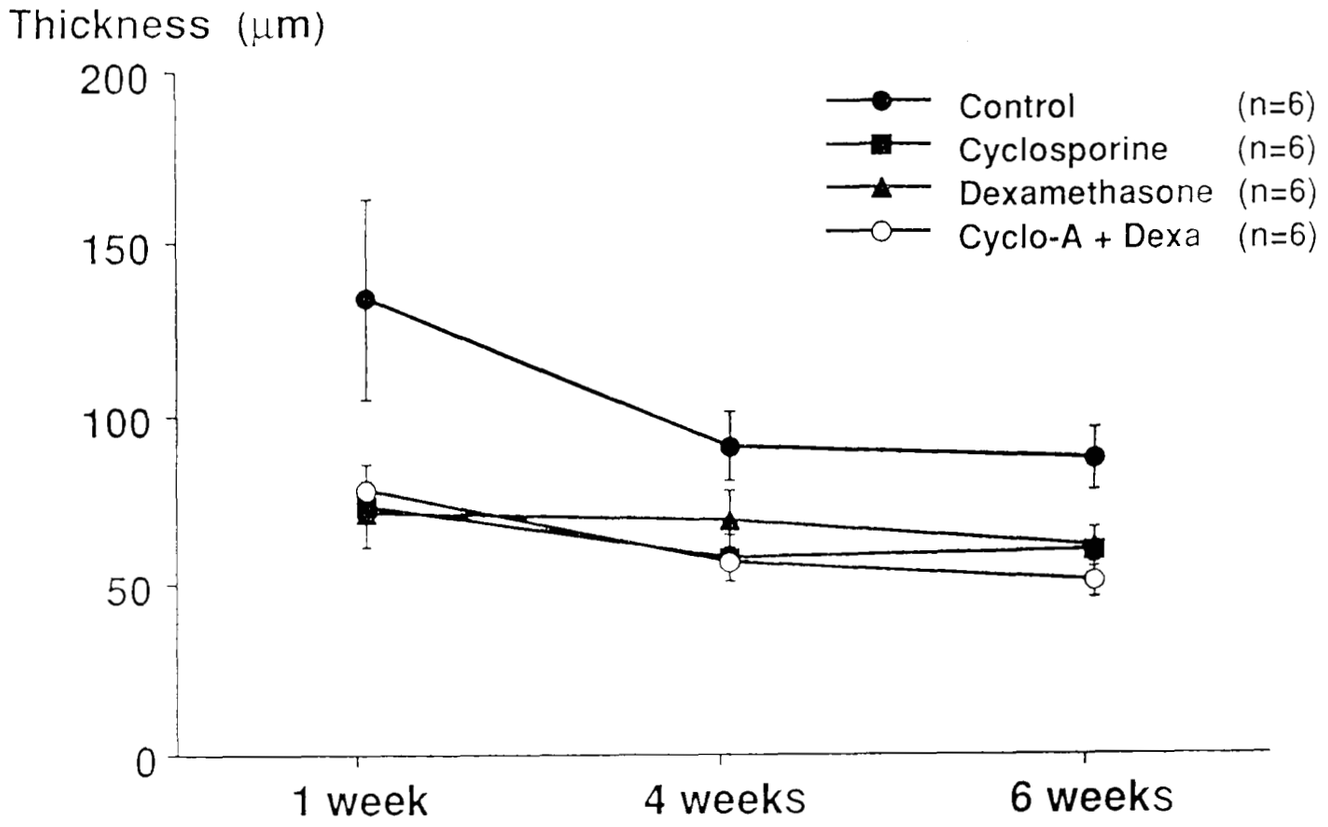

Fig. 2. Chronologic change of measured thickness of tunica media.

