PERMEABILITY TO EVANS BLUE AND HORSERADISH PEROXIDASE AND MORPHOMETRY ON STRESS FIBERS IN NORMAL AND REGENERATED RAT AORTIC ENDOTHELIUM FOLLOWING SEGMENTAL BALLOON INJURY

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ABSTRACT

Studies on normal and regenerated rat aortic endothelium at 1, 2, 3 and 4 weeks following segmental balloon denudation injury were carried out to evaluate: (1) the permeability to intravenously injected Evans blue (EB) and horseradish peroxidase (HRP) using en face aortic preparations and (2) the volume density of stress fibers by morphometry using thin section electron microscopy. The results of these studies indicated that: (1) the permeability of the regenerated endothelium to both EB and HRP was identical to normal endothelium at all time points studied and (2) stress fiber volume density significantly increased in regenerated endothelium at 1 week as compared to control, however, returned to and remained at normal value at and after 2 weeks following segmental balloon injury. These results are consistent with the view that structural and functional changes in regenerated vascular endothelium, if present, are transient in nature and the integrity of endothelial monolayer is eventually reestablished during the repair process that follows a single injury.

RÉSUMÉ

Des études sur l'endothélium aortique de rat normal et régénéré ont été effectuées 1, 2, 3, et 4 semaines après avoir pratiqué une lésion segmentaire de dénudation à l'aide d'un ballonnet dans le but d'évaluer: (1) la perméabilité au blue Evans (EB) et à la peroxidase de raifort (HRP) injectés par voie intraveineuse en utilisant des préparation aortiques en face et (2) la densité volumique des "stress fibers" par morphométrie en microscopie électronique sur coupes fines. Les résultats de ces études indiquent: (1) que la perméabilité de l'endothélium régénéré au blue Evans et à la peroxydase de raifort est identique à celle de l'endothélium normal à toutes les phases de l'étude et (2) que la densité volumique des "stress fibers" qui la première semaine, accusait une nette augmentation dans l'endothélium régénéré par rapport au sujet témoin, était redevenu normal 2 semaines après la lésion par ballonnet et qu'il l'est demeuré dans les semaines qui ont suivi. Ces résultats corroborent l'hypothèse voulant que les modifications structurelles et fonctionnelles de l'endothélium vasculaire régénéré, quand elles se produisent, revêtent un caractère transitoire et que l'intégrité de la monocouche endothéliale se rétablit au cours du processus de réparation qui suit une lésion unique.

ACKNOWLEDGEMENTS

The first and foremost thanks to my thesis supervisor Dr. Istvan Hüttner, Professor of Pathology, in whose laboratory the studies were carried out. Dr. Hüttner introduced me to experimental pathology in general and endothelial cell research in particular. He helped me in the selection and design of the experimental model, in the interpretation of the results and the preparation of the thesis. I would like to express my sincere gratitude to Dr. I. Hüttner, a genuinely dedicated scientist, for allowing me to have the privilege of studying under his guidance and for his understanding and continuous support.

I am most grateful to Dr. D.S. Kahn who initially motivated me to pursue my graduate studies in experimental pathology, introduced me to Dr. Hüttner and provided me with invaluable support and advice. I thank Dr. Sean Moore, Chairman of the Department of Pathology for providing a stimulating and cooperative environment in his department during my studies.

Special thanks to Mr. Howard Peters for his excellent technicial assistance in almost every phase of the experimental work. I learned much from Howard about animal operations, electron microscopy and photography.

I would like to thank Dr. C.C. Haudenschild for his advice on the "ballooning" procedure, Dr. André Blouin for lending a "back projection unit" for morphological studies, and Mr. Carey Levinton for

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Finally I wish to thank my wife Lucy for her patience, support and encouragement.

During these studies, I have performed all the animal operations, and all procedures related to permeability studies on normal and regenerated rat aortic endothelium with the technical help of Mr. Howard Peters, including injection of tracers, perfusion fixation, preparation and incubation of en face aortic preparations and evaluation of the results. I have sampled all of the specimens and processed most of them for electron microscopy, and prepared some of the semi-thin and ultra-thin sections. I have taken all electron micrographs for the morphometric studies, and organized and partly

built the set up for morphometry. I have done all of the point counting and statistical analysis of data on stress fiber volume densities using thin section electron microscopy and drawn the schemas and the graph for the thesis. I have completed the literature search, review of the literature and preparation of the thesis with the help of Dr. I. Hüttner.

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INTRODUCTION

Endothelial cells line the insides of arteries, veins, capillaries and lymphatics as a monolayer. These cells have important roles in physiological homeostasis; in the permeability of blood vessels and in the mediation of their response to a variety of physiological and pathological stimuli (Gimbrone et al, 1974; Jaffe et al, 1973; Majno and Joris, 1978). Abnormalities in the structure and function of endothelial cells may play a significant role in diseases of blood vessel walls, particularly thrombosis and atherosclerosis (Hüttner and Gabbiani, 1982; Ross, 1986; Schwartz et al, 1981; Stemerman, 1974; Thorgeirsson and Robertson, 1978). In culture, endothelial cells are different from most other cells. Growth of endothelial cells unlike that of fibroblast or smooth-muscle cells, is characterized by the formation of a highly ordered monolayer (Haudenschild et al, 1975; Jaffe et al, 1973; Vlodavsky and Gospodarowicz, 1979). This monolayer adopts a morphological appearance and differentiated properties similar to those of the vascular endothelium *in vivo*. The closely apposed and non-dividing cells of the monolayer have a distinctive membrane asymmetry; they have a non-thrombogenic luminal surface and can no longer internalize bound ligands such as low-density lipoprotein, while fibronectin disappears from the luminal surface and concomitantly accumulates close to the basal surface (Muller and Gimbrone, 1986; Vlodavsky and Gospodarowicz, 1979). Once the cells have formed this highly ordered structure, the only agents shown to be

able to stimulate growth are those that disrupt the continuity of the monolayer. This is true both in vitro and in vivo and suggests that contact inhibition of growth may be particularly important for endothelial cells when compared with other cells (Schwartz et al, 1981).

The biological behaviour of the endothelial monolayer is reflected by the extremely low basal rate or replication in normal adult arterial endothelium in vivo (Schwartz and Benditt, 1976; Schwartz and Benditt, 1977 Schwartz et al, 1980). It has been demonstrated, however, that there are focal areas of endothelium with increased replication, and that the overall replication of aortic endothelium is increased under certain pathophysiological conditions such as hypertension (De Chastonay et al, 1983; Schwartz and Benditt, 1977) and endotoxemia (Reidy, 1985; Reidy and Schwartz, 1983).

Normal quiescent arterial endothelium consists of a layer of flattened, elongated cells oriented with their long axis in the direction of blood flow. This quiescent cell layer, however has properties that are crucial to normal functioning of blood vessel wall; they fall into three major categories (Majno and Joris, 1978; Thorgeirsson and Robertson, 1978): (1) normal endothelial cells are metabolically active and highly versatile; they synthesize prostacyclin (PGI_2) and an activator of plasminogen, they produce factor VIII and von Willebrand factor, they contribute several components to the subendothelial connective tissue, and they contain receptors to a variety of vasoactive agents; (2) the normal

endothelial cell layer provides a thromboresistant surface that prevents platelet or leukocyte adherence and activation of intrinsic or extrinsic coagulation systems; (3) the normal endothelial cell layer forms a barrier to the passage of blood constituents into the artery wall; this is essential to maintain normal microenvironment and growth pattern of underlying smooth muscle cells (Ross and Glomset, 1976).

Endothelial continuity is vital to maintain all major properties of vascular endothelium particularly its thromboresistant surface and its barrier role. Thus, these cells have been the focus of intense studies both in vivo and in vitro (Cotran, 1989; Majno and Joris, 1978; Ryan, 1988; Stemerman, 1979; Stemerman et al, 1984; Thorgiersson and Robertson, 1978). Alteration of the endothelial barrier is obvious in small artery disease, particularly in hypertension (Thorgiersson and Robertson, 1978) and diabetes (Rossini and Chick, 1980), and has also been widely implicated in large artery disease (Ross and Glomset, 1976). Loss of continuity of the endothelial cell layer is the major cause of thrombosis because it exposes the highly thrombogenic subendothelial tissues to platelets, and initiates the cascade of platelet adherence, aggregation, and degranulation (Stemerman, 1979; Stemerman and Ross, 1972; Weiss, 1975). Furthermore, there is evidence that endothelial denudation leads to proliferation and migration of smooth muscle cells into the intima as a result of the release of platelet-derived growth factor at sites where endothelial continuity is disrupted (Ross and Glomset, 1976).

The ability of the vascular endothelial cell to repair a denuded surface has been well documented both in vivo and in vitro. Large wounds are repaired by migration and proliferation while smaller wounds may be repaired by spreading and migration (translocation) alone without associated proliferation (Adamson and Bowden, 1983; Bettmann et al, 1981; Boden and Gotlieb, 1983; Clowes et al, 1983; Fishman et al, 1975; Gotlieb, 1983; Gotlieb et al, 1987; Haudenschild and Schwartz, 1979; Reidy et al, 1983; Schwartz et al, 1981; Schwartz et al, 1978; Sholley et al, 1977; Wong and Gotlieb, 1988).

It is crucial to answer the question whether the repair process in vascular endothelium results in an endothelial monolayer that acquires normal structure and function or it results in an endothelial monolayer that is defective in some way thus promoting vascular disease.

There are a number of studies that demonstrated functional differences between normal and regenerated endothelium* involving particularly metabolic activities of endothelial cells, although, in most cases it was not established whether these changes were transient or permanent following injury. Specifically, injured and/or regenerated endothelium may produce increased amounts of vasoactive agents, growth factors (particularly a mitogen resembling PDGF) and growth inhibitors (such as heparin) (Barrett et al, 1984; Hansson et

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*Regenerated endothelium is defined as a newly formed endothelial cell layer covering a denuded arterial segment, regardless of the extent of the re-endothelialization.

al, 1987; Ross, 1986; Zacharias et al, 1988). Impairment of endothelium-dependent relaxation in isolated arteries with regenerated endothelium has been reported (Shimokawa et al, 1987; Shimokawa et al, 1989). An increase in intracellular von Willebrand factor was demonstrated (Reidy et al, 1989). Monoclonal antibodies recognizing new or spreading endothelium, probably by recognizing selectively expressed antigen on these cells, have been identified (Pringle and de Bono, 1988).

There have been few studies comparing the structural aspects of normal and regenerated endothelium particularly those related to continuity of the endothelial monolayer which is vital to maintain all major functional properties of vascular endothelium.

Since cell to cell interactions as well as cell to matrix interactions are essential in maintaining the continuity of the endothelial monolayer, one aspect of each of these interactions were selected in the studies presented in this thesis:

(1) The permeability of the normal and regenerated aortic endothelium to Evans blue (EB), a dye which rapidly complexes with serum albumin when injected intravenously (Schwartz et al, 1978; Stemerman et al, 1977), and horseradish peroxidase (HRP), a protein tracer of about 40,000 molecular weight (Karnovsky, 1967), following segmental denudation in <u>en face</u> rat aortic preparations was studied in time-sequence as a reflection of cell to cell interactions in the endothelial monolayer.

There are only a few reported studies dealing with the permeability characteristics of regenerated aortic endothelium and they seem to convey conflicting messages. One study describes no difference in the permeability of re-endothelialized areas of rabbit aorta to horseradish peroxidase as compared to normal endothelium (Stemerman, 1981); other studies report increased permeability of re-endothelialized intima to lipoproteins as compared to normal rabbit intima (Day et al, 1985; Schwenke and Zilversmit, 1989).

