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Response of the Arterial Wall to Endothelial Removal: An Autoradiographic Study (40373)

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We have shown in earlier studies that the intimal hyperplasia which follows deendothelialization of rabbit aortas is self-limited even in the absence of restored endothelial cell cover (1). Thus, the hyperplastic response reaches a maximum by about two months after the insult, although reendothelialization may be incomplete for 6 months or longer. The present studies were designed to study the course of this proliferative response as reflected by incorporation of tritiated thymidine into the nuclei of vascular smooth muscle cells, as a function of the interval after deendothelialization. The findings indicate that the proliferative response subsides with remarkable rapidity.

Methods and materials. Experimental animals were male New Zealand rabbits, weighing 2–4 kg. Surgical procedures were performed under light sodium pentobarbital anesthesia, supplemented with ether as necessary. Deendothelialization of the aorta was performed as previously described (2).

Groups of three animals were sacrificed at 3, 6, 14, or 28 days after injury; sham operated animals served as controls. One hour prior to sacrifice, each animal received 4 mCi of [^{3}H]thymidine (New England Nuclear Co.) intravenously. One-half hour later 4 ml. of Evans' blue dye were similarly administered thereby defining blue areas where an endothelial cover was absent (2). Sacrifice and perfusion fixation was accomplished as previously described (2). Following perfusion, aortae were excised and quickly cleaned of their adventitia by sharp dissection. The ventral surfaces were sliced open, and the arteries pinned out as *en face* preparations for photography with a Polaroid MP4 camera. This revealed macroscopic regions which were blue or white. Tissues were then fixed for 1 hr in the perfusate before being placed overnight in 7% sucrose–0.1 *M* cacodylate buffer. Sections of small intestine were obtained to provide controls for [^{3}H]thymidine labeling.

Four segments from each aorta were then chosen for study, selected to provide representative sampling of both blue and white areas. Particularly in vessels after 2 or more weeks of healing, sections completely stained or free of staining could not be obtained. Accordingly, many sections examined had both blue and white components. Cross sections were excised by sharp dissection and embedded in paraffin. The segments chosen were marked on the Polaroid photographs and given a code number. These were then cut, mounted on slides, and coated with Kodak NTB2 emulsion as described by Sparagen *et al.* (3). Slides were incubated for 7 days at 4° in the dark, developed, and counterstained with hematoxylin and eosin.

Labeled cells were defined as those having at least five grains per nucleus. Counts were made microscopically with 450× magnification and an eye piece reticule micrometer.

Each slide consisted of 4 serial cross sections from the designated areas of each aorta. Counting was done without knowledge of origin of either section, Evans' blue staining, or animal. In each cross section the following variables were evaluated by direct counting: (a) Total number of intimal cells, (b) total number of labeled intimal cells and (c) total number of labeled medial cells. The total number of intimal cells present per cross section ranged from about 30 in control animals to about 500 in 28-day animals. For purposes of more exact localization of labeled cells, the media was divided into four approximately equal levels, with the first being immediately beneath the IEL and the fourth adjacent to the adventitia. The number of labeled cells in each level was then enumerated. Finally, the intimal cells oriented on the vessel lumen in a manner similar to endothe-

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lial cells were counted, and their location in relation to the blue–white junctions was noted. The total number of medial cells in one section of each slide was then counted. Since the size of the media was constant and did not change following injury, this number of about 500 cells was taken to be an estimate of the total medial cells of each cross sectional area. Data from all sections from all four segments studied in each group of three animals were pooled and tabulated, to give the incidence of labeled cells in each region of the vessel (lumenal intima, total intimal, medial levels 1–4) per 1000 nuclei counted ± SE of the mean.

Following this initial analysis the code was broken, and the slides were reexamined to evaluate mitotic activity in blue and white areas respectively. This was done by comparing slides with the colors (blue or white) on the photographs which were taken prior to their embedding. Further comparison was made between the cross sections on the slides with the cross sections as they appeared on the uncut remainder in paraffin blocks. Here, the vessel cross section could be plainly seen as blue, white or a mixture. Blue and white areas had their thymidine indices determined as labeled cells per 100 nuclei in the intima or media of each group. Blue and white areas in each cross section were then gauged with respect to intimal thickness by number of cell layers, and actual widths as determined with the optical micrometer. Finally, the presence of [^{3}H]thymidine labeling in sections of gut from each animal was ascertained to verify that systemic exposure to the reagent had actually occurred.

Results. Identification of labeled cells presented no difficulty, since background counts were negligible. Each animal showed uptake of radioactive label in sections of intestine. The degree of aortic intimal hyperplasia and cell labeling varied among different areas in a single cross section in both blue and white regions. In conformity with previous findings (2), there was also variation in the hyperplastic response along the length of the vessel and among different animals within a group. Whether this reflected an artifact of technique or spontaneous variability is unclear.