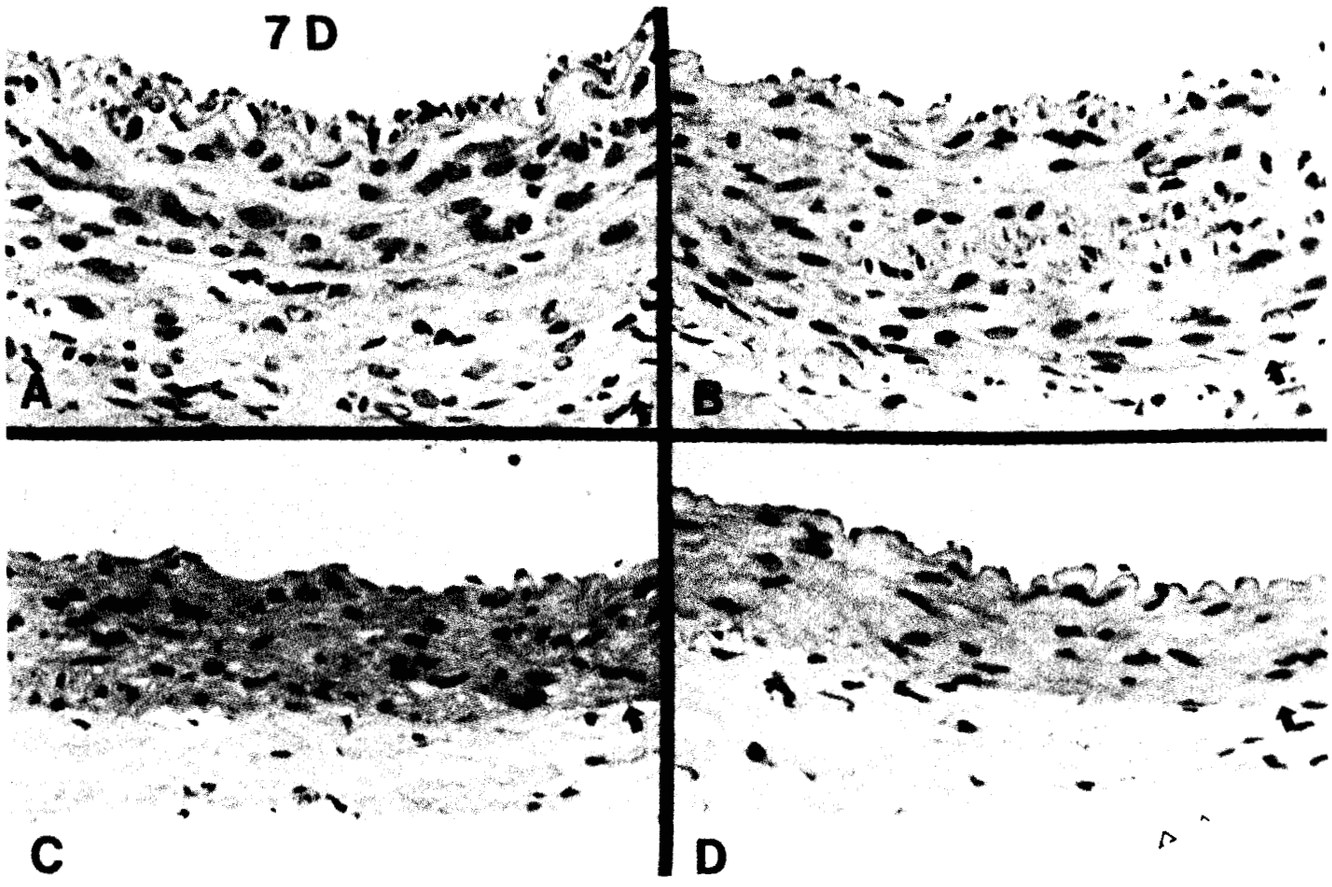

Fig. 3. Microscopic finding of the experimental groups 1 week after injury; A control, Medial thickening and intimal proliferation is prominent; B, CYA group; C, DEXA group; D, CYA and DEXA group. BCD show less thickened media and mild intimal proliferation. Arrow indicates outer smooth muscle layer (Hematoxylin-Eosin stain, x 400).