(2) The volume density of stress fibers (organelles implicated in cellular attachment of endothelial cells to subendothelium) in normal and regenerated rat aortic endothelium following segmental denudation was studied in time-sequence by morphometric analysis using thin section electron microscopy as a reflection of cell to matrix interactions in the endothelial monolayer.

It is well established that endothelial cells of regenerated aortic endothelium in vivo contain a strikingly increased number of stress fibers as compared with those of quiescent normal endothelium (Gabbiani et al, 1983; Hüttner et al, 1985). However, the stress fiber volume density of regenerated endothelium has not been studied in time-sequence. A recent study published during the writing of this thesis, has demonstrated qualitatively by immunofluorescent microscopy using anti-platelet myosin antibody that the stress fiber expression in regenerated rat and rabbit aortic endothelium is reversible (White et al, 1988).

MATERIALS AND METHODS

Experimental Procedures

The experimental protocol for the studies of the segmental aortic endothelial denudation and regeneration in rats is shown schematically in Figure 1. The experiments were carried out in 36, two-month old male Wistar rats (Charles River, Canada, Inc., Montreal, Quebec, Canada), weighing 250 to 300 gm. The animals were purchased through the McGill University Animal Center and housed and cared for in their facilities throughout the experiments. The rats were fed Rat Chow #5012 (Purina Mills Inc, St. Louis, MO, U.S.A.) and water, both ad libitum except the overnight period preceding the operations described below when they were deprived of food and water. The animals were divided into two series of 18 rats, one series serving for the permeability studies using EB dye and the other for the permeability studies using HRP. The HRP series also served, subsequently, for the morphologic and morphometric studies using thin section electron microscopy. Three rats in each series were utilized as non-operated control animals and the remaining 15 underwent a segmental denudation of the aortic intimal surface to remove the endothelium with a saline-filled embolectomy catheter (2F Fogarty, Edwards Laboratories Inc., Santa Ana, California, U.S.A.) applying the method of Baumgartner and Studer (Baumgartner and Studer, 1966) with minor modifications (Gabbiani et al, 1982, Reidy et al, 1983).

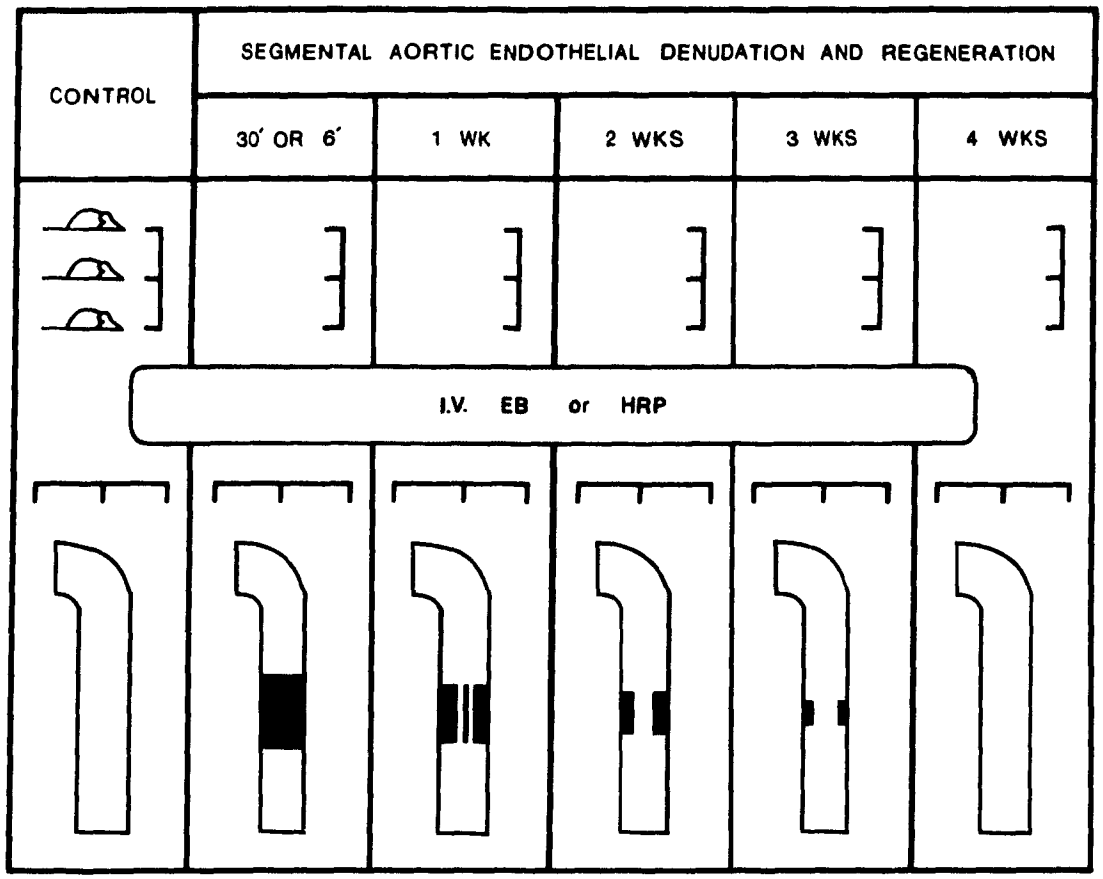


Figure 1: Schematic representation of the experimental protocol to visualize the permeability of the denuded and re-endothelialized rat aortic segments to intravenously injected EB or HRP following balloon injury. A total of 36 adult male Wistar rats, 3 animals per group providing 18 animals for the EB series and 18 animals for the HRP series, were used for the experiments.

Segmental denudation of aorta was selected as opposed to denudation of the full length of the aorta to obtain completely regenerated endothelium in a relatively short time.

The animals were anesthesized by intraperitoneal pentobarbital (Somnotol, M.T.C. Pharmaceuticals, Mississauga, Ontario, Canada) administered at a dose of 65 mg/kg body weight for the operations. A 3 cm long inferior midline neck incision was made and the left common carotid artery was isolated and ligated at the cephalad side. The embolectomy catheter was inserted caudally into the artery as a small transverse incision in the vessel was held open by microdissecting tweezers (Dumont No. 7 JB EM Services Inc., Montreal, Quebec, Canada). The tip of the catheter was further advanced into the thoracic aorta to a distant most point of 5 cm measured from the caudad angle of the left muscular triangle in the neck. The balloon of the catheter was inflated to a diameter of 4 mm with a predetermined amount of saline (about 0.05 cc). A 1.0 ± 0.1 cm long segment of the thoracic aorta between the first and fourth pairs of intercostal artery openings was denuded of the endothelium by moving the catheter back and forth six times. After the completion of the denudation, the balloon was deflated and withdrawn. The carotid artery was ligated caudally to the catheter insertion site after removal of the catheter. The skin incision was closed with 11 mm Michel clips. The operation was completed by punching an accession number in the ears of the rats for identification. Following the operation, the animals were separated into groups of 3 rats for

time-sequence studies on the regenerated aortic endothelium both in the EB and HRP series. The selected time points comprised of 30 minutes, 1 week, 2 weeks, 3 weeks, and 4 weeks following the surgery for the EB series and 6 minutes, 1 week, 2 weeks, 3 weeks and 4 weeks following the surgery for the HRP series.

Permeability Studies using EB and HRP at Macroscopic Level

EB Series. All rats in the EB series, 15 operated and 3 non-operated control, under intraperitoneal pentobarbital anesthesia, were injected through the left femoral vein with 1 cc of 1% EB dye (Allied Chemical Corp., New York 6, New York, U.S.A.) in 0.9% sodium chloride solution 30 minutes prior to their sacrifice (Clowes et al 1983; Clowes et al 1985; Haudenschild and Schwartz, 1979) by simultaneous exsanguination from the thoracic segment of the inferior vena cava and perfusion fixation through the ascending aorta preceeded by a 30 second flush by a 0.9% sodium chloride solution. The perfusion fixation was initiated by modified Karnovsky's fixative (Hüttner et al, 1973a; Hüttner et al, 1973b) containing 1% paraformaldehyde and 1.25% gluteraldehyde in 0.1 N sodium cacodylate buffer (pH 7.4) with 5% sucrose (final osmolality: $\sim$ 750 mOsm) for 5 minutes. The perfusion was continued with a second fixative solution containing 2% paraformaldehyde and 2.5% glutaraldehyde with the same buffer (final osmolality: $\sim$ 900 mOsm) for 10 minutes. The flush and fixative solutions prepared at room temperature were administered

through a canula placed in the aortic arch at 120 mmHg pressure while the solutions were allowed to escape from the inferior vena cava.

After the perfusion fixation, the aorta, including the arch as well as the thoracic and abdominal segments, was removed en bloc using sharp dissection and further fixed for 2 hours by immersion in the second fixative solution then stored overnight in 0.1 N sodium cacodylate buffer (pH 7.4) containing 11.25% sucrose (final osmolality: ∿ 380 mOsm) at 4° C. The fixed aortas were then cleaned of their adventitial elements under a dissecting microscope, trimmed, opened longitudinally and pinned out on a silicone rubber pad with stainless steel minutien pins (Fine Science Tools Inc., Belmont, California, U.S.A.). The specimens were grossly examined first unaided then under a magnifying lens, and photographed.

HRP Series. All rats in the HRP series, 15 operated and 3 non-operated control, under intraperitoneal pentobarbitol anesthesia, were injected through the left femoral vein with HRP (Horseradish peroxidase, Type II, Sigma Chemical Company, St. Louis, Montana, U.S.A.) at a dose of 10 mg/100 gm body weight dissolved in 0.5 cc of 0.9% sodium chloride solution 6 minutes prior to perfusion fixation and sacrifice in the same manner as in the EB series. The dose of 10 mg/100 g body weight and the circulation time of 6 minutes for HRP were selected as they resulted in a reproducable overall white background with only focal light brown patches in the aortas of non-operated Wistar rats [this contrasts previously reported findings in Sprague-Dawley rats where the same dose and circulation time for

HRP resulted in a considerably dark background staining (Hüttner et al, 1973 a; Hüttner et al, 1973 b) presumably related to histamine and serotonin release (Cotran and Karnovsky, 1967)].

The same fixative solutions as for the EB series were used, however the 30 second flush was with Krebs-Ringer-Bicarbonate (KRB) containing 8 mg heparin sodium (Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, New Jersey, U.S.A.) per 100 ml KRB solution (Nagy et al, 1983). After the perfusion fixation, the aorta, including the arch as well as the thoracic and abdominal segments, was removed en bloc using sharp dissection and further fixed for 2 hours by immersion in the second fixative solution and stored overnight in 0.1 M sodium cacodylate buffer (pH 7.4) containing 11.25% sucrose (final osmolality: $\sim$ 380 mOsm) at 4^{o} C with the same composition as the one used for the EB series.

The fixed aortas were processed to demonstrate the presence and the localization of the intravenously injected HRP by incubating the aortas en bloc in dark and gently agitating for 1 hour at room temperature in Graham-Karnovsky medium [10 ml of 0.05 M tris-HCl buffer, pH 7.6, containing 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.1 ml of 1% H_2O_2] (Graham and Karnovsky, 1966). After the HPR-DAB chromogen reaction had been completed the aortas were cleaned of their adventitial tissue under a dissecting microscope, trimmed, opened longitudinally and pinned out on silicone rubber pads with stainless steel minutien pins as en face preparations. The specimens were then grossly examined, first unaided

then under a magnifying lens, and photographed.

Morphologic and Morphometric Studies on Endothelial Stress Fibers using Thin Section Electron Microscopy

Tissue Preparation for Electron Microscopy. The aortas fixed by perfusion then immersion were sectioned, using sharp and clean razor blades, under a dissecting microscope to obtain about 0.2 x 0.1 x 0.05 cm longitudinal tissue blocks of the vessel wall including the intima and media. The sections were always taken from the dorsal aspect of the aorta between the intercostal artery openings to obtain regenerated endothelium at various time points during the experiments.