Intimal hyperplasia, as measured by the parameters of thickness and total number of intimal cells per cross section, increased with time after injury (Fig. 1). Mitotic activity, as reflected by [^{3}H]thymidine cell labeling, was maximal in the first week post-injury; it rapidly and progressively decreased thereafter (Table I, Fig. 2). Few intimal cells were present 3 days after injury, but those present showed significant mitotic activity. By 6 days a characteristic labeling pattern had become evident: The closer to the lumen the greater the incidence of labeled cells. In the subsequent periods, this relationship was maintained in the face of the reduced proliferative activity, and the lumenal cells were the last to return to baseline levels. Of additional interest is the rate of this reduction, which appears to approximate linearity on the semilogarithmic plot, and gives a halving time of about 3 days.

The relationship between continued proliferative activity and restoration of endothelial cover was evaluated by systematic counting of labeled cells from blue or white areas. Although no significant and consistent differences were observed, it should be noted that the area of reendothelialization up to 2 weeks postinjury was small, thus preventing collection of meaningful data, and no white area counted was more than 2 mm from a blue area. Perhaps this represented lateral diffusion of blood borne mitogens in the vessel wall. Re-endothelialization was more extensive in the 28-day animals, but in these, the labeling index was too low for meaningful comparisons. These data are presented in Table II. Cell proliferation appeared to depend primarily upon time after injury rather than location in blue or white areas. Thus, the thymidine labeling index in blue areas was only slightly higher than in white areas (Table II). Variations in intimal thickness were not associated with differences in mitotic activity in either the intima or media. At 28 days, the vessel walls were almost completely devoid of labeled cells throughout.

Discussion. The vascular endothelium, and medial smooth muscle cells, represent relatively stable populations of cells under normal conditions. In the uninjured rabbit aorta the incidence of [^{3}H]thymidine labeled cells in the intima and media are about 0.8 and 0.03 per thousand respectively (4). Increased cell turnover and associated mitotic activity

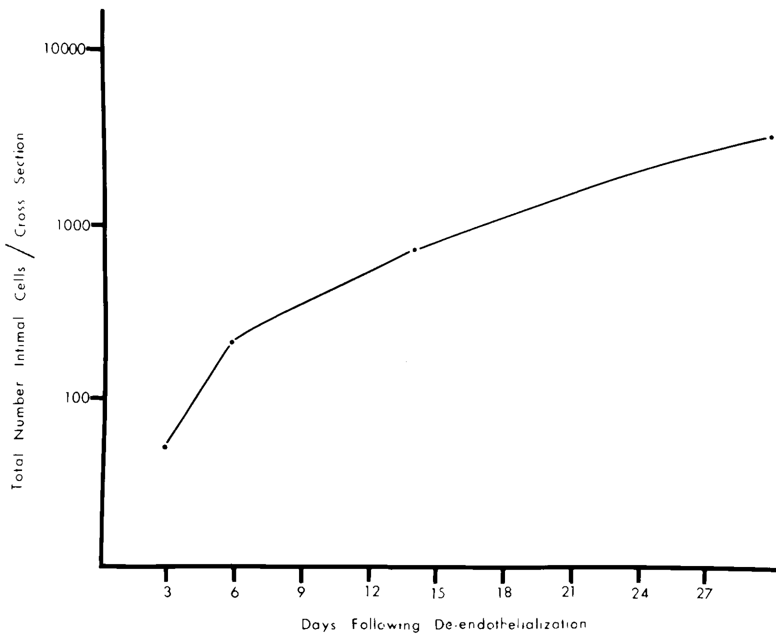

FIG. 1. Intimal hyperplasia following deendothelialization of rabbit aortae from animals sacrificed at varying intervals following endothelial removal with a balloon catheter. Extent of proliferation is expressed as mean number of intimal cells per cross section.

TABLE I. MITOTIC ACTIVITY OF INJURED RABBIT AORTAE.

	Incidence of labeled cells per 1000 cells ± S.E.M. Days after injury				
Location	Control	3	6	14	28
Lumenal Intima	0.3	160 ± 35	250 ± 66	40 ± 10	2 ± 2
Total Intima	—	114 ± 32	138 ± 32	17 ± 1	1 ± .4
Media Level 1	0	12 ± 7	7 ± 5	1 ± 1	0
Media Level 2	0	6 ± 5	5 ± 5	0.5 ± 0.5	0
Media Level 3	0	6 ± 6	4 ± 5	0.4 ± 0.4	0
Media Level 4	0	6 ± 6	3 ± 2	0.3 ± 0.3	0

is seen as a response to various insults in the aortae of rabbits such as atherogenic diets (3), hemodynamic stresses (5), or physical trauma (6).

Medial smooth muscle cells constitute the source of neo-intimal SMC's in the regenerating intima of injured arteries, but cells from the entire breadth of the media are stimulated to divide. Some of these then migrate to the intima and continue dividing. Intimal mitotic activity was typically greater and persisted longer than that of the media. Thus, the majority of neo-intimal cells are generated within the intima itself from a starting pool of SMC's originating in the media. The consequence of these events is preservation of medial thickness in the presence of intimal hyperplasia.