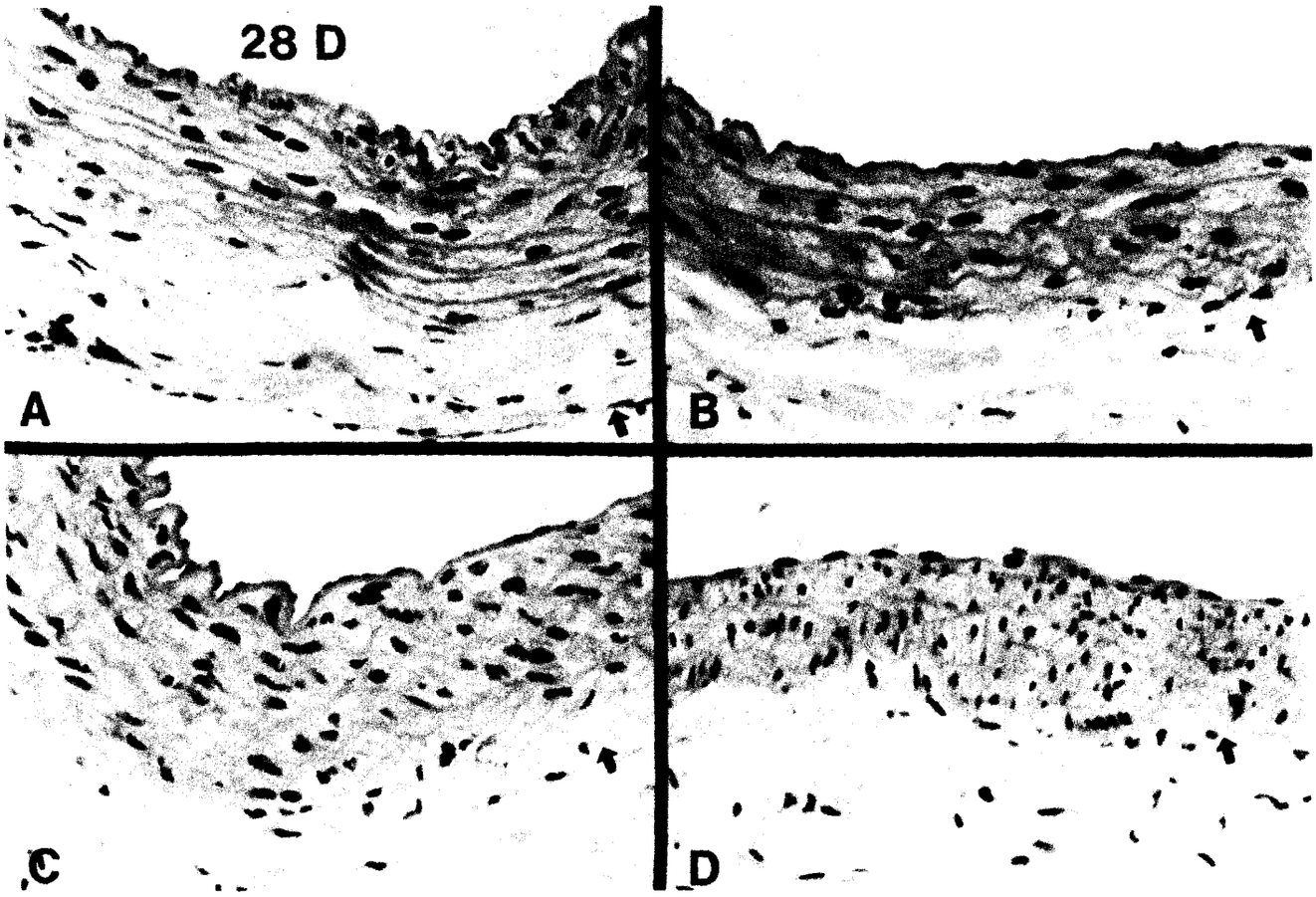

Fig. 4. Microscopic finding of the experimental groups 4 weeks after injury; A control; B, CYA group; C, DEXA group; D, CYA and DEXA group. Intimal proliferation is still seen in A. Arrow indicates outer smooth muscle layer (Hematoxylin-Eosin stain, x 400).