The tissue blocks were postfixed for 90 minutes with ferrocyanide reduced 1% osmium tetroxide (Karnovsky, 1971) in Palade buffer (pH 7.4) containing 4.9% sucrose (final osmolality: ∿ 430 mOsm) for 90 minutes at room temperature. The tissue blocks were then dehydrated in graded ethanol and embedded in Epon 812. One micron thick sections were cut with glass knives by an LKB III ultramicrotome and stained with toluidine blue for light microscopy. Thin sections, about 80 nm thick, were cut, using diamond knives from the areas selected on the basis of light microscopy, and double stained with uranyl acetate and lead citrate then examined in Philips EM 300 and Philips EM 200 electron microscopes at 60 kv for qualitative and quantitative studies respectively.

Morphometric Estimation of Stress Fiber Volume Densities.

Morphometric estimation of stress fiber volume densities in normal and regenerated rat aortic endothelium were carried out in the HRP series using a stratified random sampling approach as illustrated schematically in Figure 2.

A total of 15 rats were included in morphometric studies. The non-operated control group was represented by 3 rats. Similarly, each of the experimental groups except, the 6 minute group where there was no grossly identifiable regenerated endothelium (see Figure 4), was represented by 3 rats. Four longitudinally sectioned tissue blocks of aortic wall, including the intima and media, obtained from the dorsal aspect of each aorta between the second and fourth pairs of the intercostal artery openings were selected. The tissue blocks were processed and a single double-stained grid was prepared from each block for thin section electron microscopy, as described above. Random electron micrographs of the endothelium were taken by reference to the frame of the supporting grid. A maximum of 2 micrographs from each "square" of the grid and a total of 10 consecutive pictures from a grid were recorded on 35 mm film strips (Kodak, Catalogue No. 157 6073), at the primary magnification of X 3210, calculated by using a carbon grating replica (JB EM Services Inc., Montreal, Quebec, Canada). A positive contact print of each film strip was made using a long "light box" (Weibel, 1979). The positive films were projected in a "back projection unit" and the image was superimposed on a screen equipped with a short-line multipurpose test system with a test point

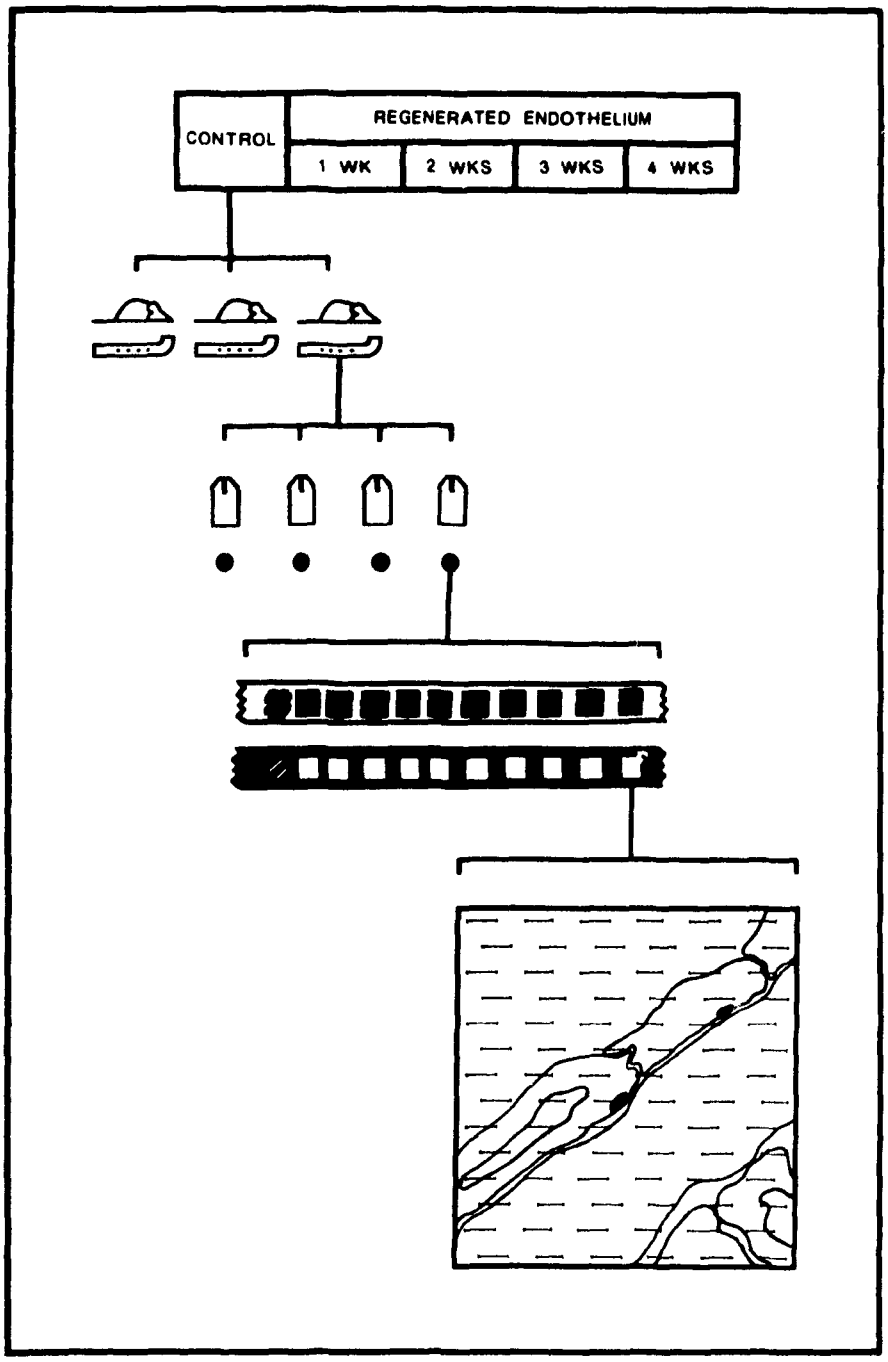


Figure 2: Hierarchy of sampling for morphometric evaluation of stress fiber volume densities in control and regenerated rat aortic endothelium in various experimental groups.

number of 168 (P_T = 168) and a test line length of 168 cm (L_T = P_T x d/2 = 168 cm). The final magnification on the screen was X 31959 calculated in the same fashion as described above.

The volume density of the stress fibers in reference to the endothelial cell layer ($V_{Vsf,el}$) was calculated by point counting, according to the method of Weibel (Weibel, 1979), for each tissue block with the following equation:

$$V_{Vsf,el} = \frac{\sum_{i=1}^{n} Psf(i)}{\sum_{i=1}^{n} Pel(i)}$$

where, the numerator and denominator were the sums of the points falling on the stress fibers and endothelial cell layer, respectively.

Each animal was represented by the mean stress fiber volume density of 4 tissue blocks and each experimental group, in turn, was represented by the mean stress fiber volume density of 3 animals. The results were evaluated by one-way analysis of variance (ANOVA) (Kleinbaum and Kupper, 1978), and multiple comparisons were performed applying Tukey's method. On account of the comparative nature of the statistical analysis on the morhometric data, the values of the stress fiber volume densities were not corrected for errors due to finite section thickness (the Holmes effect), section compression and tissue shrinkage.

RESULTS

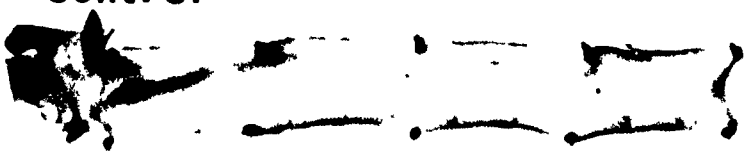
Permeability of Normal and Regenerated Rat Aortic Endothelium to EB as Evaluated by Macroscopic Examination of En Face Aortic Preparations

Representative en face aortic preparations from control rat and rats 30 minutes to 4 weeks following segmental endothelial denudation subjected to intravenous injection of 1 cc of 1% EB 30 minutes prior to sacrifice are shown in Figure 3.

Control rats injected intravenously with EB showed uniformly white luminal (endothelial) surfaces similar to non-EB injected animals. At 30 minutes following segmental balloon injury a 1.0 ± 0.1 cm long sharply demarcated blue stained segment, measured axially, was evident in the thoracic aorta between the first and fourth pairs of the intercostal artery openings representing the de-endothelialized aortic segment permeable to EB. As shown in Figure 3, an approximately 0.1 cm wide blue strip was seen in the luminal surface of the cephalad portion of the thoracic aorta in each animal at 30 minutes, obviously related to the insertion of the catheter into the segment to be denuded as well as back and forth movement of the catheter during denudation. A short blue tail, about 0.3 cm long and 0.1 cm wide, was also seen at the caudad margin of the denuded segment presumably caused by the tip of the catheter extending beyond the inflated balloon. These inadvertent catheter related injuries were not seen at later time points. At 1 week, 2 weeks and 3 weeks following segmental

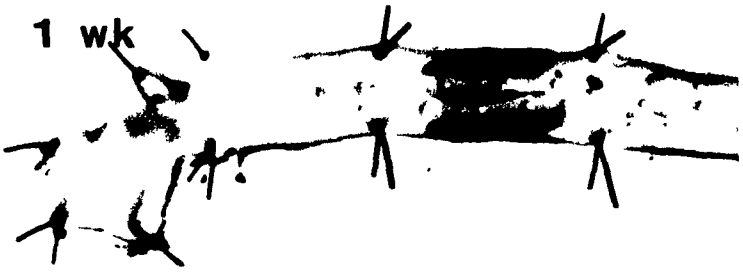
Figure 3: En face view of rat aortas following intravenous EB injection. All but the control animals have undergone an aortic ballooning procedure for segmental endothelial denudation 30 minutes to 4 weeks prior to EB injection. The blue areas (appear black in Black and White photographs) represent de-endothelialized aortic surface permeable to EB. The re-endothelialization has started and advanced from the non-denuded endothelium of the cephalad and caudad margins as well as of the intercostal arteries. This is evidenced by the progressive diminution of the size of the denuded blue areas during the course of the experiments. The re-endothelialization was complete by 4 weeks. Note that the 30 minute and 1 week aortas have been opened ventrally while the control, 2 week, 3 week, and 4 week aortas have been opened dorsally in order to better visualize the final stages of regeneration. The permeability of the regenerated endothelium to EB at each time point studied is identical to that of the control endothelium as evidenced by the white color of both the control and regenerated endothelium. x 2.3.

control

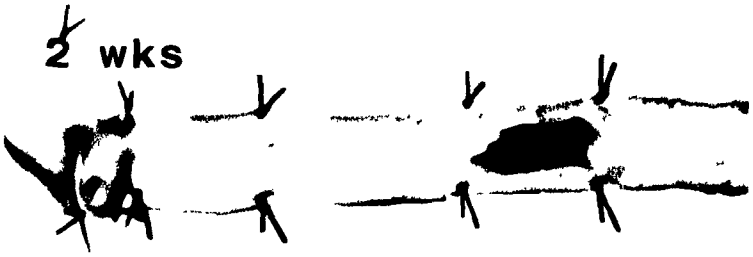


30 mins

1 wk

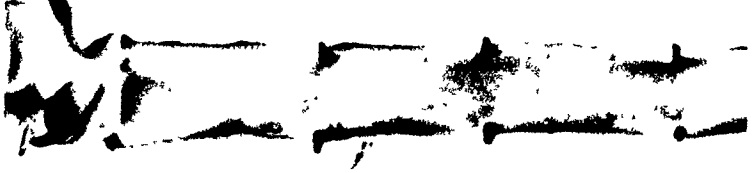


2 wks



3 wks

4 wks



balloon injury there was progressive decrease in the size of the denuded areas permeable to EB. This was characterized by the appearance of white patches around the openings of the intercostal arteries followed by the coalescence of these patches resulting in white strips running in-line with the openings of the intercostals. The expansion of the white areas around the openings of the intercostals was accompanied by extension of white fields from both the cephalad and caudad directions resulting in shortening of the denuded segment permeable to EB. The pattern of the progressive decrease in the size of the denuded aortic surface areas correlate well with the well-established knowledge that the endothelial regeneration in denuded rat aortas advances both from the endothelium of the intercostal arteries and from the endothelium of the non-denuded cephalad and caudad aortic segments (Haudenschild and Schwartz, 1979; Poole et al, 1958; Schwartz et al, 1978).