The present data are in conformity with the earlier observations of Hassler who subjected carotid arteries and aortae to mechanical trauma (7), and with the findings of Webster *et al.* (8) in the rabbit aorta. However, in those earlier experiments, the relationship between moderation of the proliferative response and reendothelialization was not characterized, and no hypothesis was developed concerning the mechanisms involved.

The stimulus for the initial migration and proliferation of the medial smooth muscle cells is presumably based upon the mechanisms suggested by Ross and his colleagues (9), whereby platelets adhering and aggregat-

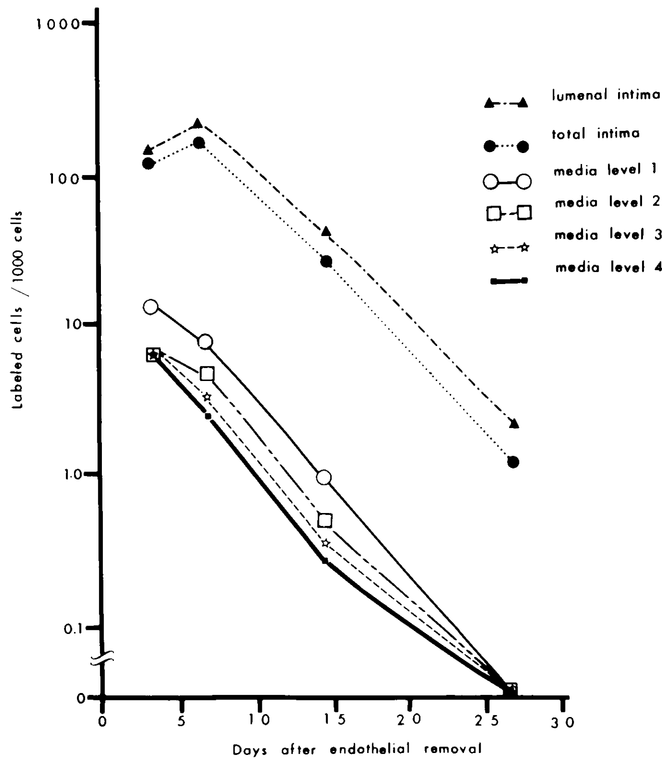

FIG. 2. Intimal medial mitotic activity of rabbit aortae following endothelial removal.

TABLE II. MITOTIC ACTIVITY OF INJURED RABBIT AORTAE TREATED WITH EVANS' BLUE DYE.

	Thymidine index (% + S.E.M.)[a] Days after injury				
Location	Control	3	6	14	28
Intima:					
Blue areas	—	10.9 ± 5	14.1 ± 6	3.2 ± 2	0.1 ± 0.1
White areas	0	—	13.3 ± 5	2.1 ± 3	0.1 ± 0.1
Media:					
Blue areas	—	1.0 ± 0.5	1.1 ± 0.3	0.1 ± 0.2	0
White areas	0	—	1.0 ± 0.4	0.1 ± 0.2	0

[a] Based on total number of intimal and medial cells present on each cross section.

ing at the sites of exposed subendothelial connective tissue undergo a release reaction, and flood the vessels cells with a mitogenic protein. Several possibilities could account for the transient nature of this response. One of these could be a rapid decline in delivery of platelet mitogen to the vessel, and this would be consistent with the findings of Groves *et al.* (10), who demonstrated a dramatic reduction of platelet turnover shortly after deendothelialization of rabbit aortae by techniques similar to those used by us. Whether platelet turnover is reduced sufficiently to produce the observed changes remains to be determined: We have found well preserved and presumably recently adhering platelet in significant numbers on blue areas for many months after injury (2). Blue areas in the balloon deendothelialized rabbit have been shown to represent the virtual absence of an endothelial cell cover (2). Additionally, even in the presence of sustained turnover, platelets participating at later intervals following injury might undergo a diminished release reaction, and thus make correspondingly less mitogen available. Alternatively,

the smooth muscle cells themselves might become progressively less responsive to available mitogen as a consequence of their previous proliferative history. In any event, the phenomenon of the limited hyperplastic response is clearly adaptive: Persistence of the initial proliferative rate would rapidly produce a mass of tissue that would rapidly become occlusive to the lumen whenever major endothelial loss occurred.

Summary. The proliferative response of the balloon deendothelialized rabbit aorta was followed by tritiated thymidine labeling. Peak labeling was seen by 6 days after the procedure, with progressively decreasing activity with increasing distance from the lumen. The proliferative response rapidly subsided, so that base-line values were achieved by a month after the vessel insult. The decrease in proliferation occurred even in areas which were not re-endothelialized. The mechanism of this moderating response is presently not explained, but it appears to have adaptive value in preventing excessive lumen occlusion following vascular injury.

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