lishes the consistency of depth of this experimental lesion. The data of the measured thicknesses of the tunica media are shown in Table 1 and Figure 2. In the control group the injured iliac artery had significant medial thickening when compared to that of the noninjured 1 week after injury ($p<0.0001$). These effects were decreased 4 weeks, and persisted 6 weeks, after injury. At 1 week after injury, endothelial regeneration was complete and significant thickening of medial layer was seen in all animals on the injured arterial side(Fig 3A). The medial thickening was from the smooth muscle proliferation as evidenced by increased number of SMCs, darkened nuclear staining, and mitosis. Lymphocytes, monocytes, and macrophages were seen frequently. Such findings were mildly observed in drug-treated groups and medial thickening was less prominent (Fig 3. B,C,D). At 4 and 6 weeks after injury, medial thickening and intimal proliferation were less prominent than in the 7 days group. Intimal proliferation was still noted in the control group (Fig. 4 A and Fig. 5 A). Injured arteries treated with drugs showed significantly less medial thickening when compared to controls ($P<0.001$) but no significant difference was noted among drug treated groups at 1 and 4 weeks of treatment. At 6 weeks, however, medial thickness in the CYA and DEXA group was significantly less than that of the CYA or DEXA group ($P<0.05$).

Intimal and medial changes are minimal and are nearly normal in the CYA and DEXA group at 6 weeks of treatment. Whole blood CYA levels are shown in Table 2. All rats had demonstrable levels of CYA with variation.

Table 1. Measured thickness of the tunica media

	1 week	4 weeks	6 weeks
Control	131±29 (49±5)	88±10	84±9
Cyclosporine	70± 5* (51±3)	55± 7	57±7
Dexamethasone	68±10* (41±4)	66± 9	58±6
CYA and DEXA	75± 8* (47±4)	53± 5	48±5**

Data are expressed as mean microns±SEM and six rats per each experimental group (N=6). Numbers in parenthesis are the value of uninjured artery, left.

* $p < 0.001$, versus control

** $p < 0.05$, versus CYA or DEXA group

Table 2. Cyclosporine levels in whole blood

	Cyclosporine group	Cyclosporine and dexamethasone group
1 week	789±100	380±180
4 weeks	113± 20	128± 24
6 weeks	134± 49	190± 64

Data are expressed as mean (ng/ml) ± SEM, and cyclosporine levels were measured at the time the animals were killed.