At 4 weeks, the re-endothelialization was complete as evidenced by the absence of any aortic surface areas permeable to EB.

The rate and pattern of progressive decrease in the size of the denuded aortic surface areas permeable to EB were similar in all aortas within each group representing a given time point.

The permeability of the re-endothelialized areas was identical to the endothelium of the non-operated control aortas as well as of the non-denuded segments of the experimental aortas in all time points studied.

Permeability of Normal and Regenerated Rat Aortic Endothelium to HRP as Evaluated by Macroscopic Examination of En Face Aortic Preparations

Representative en face aortic preparations from control rat and rats 6 minutes to 4 weeks following segmental endothelial denudation, subjected to intravenous injection of 10 mg/100 g body weight HRP 6 minutes prior to sacrifice, are shown in Figure 4.

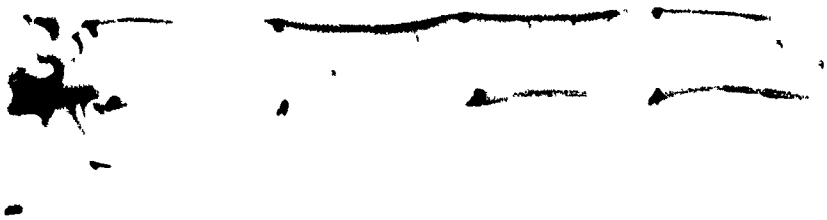
Control rats injected intravenously with HRP showed uniformly white luminal (endothelial) surfaces similar to non-HRP injected animals. At 6 minutes following segmental balloon injury a 1.0 $\pm$ 0.1 cm long sharply demarcated brown colored segment, meausured axially, was evident in the thoracic aorta between the first and fourth pairs of intercostal artery openings representing the de-endothelialized aortic segment permeable to HRP. As shown in Figure 4, an approximately 0.1 cm wide brown colored strip was seen in the luminal surface of the cephalad portion of the thoracic aorta in each animal at 30 minutes obviously related to the insertion of the catheter into the segment to be denuded and movement of the catheter during denudation. A short brown tail about 0.3 cm long and 0.1 cm wide, was also seen at the caudad margin of the denuded segment presumably caused by the tip of the catheter extending beyond the inflated balloon. These inadvertent catheter related injuries were not seen at later time points. At 1 week, 2 weeks and 3 weeks following segmental balloon injury there was a progressive decrease in the size of the denuded areas permeable to HRP. This was characterized by the

(page 22 on reverse)

(Figure 4: see legend overleaf)

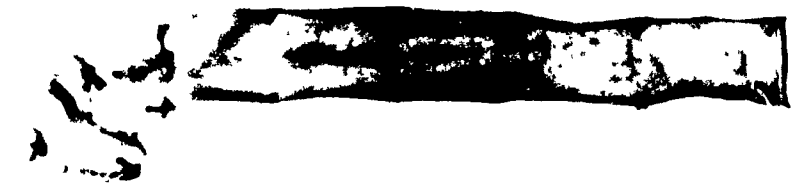
Figure 4: En face view of rat aortas following intravenous HRP injection and en bloc incubation of aortas with DAB and hydrogen peroxide. Similar to the EB experiments all but the control animals have unergone an aortic ballooning procedure for segmental endothelial denudation 6 minutes to 4 weeks prior to HRP injection. The brown areas (appear black in Black and White photographs) produced by HRP-DAB chromogen reaction represent the de-endothelialized aortic surface permeable to HRP. Note that the control, 6 minute, 1 week and 2 week aortas have been opened ventrally while the 3 week and 4 week aortas have been opened dorsally in order to better visualize the final stages of regeneration. The permeability of the regenerated endothelium at each time point studied is identical to that of the control endothelium as evidenced by the white color of both the control and regenerated endothelium. x 2.3.

control



6 mins

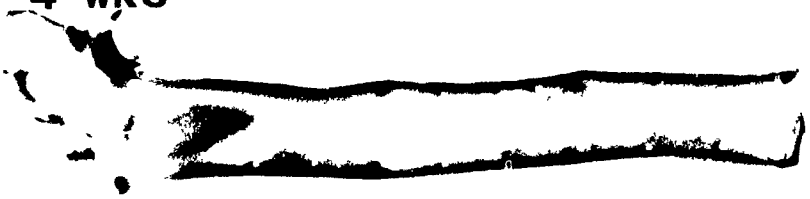
1 wks



2 wks

3 wks

4 wks



appearance of white patches around the openings of the intercostal arteries followed by the coalescence of these patches resulting in white strips running in-line with the openings of the intercostals. The expansion of the white areas around the openings of the intercostals was accompanied by extension of white fields from both the cephalad and caudad directions resulting in shortening of the denuded segment permeable to HRP. At 4 weeks there were no aortic surface areas permeable to HRP indicating complete re-endothelialization of the denuded aortic segment. The rate and pattern of progressive decrease in the size of the denuded aortic surface areas permeable to HRP were similar in all aortas within each group representing a given time point. Furthermore, the pattern of progressive decrease in the size of the denuded aortic surface areas was similar to the pattern observed in the EB injected animals, thus correlating with well-established observations on endothelial regeneration of denuded rat aortas (Haudenschild and Schwartz, 1979; Poole et al, 1958; Schwartz et al, 1978). However, as compared to aortas of the EB injected animals (where both the non-denuded endothelial surface and the re-endothelialized areas appeared uniformly white) aortas of the HRP injected animals showed poorly defined small patches of brownish discoloration of various intensity both in the non-denuded endothelial surface and in the re-endothelialized areas. These brown patches were clearly lighter than the brown areas representing denuded aortic segments, and occured in all groups, most prominently at early time points after balloon

injury. They probably corresponded to areas of increased permeability to HRP, related to operative stress and/or regional differences of endothelial permeability reported also in aortas of non-operated control animals (Stemerman, 1981).

Morphology of Stress Fibers in Normal and Regenerated Rat Aortic Endothelium using Thin Section Electron Microscopy

The general fine structure of the endothelial cell layer of control rat aorta and regenerated endothelium of rat aortas 1 week, 2 weeks, 3 weeks and 4 weeks following balloon injury is shown in the composite picture of low power electron micrographs in Figure 5.

As compared to control endothelium, endothelial cells of the regenerated endothelium appeared larger and contained more organelles such as prominent Golgi apparatus and rough endoplasmic reticulum. In particular, stress fibers, organelles that are implicated in cellular adhesion to the subendothelial matrix (Burridge, 1981; Byers and Fujiwara, 1982; Byers et al, 1984; Fujiwara et al, 1986; White et al, 1983; Wong et al, 1983) were seen infrequently in control aortic endothelium while they were frequent in regenerated endothelium, most prominently at 1 week after segmental denudation of endothelial cell layer. These observations are in agreement with well-established knowledge on the presence of an increased number of stress fibers in aortic endothelial cells during the regeneration that follows experimental endothelial denudation demonstrated by immunofluorescence

and morphometric techniques (Gabbiani et al, 1983; Hüttner et al, 1985). Longitudinal and transverse views of individual stress fibers from regenerated endothelial cells are illustrated in Figures 6 to 8.

The stress fibers are made up of bundles of cytoplasmic microfilaments with periodic dense bodies. They are oriented axially in endothelial cells and attached exclusively to the abluminal plasma membrane where they show coaxial alignment with extracellular matrix fibers of the subendothelium (Hüttner et al, 1985; White and Fujiwara, 1986). In the cytoplasm of endothelial cells, adjacent to the attachment domain of stress fibers, there is an electron dense zone which has been named "subplasmalemmal microfilament condensation" (Hüttner et al, 1985) and is structurally similar to "dense bands" considered to be an integral part of force transmissions apparatus in smooth muscles (Gabella, 1984; Hüttner, et al, 1989).

Morphometric Analysis on Stress Fiber Volume Densities in Normal and Regenerated Rat Aortic Endothelium using Thin Section Electron Microscopy

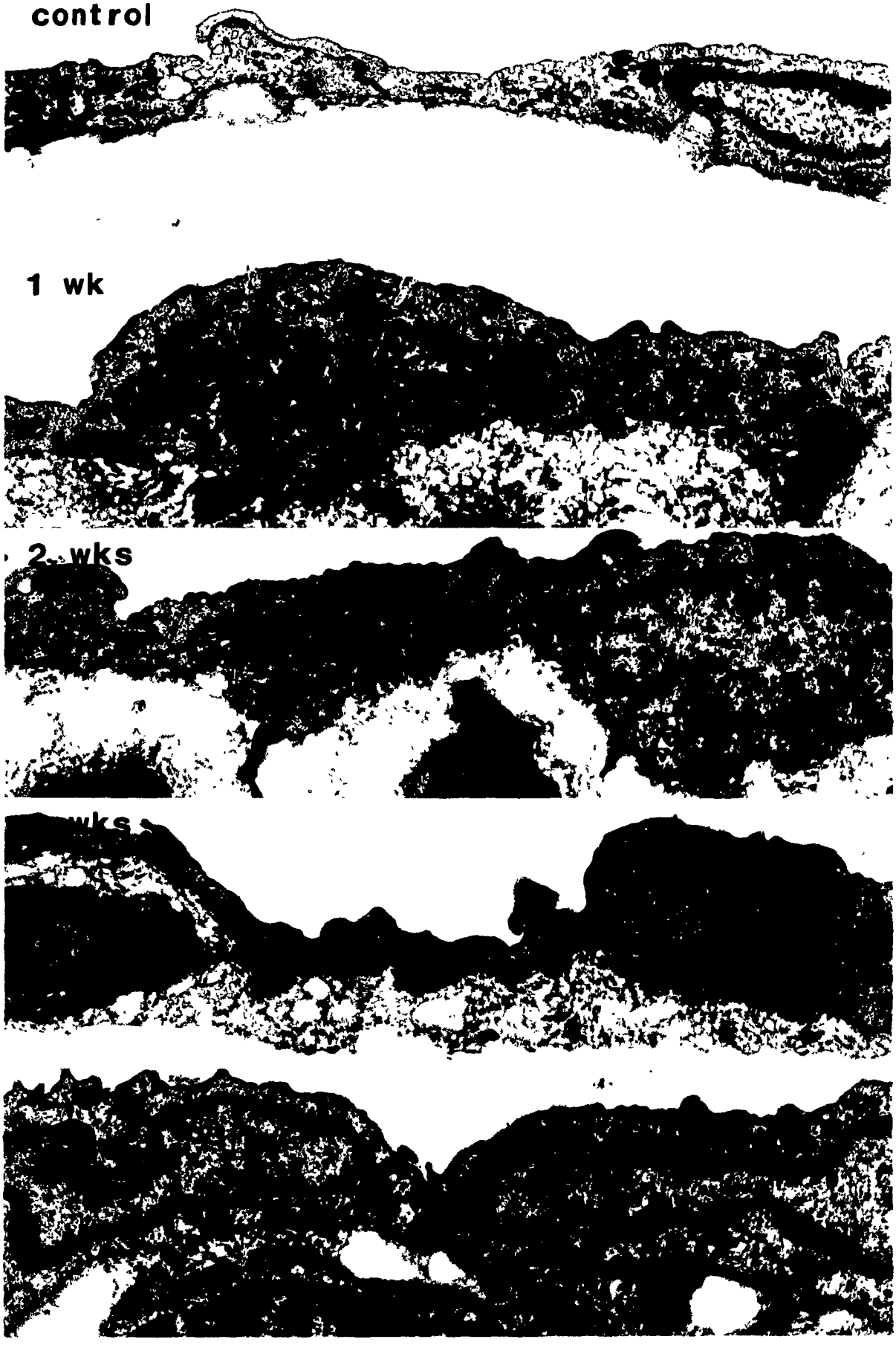
The results of morphometric analysis on stress fiber volume densities are summarized in Table 1 and depicted graphically in Figure 9. An ANOVA F test (which compares the variability between groups to the variability within groups) showed statistically significant differences ($p < 0.01$) among the mean stress fiber volume densities of

(continued on page 37)

(page 27 on reverse)

(Figure 5: see legend overleaf)

Figure 5: Composite picture of thin section electron micrographs of endothelium from control rat aorta and regenerated endothelium from rat aortas 1 week, 2 weeks, 3 weeks and 4 weeks following balloon injury. Note that regenerated endothelial cells at 1 week, as compared to control, appear larger, contain more organelles such as prominent Golgi apparatus and rough endoplasmic reticulum and particuarly are equipped with a large number of well developed stress fibers (asterisks). At 2 weeks, 3 weeks, and 4 weeks the endothelial cells appear still larger than control but stress fibers are less numerous than at 1 week. Note that stress fibers appear cut transversally in all micrographs as endothelial cells of all aortas have been cut transversally. x 19,000.


control
1 wk
2 wks

(page 29 on reverse)

(Figure 6: see legend overleaf)

Figure 6: Thin section electron micrograph of an endothelial cell from regenerated endothelium of rat aorta at 1 week after balloon injury showing a prominent stress fiber cut longitudinally. The stress fiber is made up of bundles of microfilaments with periodic dense bodies. The microfilaments are attached to the abluminal plasma membrane (seen here tangentionally cut) and show coaxial alignment with extracellular matrix fibers of the subendothelium. The close association between the cytoplasmic stress fibers (predominantly actin) and extracellular matrix fibers (predominantly fibronectin) has been named fibronexus in other in vivo and in vitro systems (Singer, 1979; Singer et al, 1984). X 59,000.

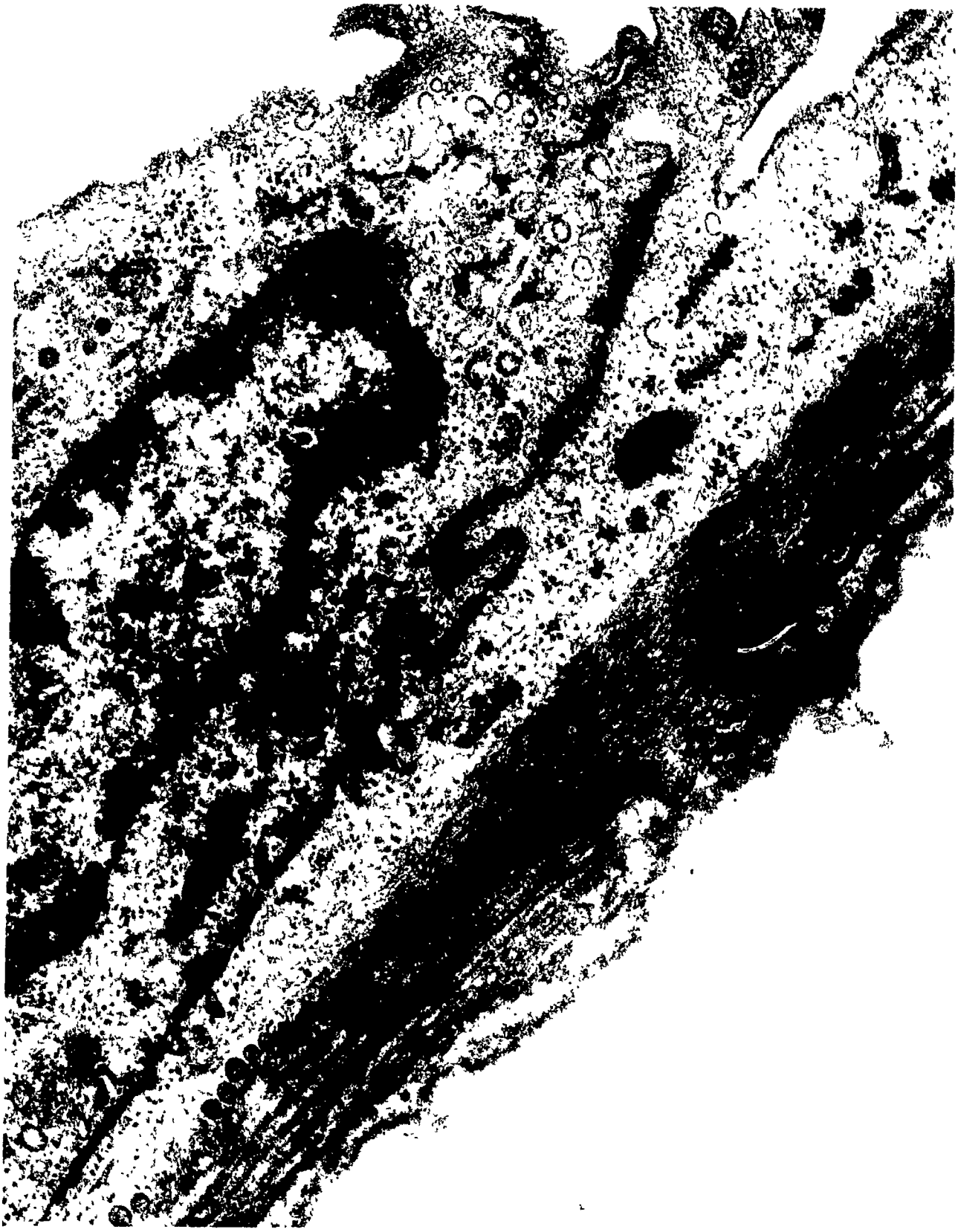
M.S. COKAY-30

(page 31 on reverse)

(Figure 7: see legend overleaf)

Figure 7: This thin section electron micrograph of endothelial cells from regenerated endothelium of rat aorta at 1 week after balloon injury shows a longitudinally cut stress fiber, similar to that seen in Figure 6. Here however, the stress fiber-membrane attachment domain is better visualized as the plasma membrane is favourably oriented. The electron dense cytoplasmic zone adjacent to the attachment domain (arrows) has been named "subplasmalemmal microfilament condensation" (Hüttner et al 1985) and is identical to "dense bands" considered to be an integral part of the force transmission apparatus in smooth muscle (Gabella 1984). Note the absence of plasmalemmal vesicles at the stress fiber-membrane attachment domain, contrasting the numerous plasmalemmal vesicles present in the endothelial plasma membrane not specialized for cell to matrix attachment. X 59,000.

M.S. COKAY-32



(page 33 on reverse)

(Figure 8: see legend overleaf)

Figure 8: Thin section electron micrograph of endothelial cells from regenerated endothelium of rat aorta at 1 week after balloon injury showing a large stress fiber cut transversally. Note cross section of extracellular matrix fibers in the subendothelium adjacent to the stress fiber. The diameter of the extracellular matrix fibers is larger than that of the microfilaments composing the stress fiber. x 93,000.

M.S. COKAY-34

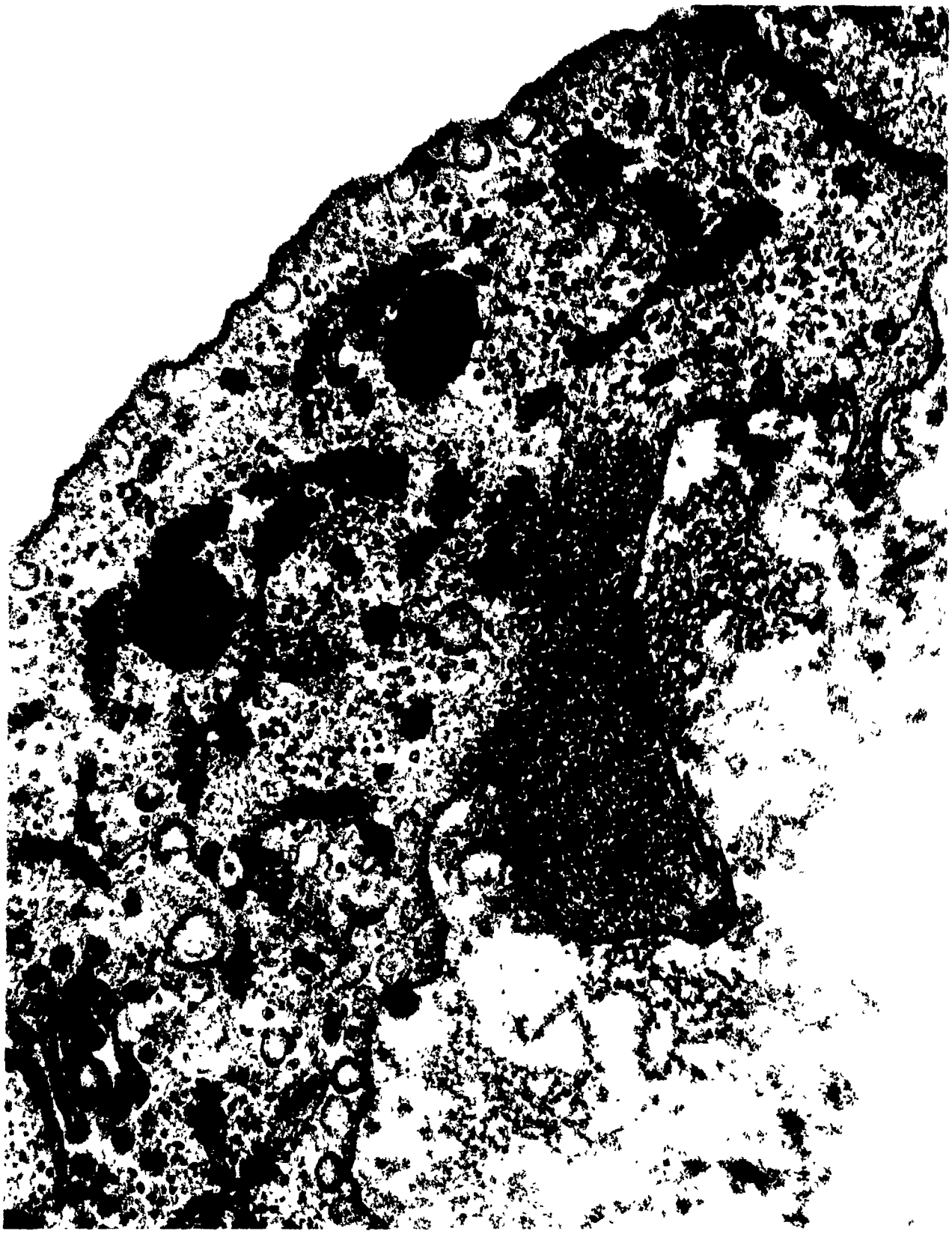


Table 1

Stress fiber volume densities in control and regenerated rat aortic endothelium following segmental balloon catheter injury in various experimental groups*.

		Volume Densities (cm°)				
			Regenerated Endothelium			
		Control	1 wk	2 wks	3 wks	4 wks
Animals	1	0.01511	0.04435	0.02122	0.02338	0.03081
	2	0.02057	0.04001	0.01230	0.00090	0.02142
	3	0.01604	0.05617	0.01249	0.02396	0.01183
Mean†		0.01724	0.04684‡	0.01534§	0.01608§	0.02135§
SEM‖		0.0020655	0.0059139	0.0036034	0.009298	0.0067105

* Stress fiber volume density is the ratio of the volume of endothelial stress fibers to the volume of endothelial cell layer.