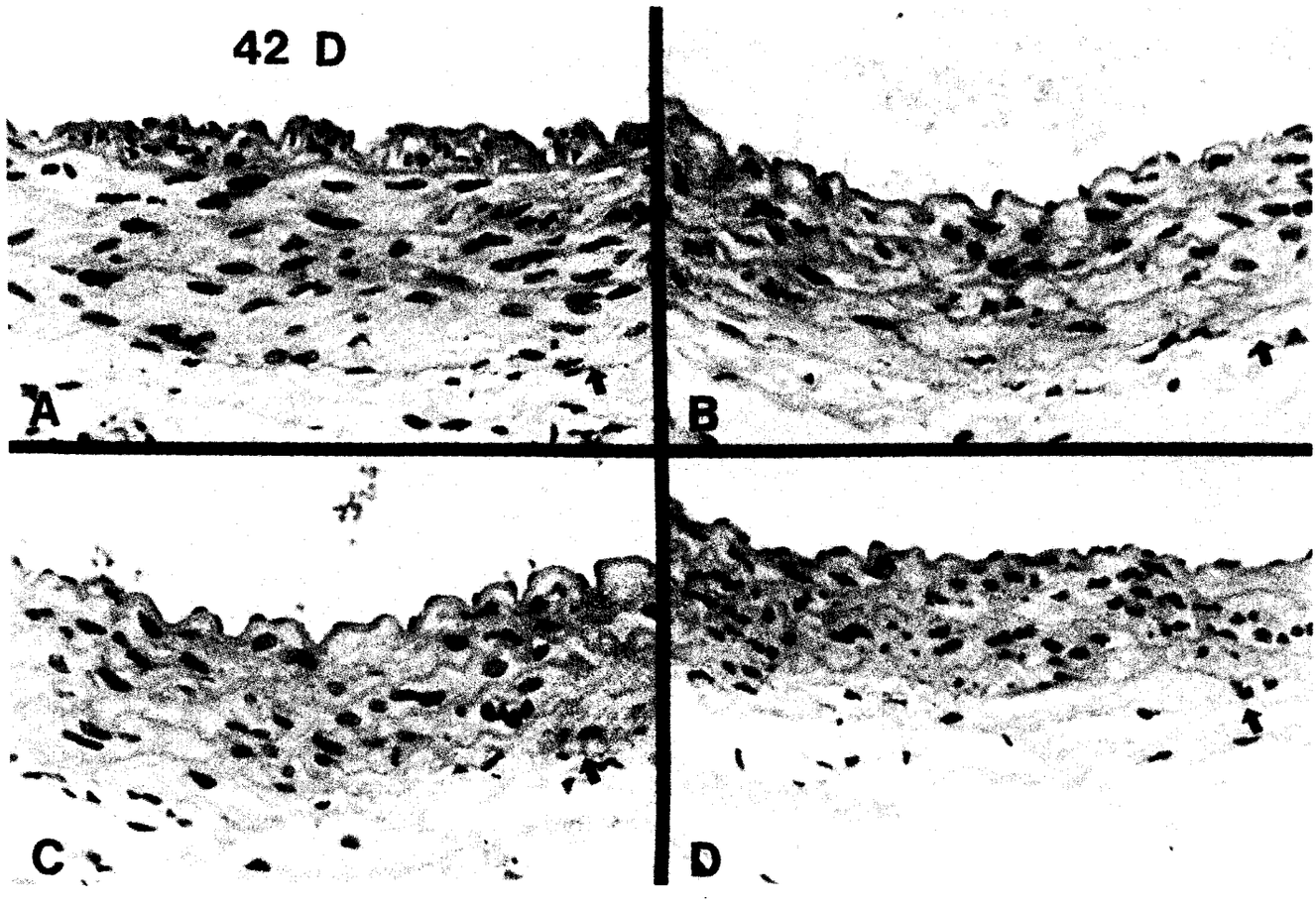

Fig. 5. Microscopic finding of the experimental groups 6 weeks after injury. A control; B, CYA group; C, DEXA group; D, CYA and DEXA group. Intimal and medial changes are minimal and are nearly normal in D. Arrow indicates outer smooth muscle layer (Hematoxylin-Eosin stain, x 400).

DISCUSSION

Arterial reconstruction has become a treatment of choice in occlusive arterial disease. Techniques were previously limited to large branch vessels but have been extended to small vessels according to technical improvement. Also balloon angioplasty and arterectomy has been widely performed with excellent outcome at localized stenosis regardless of lower morbidity. But this vascular reconstructive surgery has been plagued by the problems of

graft occlusion and recurrent arterial stenosis. This occurs in coronary arteries subjected to balloon angioplasty (Holmes *et al.* 1984), vein grafts used for peripheral arterial bypass (Whittemore *et al.* 1981), and carotid endarterectomy (Callow 1982). Although this mechanism leading to stenosis or occlusion has not been characterized, myointimal hyperplasia is the typical fibromuscular response of a vascular system to injury. The 'reaction to injury' hypothesis(Ross 1986) as originally stated suggests that the initial injury and finally myointimal thickening might be linked by the early thrombotic events and the release of smooth muscle growth factors from adherent platelets into the damaged artery.

The important cells in this response to injury are the SMCs, which can respond to a number of chemotactic factors. Replication of the SMCs begins intially in the media and then migrates to the luminal surface through fenestrae of the internal elastic lamina. Myointimal SMCs proliferation occurs with elaboration of connective tissue elements contributing, in part, to the intimal thickeness. But when the subendothelial layer is established again, SMC proliferation definitively ceases. So length of endothelial injury is an important conclusive factor leading to myointimal thickening. In some circumstances, no SMCs proliferation or myointimal thickening was observed at limited endothelial denudation. Selective removal of endothelium with a fine wire produces detectable gaps that are healed by migration and proliferation of adjacent endothelial cells(Reidy 1985). Nevertheless even in examples of this kind of injury, which require 7 days for full repair, SMC proliferation in the media is not evident. It is apparent that denudation, such as injury to the underlying media and distension of the vessel, must be important.

This experiment standardized the extent of injury by rotating a 1 mm coronary dilator four times around the inside of the right iliac artery and internal elastic lamina remained essentially intact in all specimen.