† ANOVA F test showed significant differences among the mean stress fiber volume densities for the five groups ($P < 0.01$).

‡ Significantly increased over each of the four other means (by Tukey's method with overall significance level $\alpha = 0.05$).

§ Not significantly different from the control value (by Tukey's method with overall significance level $\alpha = 0.05$).

‖ Standard error of mean.

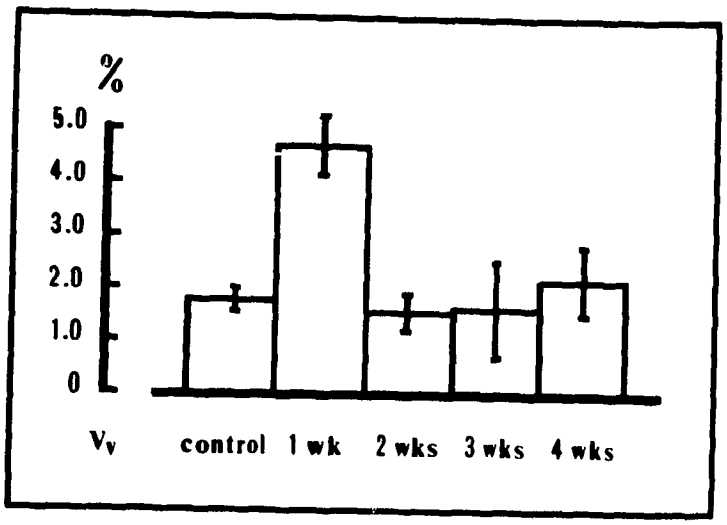


Figure 9: Morphometric data on mean stress fiber volume densities in control and regenerated rat aortic endothelium.
Based on Table 1. Bars represent ± SEM.

(continued from page 26)

the normal and regenerated rat aortic endothelium from the 5 groups tested, namely the non-operated control, 1 week, 2 weeks, 3 weeks and 4 weeks groups. This indicated that most of the total variability was due to differences between groups rather than to differences within groups, implicating true biological variation as the source of variation as opposed to measurement error variation. In order to determine what specific differences there were between the various groups, multiple pairwise comparisons of the means, applying Tukey's method, were performed.

There was a significant increase in stress fiber volume density in the regenerated rat aortic endothelium at 1 week after the segmental balloon injury as compared to the stress fiber volume density in control endothelium from non-operated animals ($p < 0.01$). The stress fiber volume density in regenerated rat aortic endothelium at 2 weeks following the aortic segmental balloon injury returned to near control value and remained low at 3 weeks and 4 weeks. These changes were reflected in statistical analysis as the mean stress fiber volume density in regenerated rat aortic endothelium at 1 week after the segmental balloon injury was significantly increased as compared to the stress fiber volume densities in regenerated endothelium at 2 weeks, 3 weeks, and 4 weeks ($p < 0.01$, $p < 0.05$ and $p < 0.05$ respectively). Furthermore, the mean stress fiber volume densities in regenerated rat aortic endothelium at 2 weeks, 3 weeks and 4 weeks following aortic segmental balloon injury were not significantly

different from the mean stress fiber volume density in control endothelium ($p > 0.05$ for all three pairwise comparisons). A second ANOVA F test excluding the 1 week group was also performed which showed no significant differences ($p > 0.05$) among the mean stress fiber volume densities for the remaining four groups (namely the control, 2 weeks, 3 weeks, and 4 weeks groups), further indicating that the 1 week group had the largest contribution in the test.

CURRENT STATE OF KNOWLEDGE AND DISCUSSION OF RESULTS

Permeability Pathways in Vascular Endothelium

The ultimate purpose of the circulatory system is to allow continuous exchange of substances between blood and tissues. Because of its strategic location, the endothelial cell layer in most areas assumes the role of a barrier regulating this exchange. In arteries, the endothelial cell layer acts as a selective permeability barrier and is essential to regulate the influx of plasma macromolecules into the the arterial wall and particularly to maintain the normal microenvironment and growth pattern of underlying smooth-muscle cells (Ross, 1986).

Structurally, a continuous endothelial cell layer provides a number of potential routes to the subendothelial space, via channels along intercellular tight junctions, via plasmalemmal vesicles and/or transendothelial channels and in the lipid phase of the endothelial plasma membrane.

Intercellular Tight Junctions. Open or leaky tight junctions are well established as pathways of water-soluble macromolecules across continuous endothelium at certain segments of the microvascular bed, notably the venous ends of the capillaries and/or in the post-capillary venules under both physiological and pathological conditions (Majno, 1965; Majno and Palade, 1961; Majno et al, 1969; Simionescu N et al, 1978). The permeability characteristics of tight

junctions in arterial capillaries (Karnovsky, 1967; Michel, 1979; Palade et al, 1979; Renkin, 1979; Simionescu N et al, 1975), and in arteries (Florey et al, 1970; Giacomelli and Wiener, 1974; Hüttner et al, 1973c; Hüttner et al, 1973d, Schwartz and Benditt, 1972; Stein and Stein, 1972), however, are a matter of some controversy. Nevertheless, it appears that the tight junctions of arterial endothelium are normally impermeable or have low permeability to macromolecules in most regions. They are, however, relatively labile structures that may widen under the influence of hemodynamic factors such as high blood pressure and possibly of vasoactive agents (Hüttner et al, 1973d; Nagy et al, 1979; Thorgeirsson and Robertson, 1978).

The leakiness of the endothelium in the venous capillaries and post-capillary venules to macromolecules correlates with the presence of single-stranded and discontinuous tight junctions, while the relative impermeability of the endothelium in the arterial end of capillaries and of arterioles correlates with the presence of multistranded and continuous belts of tight junctions between adjacent endothelial cells in these vascular segments (Simionescu M et al, 1975; Simionescu N et al, 1978). Structurally discontinuous, mechanically weak tight junctions between adjacent endothelial cells in the venous ends of the microcirculation may provide sites for a small number of leaks allowing the low level non-selective passage of macromolecules across the endothelial cell layer under normal conditions (Bundgaard and Frøkjaer-Jensen, 1982). They are obvious sites of endothelial cell separation which results in a large increase

in protein efflux and elevated lymph-to-plasma total protein ratio in inflammation (Grega, 1986). This view of microvascular permeability is supported by recent physiological data (Grega, 1986; Grega et al, 1982; Olesen and Crone, 1984; Olesen and Crone, 1986) and is consistent with the notion that all transcapillary solute transport occurs by passive processes in accordance with the original pore theory (Bundgaard et al, 1979; Pappenheimer, 1953).

In large arteries, such as the aorta, the complexity of endothelial tight junctions lies generally between "leaky" venous-type and "tight" capillary-type tight junctions, being characterized by mostly continuous single- or double-stranded junctional belts (Hüttner et al, 1973b; Hüttner et al, 1973c; Hüttner et al, 1982; Hüttner and Peters, 1978; Hüttner et al, 1985; Simionescu M et al, 1976). There is, however, considerable heterogeneity in tight junctional structure from cell to cell even within the same aortic segment, with discontinuous tight junctions also occurring focally in normal aortic endothelium (Hüttner et al, 1982; Hüttner and Peters, 1978). Cell junction heterogeneity correlates with areas of different permeability in this cell layer as evidenced by preferential labelling of some interendothelial clefts following in vivo injection of ultrastructural tracers both under normal and under pathophysiological conditions (Hüttner et al, 1973b; Hüttner et al, 1973c; Hüttner et al, 1973d). It correlates also with observations made on aortic endothelium following addition of ruthenium red, after fixation of the endothelial cell layer. This cationic extracellular tracer was observed to

penetrate the entire length of intercellular spaces between some endothelial cells but it was stopped by tight junctional elements between other endothelial cells (Martinez-Palomo, personal communications). Preferential penetration of some interendothelial clefts by silver salts has also been observed in aortic endothelium (Majno et al, 1985; Zand et al, 1982). Thus, large-vessel endothelium with its "tight" and "leaky" regions seems to mirror the situation in the microcirculation with its juxtaposed "tight" and "leaky" segments, where the mechanically weak "leaky" regions react primarily to various hemodynamic and chemical stimuli (Hüttner et al, 1973d).

Plasmalemmal Vesicles and/or Transendothelial Channels. The large population of plasmalemmal vesicles in continuous endothelium is its most conspicuous anatomical feature (Bruns and Palade, 1968; Wagner and Robinson, 1984). It has been suggested, on the basis of studies with fine structural tracers, that these vesicles and/or transendothelial channels represent the sole avenue for exchange of water-soluble macromolecules across capillary endothelium under physiological conditions (Palade et al, 1979; Simionescu N, 1983; Simionescu N et al, 1975). It has further been suggested that vesicular transport across the microvascular endothelium occurs in the perfused rat heart (Boyles et al, 1981) and in frog mesenteric capillaries (Clough and Michel, 1981; Turner et al, 1983) and vesicular transport has also been implicated as a mechanism responsible for increased permeability of arterial endothelium in

various pathophysiological situations (Thorgeirsson and Robertson, 1978).

Recent investigations have identified a number of distinct properties of the membrane of endothelial plasmalemmal vesicles, including the paucity or absence of anionic sites detected by cationized ferritin binding, suggesting that these structures may favour the penetration of anionic molecules (such as the majority of plasma proteins) for diffusion or vesicular transport (Simionescu M et al, 1985), the high concentration of lectin receptors (Simionescu M et al 1982) and of binding sites for albumin (Ghitescu et al, 1986) as well as the presence of a specific antigen, detected by its monoclonal antibody (Schlingemann et al, 1985) only on vesicles and not on the adjacent plasmalemma proper. A characteristic striped surface structure has been also observed on the cytoplasmic aspect of plasmalemmal vesicles, distinct from that of coated pits and plasmalemma proper (Peters et al, 1985). Quick-freeze, deep-etch studies (Bearer et al, 1985) have identified furthermore an internal structure in the diaphragm of plasmalemmal vesicle, similar to that of fenestral diaphragms (Clementi and Palade, 1969). All these data seem to rule out derivation of plasmalemmal vesicles by random invaginations from the plasmalemma and suggest their potential role in macromolecular transport. Vesicular transport of various plasma constituents such as low-density lipoproteins (Vasile et al, 1983), glycosylated albumin (Williams et al, 1981), albumin and fibrinogen (Bendayan, 1980; Yokota, 1983), transferrin (Jefferies et al, 1984)

and insulin (King and Johnson, 1985) has been suggested in various in vitro and in vivo systems.

Although these data certainly implicate plasmalemmal vesicles in receptor-mediated transfer of ligands across vascular endothelium and are consistent with observations on the uptake at the luminal surface and discharge at the tissue front of cationized ferritin by vesicles, the role of plasmalemmal vesicles and/or transendothelial channels in passive, non-selective permeation of macromolecules across the endothelial cell layer is questionable. Studies using a rapid freezing-substitution method of fixation suggest that the number of plasmalemmal vesicles and the fusion of vesicles to form transendothelial channels may be related to a slow influence of chemical fixatives (Mazzone and Kornblau, 1981; McGuire and Twietmeyer, 1983; Robinson et al, 1984; Wagner and Andrew, 1985; Wagner and Robinson, 1982). An extreme example illustrating how chemical fixatives may effect the plasma membrane is the extensive vesiculation that occurs, including formation of plasmalemmal vesicles and transendothelial channels in endothelial cells fixed with glutaraldehyde, in the presence of the membrane detergent dimethylsulphoxide. The electron microscopic studies of frog mesenteric capillaries with tannic acid as a mordant imply that most of the endothelial vesicles are permanent or semipermanent structures which may be labelled by macromolecular tracers through diffusion rather than through transport (Bundgaard, 1980; Bundgaard and Frøkjaer-Jensen, 1982; Bundgaard et al, 1979; Bundgaard et al, 1983;

Frøkjaer-Jensen, 1980). In this context, extensive labelling of endothelial vesicles by ultrastructural tracer molecules reported in various pathophysiological situations may imply that these tracers diffused into the vesicles from the subendothelial space after they had traversed focally opened endothelial tight junctions (Hüttner and Gabbiani, 1982).

Lipid Phase of the Endothelial Plasma Membrane. Diffusion in the lipid phase of the endothelial cell membrane has been proposed as a mechanism of lipid transport across continuous endothelium (Scow et al, 1976; Scow et al, 1980). According to this model of transport, the relatively water-insoluble products of lipolysis (diglycerides, monoglycerides and free fatty acids) would preferentially dissolve in the outer layer of the endothelial plasma membrane. Having entered this lipid phase, they could rapidly diffuse along the cell surface to reach the abluminal side, where binding to extracellular matrix or transfer to other cells might occur (Scow et al, 1976; Scow et al, 1980). Lipoprotein lipase, an enzyme which hydrolyses the triglyceride component of plasma chylomicrons, appears to be bound to the glycoprotein coat of the luminal endothelial surface (Dicorleto and Zilversmit, 1975), thus this hypothetical scheme indicates the potential for active involvement of endothelium in the transport of complex lipids into the arterial wall. Another mechanism whereby lipoproteins may cross the endothelial cell layer is receptor-mediated uptake and discharge (Vasile et al, 1983) by the endothelial cells as noted above. In this context, receptor density on the endothelial

cell surface may influence lipid deposition in the arterial wall under various pathophysiological conditions (Day et al, 1985). The fate of plasma lipids and other macromolecules which have reached the subendothelial space may also be influenced by endothelium-associated connective tissue elements. For example, certain glycosaminoglycans can selectively bind low-density lipoproteins (Iverius, 1972), thus changes of intimal glycosaminoglycans synthesized by the endothelial cells and/or smooth muscle cells may be an important factor contributing to intimal lipid accumulation (Alavi et al, 1983; Alavi et al, 1989; Falcone et al, 1984; Kinsella and Wight, 1986; Minick et al, 1977; Moore, 1989; Moore et al, 1982; Moore and Richardson, 1985; Richardson et al, 1980).

As it is reflected in the discussion above, there is no generally accepted transport pathways across vascular endothelium. However, currently available information is consistent with the view that while normal rat aortic endothelium transports slight amounts of proteins through plasmalemmal vesicles, the overall permeability of the endothelial cell layer to water-soluble substances including proteins depends on the complexity of tight junctions interconnecting adjacent endothelial cells. There is heterogeneity in tight junction structure between the endothelium of various normal rat arterial segments and considerable heterogeneity in tight junction structure from cell to cell even within the same normal rat aortic segment. Cell junction heterogeneity correlates well with areas of different permeability in rat aortic endothelium both in normal and pathophysiological

conditions (Hüttner et al, 1973b; Hüttner et al, 1973c; Hüttner et al, 1973d; Hüttner et al 1989).

Permeability of Regenerated Aortic Endothelium

There are only a few reported studies dealing with permeability characteristics of regenerated aortic endothelium. Using EB-protein complex as a marker of denudation and re-endothelialization various investigators consistently demonstrated that there is no difference of permeability to EB between the non-denuded normal and regenerated rabbit aortic endothelium (Stemerman et al, 1977; Stemerman, 1981) although some studies describe areas of increased permeability to EB in areas with presumed increased hemodynamic stress, particularly in the aortic arch . Using HRP as a more sensitive marker for endothelial permeability on en face aortic preparations, one study demonstrated focal areas of increased permeability appearing as brown spots both in normal and regenerated rabbit aortic endothelium, however, the percentages of surface areas spotted brown were not significantly different between normal and regenerated endothelium (Stemerman, 1981).

As far as the structural aspects of regenerated rat aortic endothelium is concerned, morphometric studies using freeze-fracture electron microscopy (during regeneration that follows experimental balloon denudation in a setting similar to the studies presented here) furnished evidence that tight junctions acquire an increased degree of

complexity in regenerated as compared to normal rat aortic endothelium. The increased complexity of tight junctions in regenerated endothelium was interpreted as a reflection of increased endothelial cell turnover. It is well established that endothelial cells following injury move, while maintaining a monolayer sheet; this probably requires a continuous breakdown and rebuilding of their junctions (Hüttner et al, 1985). Although variation in the number of junctional elements provides up to now the best correlation with observed differences in tight junction permeability (Claude and Goodenough, 1973; Easter et al, 1983; Friend and Gilula, 1972; Simionescu N et al, 1978), the functional significance of these findings was not clear as these structural studies were not accompanied by permeability studies on the regenerated endothelium. Also these structural changes in regenerated endothelium were not followed in time-sequence studies.

The studies presented in this thesis were designed to compare the permeability of the normal and regenerated rat aortic endothelium to intravenously injected EB and HRP in en face aortic preparations at various time points during regeneration that follows balloon denudation of rat aortic endothelium.

The results of these studies indicated that the permeability of the re-endothelialized areas to both EB and HRP was identical (at the level of the sensitivity of techniques utilized i.e. gross evaluation, unaided and under a magnifying lens, of en face aortic preparations) to the endothelium of non-operated control aortas as well as of the

non-denuded segments of the experimental aortas in all time points studied from the early stages of regeneration to complete re-endothelialization of the denuded aortas, that is from 1 week to 4 weeks following ballon injury. These observations suggest that healing in vascular endothelium following a single denudation injury results in an endothelial monolayer that acquires and maintains normal permeability to water-soluble substances. The results of the time-sequence studies presented here are in agreement with those of others testing regenerated aortic endothelium at a single time point with intravenously injected EB and/or HRP (Stemerman et al, 1977; Stemerman, 1981), and also provide indirect support for the view that the endothelial monolayer during the process of regeneration may maintain normal permeability in the face of high cell turnover by increased turnover and complexity of the tight junctions interconnecting neighboring cells in the monolayer (Hüttner et al, 1985). The overall increase in tight junction complexity probably compensates for leaky sites that may occur in increased numbers in regenerated as compared to normal aortic endothelium (Hüttner et al, 1985; Schwartz et al, 1975).

These results do not necessarily contradict the reported increase in permeability of re-endothelialized intima to lipoproteins as compared to normal rabbit intima (Day et al, 1985; Schwenke and Zilversmit, 1989). Diffusion in the lipid phase of endothelial cell membrane (Scow et al, 1976) or receptor-mediated uptake and discharge (Vasile et al, 1983) depending on receptor density on the endothelial

cell surface (Day et al, 1985) may play a role in lipoprotein transport during the process of regeneration independently of pathways available to water-soluble substances.

Stress Fibers in Normal and Regenerated Rat Aortic Endothelium

Stress fibers are bundles of actin filaments containing myosin and alpha-actinin that are found in many cultured cells, but they are present only in vascular endothelial cells among non-muscle cells in living organisms; their function is probably related to isometric contraction and adhesion (Abercrombie and Dunn, 1975; Buckley and Porter, 1967; Burridge, 1981; Byers and Fujiwara, 1982; Byers et al, 1984; Fujiwara and Pollard, 1976; Fujiwara et al, 1986; Geiger et al, 1984b; Gordon et al, 1982; Lazarides and Weber, 1974; Lloyd et al, 1977; Mangeat and Burridge, 1984; Norton and Izzard, 1982; Weber and Greoschel-Stewart, 1974; Wong et al, 1983). Stress fibers have also been called central actin microfilament bundles as opposed to the network of actin microfilaments located at the periphery of endothelial cells (Kim et al, 1989). Stress fibers are relatively rare in normal vascular endothelium in vivo (Gabbiani et al, 1983), but are more prominent in endothelial cells of regions exposed to high velocity flow, such as the left ventricle, aortic valve and aorta (White and Fujiwara, 1986; Wong et al, 1983). Even within the aorta there are marked differences in stress fiber expression between the thoracic and abdominal segments, the stress fibers being more numerous

in the abdominal aorta where branching is more pronounced that could lead to more non-laminar flow (White et al, 1988; White and Fujiwara, 1986). Furthermore, in the thoracic segment they occur mainly in endothelial cells located immediately below intercostal artery branches, sites obviously subjected to different flow pattern than the rest of the aortic luminal surface (Gabbiani et al, 1983).

Stress fibers in vascular endothelial cells in vivo become numerous during hypertension (Gabbiani et al, 1975; Gabbiani et al, 1979; White and Fujiwara, 1986; White et al, 1983), in areas of experimentally elevated shear stress (Kim et al, 1989) and develop in most of the aortic endothelial cells during the regeneration that follows experimental endothelial denudation (Gabbiani et al, 1983; Hüttner et al, 1985). This implies that stress fibers play a role in increased cellular adhesions to the subendothelial matrix during increased hemodynamic stress (as in branching regions of normal arteries and during hypertension in general) or possibly when the compositon of subendothelial matrix is altered, say during endothelial regeneration in vivo or in culture conditions (Hüttner et al, 1989). While stress fibers in vivo are oriented axially in the direction of the blood flow; stress fibers in cultured endothelial cells run both circumferentially along the cell boundary and across its width (Gotlieb et al, 1984; Rogers and Kalnins, 1983; Wong and Gotlieb, 1988). These changes in the cytoskeleton may be due either to the lack of proper subendothelial matrix components or the lack of blood flow or both under culture conditions. The role of subendothelial

matrix composition is further suggested by marked species differences in stress fiber expression. It is well established that the rabbit aortic endothelium contains significantly more stress fibers than that of the rat. Different organization of the subendothelial matrix in the rat and rabbit has been documented (White et al, 1984) and implicated, in addition to presumed differences in normal hemodynamic states between these two species (White et al, 1988).

It has also been postulated that stress fibers may play a role in cellular migration in situ (Gordon et al, 1982). This suggestion, however, conflicts with data from in vitro studies (Herman et al, 1981; Lewis et al, 1982) whereby it was demonstrated that stress fiber expression and cell migration are inversely related. A recent in vivo study correlates with the in vitro observations by demonstrating that the extent of stress fiber expression in the regenerating endothelium is dependent upon the location of the endothelial cells in the regenerating sheet; there is a reduced stress fiber expression at the leading edge of the regenerating sheet as compared to the rest of the regenerating endothelium (White et al, 1988). While these in vivo observations give further support for the inverse correlation between migration and stress fiber expression inferred from in vitro studies, they do not contradict the well established observations that during the early stages of regeneration endothelial cells contain a strikingly increased number of stress fibers as compared to normal endothelium (Gabbiani et al, 1983; Hüttner et al, 1985; White et al, 1988).

Stress fibers in vivo are connected exclusively to the abluminal plasma membrane of endothelial cells (Huttner et al, 1985). At the site of stress fiber attachment there is an electron dense cytoplasmic zone adjacent to the endothelial plasma membane that has been called "subplasmalemmal microfilament condensation" (Hüttner et al, 1985) (see figure 7). Subplasmalemmal microfilament condensations at the abluminal endothelial plasma membrane occur without associated stress fibers as well, both in normal and regenerated endothelial cells (Hüttner et al, 1985). The quantification of stress fiber related and unrelated subplasmalemmal microfilament condensations shows that the total surface density of these structures does not change in regenerated as compared to normal aortic endothelium, thus suggesting that endothelial cells are normally equipped with structures to which stress fibers can be connected when they increase in numbers under stressful conditions (Hüttner et al, 1985).