Investigative efforts regarding vascular proliferative lesions have focused on attempts to identify pharmacologic agents such as antiplatelets or antithrombotic agents, vasodilators, physiologic inhibitors of SMC proliferation, growth factor inhibitors, and immuosuppressants that may be used in a clinical setting to decrease incidence of myointimal hyperplasia. Especially interest about immunosuppressants is derived from the role of inflammatory cells infiltrated around vascular proliferative lesions. Generally inflammatory cells were always infiltrated around the tissue damage site by external trauma and play a destructive role as a release of toxic products, which induce endothelial cell damage. Recently a more complex view of the role of inflammatory cells in the development of intimal hyperplasia has emerged. In models of vaculitis, significant luminal intimal hyperplasia has been demonstrated adjacent to areas of an arterial wall infiltrated by inflammatory cellular infiltrates (Prescott *et al.* 1989). Also in a balloon catheter animal model monocytes were demonstrated to enter the media 42 days after a denuding injury with labeled cells seen deep within hyperplastic lesions (Lucas *et al.* 1986). Additional evidence that immunologic mechanisms may play a role in the development of vascular proliferative or atherosclerotic lesions is based on the work of Jonasson *et al.* who have shown that activated SMCs associated with experimental arterial injury or with human atherosclerotic plaques express class Ⅱ antigens of the major histocompatibility complex(MHC), whereas normal arteries express none of these antigens(Jonasson *et al.* 1986; Jonasson *et al.* 1988). They also showed that human atherosclerotic plaques contain a high number of T lymphocytes. Other groups reported that monocyte derived growth factor (MDGF) had a similar structure and function as platelet derived growth factor (PDGF).

Cyclosporine(CYA) is a cyclic polypeptide that was released as a clinical immunosuppressive agent in 1983. It has been shown to exert its primary immunomodulation by downregulatory interleukin 2 production by helper T lymphocytes. CYA at short term high

dosage inhibited the development of SMC proliferation in rat arteries initially injured with balloon catheters. (Jonasson *et al.* 1988) Wengrovitz *et al.* (1990) also showed that CYA at relative low dosage inhibited development of medial thickening and was consistently patent 2, 4, 6 weeks after injury.

Corticosteroids have been widely used clinically as a nonspecific immunosuppressive agent. Dexamethasone was chosen because of its high antiinflammatory potency, long half life (36 to 72 hours), and absence of mineralcorticoid effects. Steroid effects for myointimal thickening have mixed results but preinjury initiation of pharmacologic therapy may be important in realizing the full effect of steroid treatment (Colburn *et al.* 1992).

We decided to inject relatively high drug dosages within safety blood levels without side effects in consideration of previously studied doses in this experiment. We chose 0. 1 mg/kg/day of dexamethasone dose from the results of Colburn *et al.* (1992) and 5 mg/kg/day of cyclosporine dose from the results of Wengrovitz *et al.* (1990). Injections were started 1 day before the intimal injury to meet optimal blood concentration of drug. Injured arteries treated with drugs showed significantly less medial thickening where compared to controls but no significant differences were noted among drug groups at 1, 4 weeks. Two weeks after injury has been reported to be the time for maximal SMC proliferation and then remodelling has occurred in injured animal models. Our results showed that medial thickening of the control group at 1, 4, 6 weeks decreased with time. This seems to have the same meaning as the fact that endothelial regeneration was nearly complete in ali experimental groups at 1 week. At 6 weeks medial thickening in the combined use of CYA and DEXA group was lower than that of CYA or DEXA group. That finding suggests that the two immunosuppresants combination has a synergistic effect in suppression of medial thickening.

Significant whole blood levels of CYA were demonstrated by radioimmunoassay in the present study in the injection group. At 1 week CYA blood levels showed a wide variation, but showed relatively constant levels after 4 weeks. CYA is metabolized in the liver by the microsomal monooxygenase system. Wide variation of CYA levels at 1 week is probably related to the finding that the hepatic microsomal system is highly activated after 28 days of treatment with CYA (Cunningham *et al.* 1984).

In conclusion these data suggest that immunosuppressive agents are effective in suppressing the hyperplastic myointimal reaction to an intimal injury. Additionally these data also provide further evidence that the immunological mechanism may modulate vascular proliferative lesions. Further research will be required to know the applicability of clinical use of immunosuppressants in suppression of myointimal thickening.

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