Subplasmalemmal microfilament condensations are also seen at the lateral endothelial cell membrane in association with tight junctions (Martinez-Palomo et al, 1980; Pinto da Silva and Kachar, 1982; Shasby et al, 1982; Hüttner et al, 1985), however, these are never associated with stress fibers in vivo (Hüttner et al, 1985). Subplasmalemmal microfilament condensations at the abluminal endothelial cell plasma membrane are identical to "focal contacts" with the substratum described in various cultured cells (Abercrombie and Dunn, 1975; Heath and Dunn, 1978; Izzard and Lochner, 1976) and also to "dense bands" considered to be an integral part of force transmision apparatus in

smooth muscle (Gabella, 1984). It is interesting to note that membrane domains specialized for stress fiber attachment are different from the rest of the endothelial plasma membrane; they are resistant to formation of filipin-sterol complexes suggesting that they are probably too rigid to be easily deformed (Hüttner et al, 1985). Furthermore, intracytoplasmic actin and actin-associated proteins, particularly alpha-actinin and vinculin as well as concomitant extracellular fibronectin have been localized at cell to matrix attachment sites in various in vitro and in vivo systems (Byers and Fujiwara, 1982; Chen and Singer, 1982; Couchman et al, 1983a; Couchman et al, 1983b; Geiger, 1979; Geiger et al, 1984a; Geiger et al, 1980; Hay, 1981; Herman et al, 1984; Hynes, 1981; Hynes and Yamada, 1982; Kleinman et al, 1981; Singer, 1982; Singer and Paradiso, 1981; Tomasek et al, 1982). Recently an integral membrane protein associated with sites of microfilament-membrane attachment has been identified (Rogalski and Singer, 1985). Alpha-actinin is an actin-associated protein of molecular weight of 100, 000; it seems to act as a cross-linker and a spacer between actin filaments (Jockusch and Isenberg, 1981). A number of proteins are known to accumulate at sites of actin-membrane association where cells adhere to substrates (Weeds, 1982). Vinculin, intracellular protein with molecular weight of 130, 000 (Geiger, 1979) and the vinculin-binding protein talin (Burridge, 1981; Burridge and Connell, 1983), are found at focal contacts or adhesion plaques of non-muscle cells and also at the dense bands (but not at the dense bodies) of smooth muscle cells (Geiger et

al, 1980). These proteins localized at the cytoplasmic side of the cell membrane have been implicated in the linkage of actin filaments to the cell membrane although the structural nature of the linkage is not clear (Beckerle, 1986); a direct interaction between α-actinin and vinculin (Craig, 1985) and the role of an 82, 000 molecular weight protein (Beckerle, 1986) have been recently suggested. Fibronectin, a 200, 000 molecular weight glycoprotein, has been shown to mediate adhesion of the cell membane to the stroma in various in vitro and in vivo systems (Hynes and Yamada, 1982). Fibronectin has an affinity for collagen, glycosaminoglycans and also for the surface of many cell types; it also has a tendency towards self-association, which can result in the formation of fibrils. Studies using monoclonal antibody technology suggest that the fibronectin receptor is a transmembrane glycoprotein complex (Brown and Juliano, 1986; Hasegawa et al, 1985; Knudsen et al. 1985; Rogalski and Singer, 1985) that has an extracellular binding site for fibronectin (Horwitz et al, 1985) in addition to a domain that interacts with the vinculin-binding protein, talin (Horwitz et al, 1986). The transmembrane linkage of talin with the extracellular matrix via the fibronectin receptor provides one mechansism by which cytoplasmic components can become functionally coupled to extracellular matrix (Beckerle, 1986). These data suggest complex but specific interactions between cytoplasmic actin filaments and extracellular fibronectin fibers across the plasma membrane at cell-to-matrix attachment sites.

The microfilaments of the stress fibers attached to the abluminal endothelial plasma membrane show coaxial alignment with extracellular matrix fibers of the subendothelium (see figure 6). Using immunofluorescence and immunoelectron microscopy, similar extacellular matrix fibers have been shown in transformed fibroblasts and in myofibroblasts of granulation tissue in vivo to consist of fibronectin and the close association between bundles of cytoplasmic actin filaments (stress fibres) and bundles of extracellular fibronectin microfibrils has been named the "fibronexus", a structure specialized for increased cell-to-matrix adhesion (Singer, 1979; Singer et al, 1984). As discussed above, aortic endothelial cells also develop abundant stress fibers and adhesive structures, structurally identical to fibronexus in situations requiring their increased attachment to the subendothelium such as in hypertension (Gabbiani et al, 1975; Gabbiani et al, 1979; White and Fujiwara, 1986; White et al, 1983), in experimental conditions with elevated shear stress (Kim et al, 1989) and particularly during the regeneration that follows endothelial denudation (Gabbiani et al, 1983; Hüttner et al, 1985). While it is relatively easy to understand the need for an increased endothelial cell attachment to the subendothelial matrix (and consequently an increased number of stress fibers) in conditions with increased hemodynamic stress, the reason for a strikingly increased number of stress fibers in endothelial cells during regeneration is not clear. Altered matrix composition could explain the large number of stress fibers consistently present in cultured cells, and has been implicated

in marked species differences in stress fiber expression as discussed above.

In analogy to these situations, it is conceivable that increased number of stress fibers is needed for endothelial cell adhesion to the subendothelial matrix because the composition of the subendothelial matrix is altered during regeneration that follows balloon injury. It is unlikely that the increased number of stress fibers is related to an increased replication rate and movement in regenerating endothelium (White et al, 1988). Although a significant increase in stress fiber expression of regenerated as compared to normal endothelium is well established (Gabbiani et al, 1983; Hüttner et al, 1985), there have been no quantitative time-sequence studies on the frequency of stress fibers in aortic endothelial cells during the process of regeneration that follows endothelial denudation. A recent study, published during the writing of this thesis, has demonstrated, qualitatively by immunofluorescence microscopy, reversible changes in stess fiber expression in regenerated rat aortic endothelium (White et al, 1978).

The present studies were designed to evaluate quantitatively the frequency of stress fibers in time-sequence from the early stages of regeneration to complete re-endothelialization of segmentally denuded rat aortas. Statistical analysis of morphometric data on volume densities of stress fibers indicated a significant increase in stress fiber volume densities in regenerated endothelium at 1 week after segmental balloon injury as compared to that of normal endothelium. The stress fiber volume density in regenerated aortic endothelium at 2

weeks following segmental balloon injury returned to near normal value which was not significantly different from that of the normal aortic endothelium. Furthermore, stress fiber volume densities at 3 weeks and 4 weeks following segmental balloon injury remained near normal values which were again not signficantly different from that of the normal aortic endothelium. These observations suggest that healing in vascular endothelium following a single denudation injury results in an endothelial monolayer that acquires an increased number of stress fibers at the early stages of regeneration; however, the phenomenon of increased stress fiber expression during the process of regeneration is transient.

These results are in agreement with previous reports indicating the presence of an increased number of stress fibers in aortic endothelial cells during the regeneration that follows experimental endothelial denudation (Gabbiani et al, 1983; Hüttner et al, 1985). They correlate well and give quantitative support, furthermore, to the recent immunofluorescence study, published during the writing of this thesis, which demonstrated reversible changes in stress fiber expression in regenerated rat aortic endothelium (White et al, 1988). The transient increase in stress fiber expression may reflect a temporary demand for greater adhesive capabilities of endothelial cells until the subendothelial extracellular matrix is remodeled during the regeneration process following balloon injury. The significant increase in stress fiber expression demonstrated at 1 week in the current study contrasts the significant increase in stress

fiber expression demonstrated at 2 weeks in a previous report (Huttner et al, 1985). This discrepancy may be related to differences in experimental techniques of endothelial denudation (short versus long aortic segments) and sampling for electron microscopy (sampling from fixed site versus sampling near the advancing edge of regenerating endothelium). Although, the results presented here are based only on the evaluation of the relative volumes of stress fibers in endothelial cell layers, the direction of changes implicates that increase in stress fibers in regenerating vascular endothelium that follows a single injury is transient in nature.

On the basis of the results presented in this thesis and those of others elaborated in the discussion, it may be hypothesized that vascular endothelium attempts to maintain its structural and functional integrity during the process of regeneration on one hand by forming more complex tight junctions to maintain adequate cell to cell interactions and normal permeability in the face of high cell turnover during the early stages of endothelial regeneration and on the other hand by developing an increased number of stress fibers to provide adequate cell to matrix interactions in the face of a probably defective subendothelial matrix during the early stages of regeneration. It may be presumed that increased tight junction complexity returns to normal in parallel with the establishment of normal cell turnover in a quiescent endothelial monolayer upon completion of regeneration, in analogy to the transient nature of increased stress fiber expression that provides adequate cell to

matrix adhesion until the subendothelial matrix is remodeled to its normal state upon completion of regeneration.

Although the data presented in this thesis represent only two selected aspects of regenerated endothelium, they are consistent with the view that changes in regenerating vascular endothelium are transient in nature and the structural and functional integrity of the endothelial monolayer is eventually reestablished upon the completion of regeneration following a single injury.

SUMMARY AND CONCLUSIONS

Endothelial cells line insides of arteries, veins, capillaries and lymphatics as a monolayer. Continuity of this monolayer, particularly in arteries, is vital to maintain all major properties of endothelium such as metabolic activity, thromboresistant surface and barrier function. These properties of the endothelial monolayer, in turn, are crucial to normal functioning of blood vessel wall. Therefore, it is important to answer the question whether the repair process in vascular endothelium that follows injury results in an endothelial monolayer that acquires normal structure and function or it results in an endothelial monolayer that is defective in some way thus promoting vascular disease.

Cell to cell interactions and cell to matrix interactions were essential in maintaining the continuity of the endothelial monolayer. In the studies presented in this thesis, one aspect of each of these interactions were selected to evaluate the function of this cell layer during the process of regeneration that follows denudation injury: (1) the permeability of the normal and regenerated aortic endothelium to EB and HRP was studied in time-sequence following segmental denudation in en face rat aortic preparations as a reflection of cell to cell interactions in the endothelial monolayer, (2) the volume density of stress fibers in normal and regenerated rat aortic endothelium was studied in time-sequence following segmental denudation by morphometric analysis using thin section electron microscopy as a reflection of cell to matrix interactions in the

endothelial monolayer.

The current state of knowledge on the permeability pathways in vascular endothelium and on the morphology and function of stress fibers in normal and pathophysiological conditions were reviewed and the results of the experimental work presented in this thesis were correlated with the available relevant information.

The results of the experiments presented here indicated that: (1) the permeability of the regenerated endothelium to both EB and HRP was identical to normal endothelium in all time points studied from the early stages of regeneration to complete re-endothelialization of the denuded aortas, implicating that the endothelium maintains normal permeability to water soluble substances during the process of regeneration that follows a single denudation injury, (2) stress fiber volume density was significantly increased in regenerated endothelium at 1 week after segmental balloon injury as compared to that of normal endothelium; however, stress fiber volume density returned to and remained at normal value at and after 2 weeks following segmental balloon injury, indicating the transient nature of increased stress fiber expression during the process of regeneration.

The results presented in this thesis, although representing only two selected aspects of cell to cell and cell to matrix interactions in regenerated endothelium, are consistent with the view that structural and functional changes in regenerated vascular endothelium, if present, are transient in nature and the integrity of the endothelial monolayer is eventually reestablished during the repair process that follows a single injury.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

(1) The observations that the permeability of regenerated rat aortic endothelium to EB and HRP are identical to that of normal endothelium at all time points during the process of regeneration following a single injury [supporting previous observations made in rabbit aortic endothelium at a single time point of regeneration (Stemerman, 1981)].

(2) Quantitative evidence for the transient nature of increased stress fiber expression in regenerated rat aortic endothelium [supporting recent qualitative observations made by immunofluorescence microscopy and published during the writing of this thesis that the changes in stress fiber expression are reversible in regenerated endothelium (White et al, 1988)].

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