Endothelial cell regrowth and morphology after balloon catheter injury of alloxan-induced diabetic rabbits

Natalie K. Schiller, Alvin M. Timothy, Janet C. Rice, Donald L. Akers, Philip J. Kadowitz, and Dennis B. McNamara. Endothelial cell regrowth and morphology after balloon catheter injury of alloxan-induced diabetic rabbits. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H740–H748, 1999.—Neointimal thickening after catheter injury has been reported to be influenced by the integrity of the vascular endothelium. We have previously shown that neointimal thickening is significantly reduced in alloxan-induced diabetic New Zealand White rabbits after catheter injury compared with euglycemic rabbits. In the present study, it was hypothesized that endothelial cell regrowth, morphology, and endothelium-dependent vasoreactivity after catheter injury are improved in the diabetic rabbit (glucose \( \geq 400 \text{mg/dl} \)) compared with the euglycemic rabbit. Two weeks after catheter injury, the percent endothelial regrowth was significantly increased in diabetic animals compared with euglycemic animals (32.1 \( \pm 2 \) and 15.6 \( \pm 1 \), respectively; \( P < 0.05 \)). The endothelial cell morphology analyzed by scanning electron microscopy was also restored 2 wk after catheter injury in thoracic aortas from the diabetic animals compared with vessels from euglycemic animals. Endothelium-dependent relaxation to ACh in vessels from diabetic and euglycemic rabbits was attenuated 2 wk after injury, and, although improved by 4 and 8 wk, relaxation remained significantly depressed. These results suggest that endothelial cell regrowth and morphology in diabetic animals was improved compared with euglycemic animals; however, endothelium-dependent vasoreactivity remained impaired. Thus the attenuated neointimal thickening seen in the diabetic rabbit may be a function of the rate and degree of regrowth rather than the normalization of ACh-induced relaxation.

Neointimal thickening; nitric oxide; acetylcholine-induced relaxation; vascular smooth muscle cells; hyperglycemia.

The physiological and anatomical integrity of the endothelium has been reported to be critical in atherogenesis and neointimal thickening vascular injury (24, 25). The endothelium functions as a thrombo-resistant barrier between underlying vascular smooth muscle cells (VSMC) and mitogens or chemoattractants within the circulation (6, 10, 14, 30). Endothelial denudation reveals subendothelial, proaggregatory structures that, combined with the loss of endothelium-derived, antiaggregatory factors, promote the formation of a mural thrombus. Activated platelets at the site of injury degranulate, releasing a large number of procoagulant, vasoconstrictive, and mitogenic substances, such as platelet-derived growth factor, insulin-like growth factor I, thromboxane A2 (TXA2), thrombin, serotonin, von Willebrand factor, and ADP. These substances promote aggregation, leading to further platelet deposition, activation, and, ultimately, mural thrombus formation.

The accumulation of mitogens at the site of injury activates quiescent VSMC, transforming them from the normal contractile phenotype to one that is more proliferative and secretory. VSMC migrate through breaks in the internal elastic lamina to the intima where they proliferate and synthesize extracellular matrix. The increased production of mitogens combined with the loss of endothelium-derived inhibitory substances tips the balance toward uncontrolled neointimal growth (34). The net result is an increasing neointimal mass that decreases the luminal diameter and patency of the vessel, contributing to ischemic symptoms.

Neointimal thickening after catheter injury has been suggested to be directly related to the integrity of the vascular endothelium. Delayed reendothelialization has been suggested to have a permissive impact on VSMC proliferation. Studies have shown that, in areas where the endothelial lining rapidly regenerated after injury, neointimal thickening was less marked than in areas where regeneration occurred later (14, 30). This is presumably due, in part, to the effect of the endothelial layer on the underlying VSMC. The endothelium modulates the responses of VSMC by releasing substances, such as nitric oxide (NO; see Refs. 10 and 23) and prostacyclin, which have inhibitory influences on VSMC proliferation and mitogenesis (11, 28) as well as on platelet adhesion and aggregation (22).

We have previously shown that neointimal thickening after catheter injury is significantly reduced in diabetic animals compared with euglycemic animals (27). In the present study, it was hypothesized that the decreased neointimal thickening present in the diabetic rabbit may be due in part to an increase in the rate or degree of endothelial regrowth and/or a normalization of the endothelial cell phenotype. It was found that, after catheter injury, vessels from diabetic animals had greater endothelial regrowth and a normalization of endothelial cell morphology compared with euglycemic animals; however, endothelial cell function remained impaired. These results suggest that the restoration of anatomical integrity and factor VIII-related antigen immunoreactivity of the endothelium does not necessarily coincide with the recovery of the physiological function of the endothelial cells. Further-
more, these results support the suggestion that reendothelialization upon vascular injury provides a mechanism by which neointimal thickening may be inhibited and extend the observation to the alloxan-induced diabetic animal. These data provide additional support for the use of agents that facilitate endothelial cell growth in the prevention of fibroproliferative disorders.

**METHODS**

Male New Zealand White rabbits (3.0–3.5 kg) were treated with alloxan monohydrate (125–175 mg/kg iv) to induce the diabetic state, and glucose levels were checked routinely using an Accu-check III blood glucose monitor. Blood glucose levels of alloxan-treated animals were monitored for at least 6 wk after initial treatment. Rabbits with mean blood glucose value ≥400 mg/dl were defined as being diabetic. This is consistent with the literature (3).

Vascular injury was produced using a 4-Fr embolectomy catheter. Rabbits were anesthetized using a mixture of ketamine and xylazine (50 mg/kg im ketamine; 10 mg/kg im xylazine). Nitrous oxide was used as an inhalational anesthetic, and pentobarbital sodium was given (25 mg/kg iv). The superficial femoral artery was isolated through a short incision in the right groin. The catheter was inserted through an arteriotomy and was passed to the level estimated to be the ascending aorta. The balloon was inflated with saline and allowed to equilibrate under 4 g tension for 60 min before contraction with high-potassium Krebs solution (120 mM KCl). After a period of 10 min and when maximal contraction was attained, the high-potassium solution was washed out, and rings were allowed to reequilibrate. Phenylephrine (0.1, 0.2, or 0.3 μM) was used to precontract the rings to 60–85% of the maximal tension generated in response to the high-potassium solution. Responses to ACh and nitroglycerin (NTG) were expressed as change in relaxation in grams from 60–85% of the maximal tension generated in response to the high-potassium solution. Responses to ACh and nitroglycerin (NTG) were expressed as change in relaxation in grams. In those studies, indomethacin inhibited the vascular responses to arachidonic acid, and SQ-29,548 inhibited the vascular responses to U-46619 in the cat.

For light microscopy, rings of thoracic aorta were immersed in 30% sucrose in 0.1 M phosphate buffer overnight at 4°C for cryoprotection. Frozen sections were cut (40 μm) and mounted on glass microscope slides. For immunohistochemical localization of factor VIII, the conventional avidin-biotin complex technique was used. 3,3'-Diaminobenzidine was used as a chromagen to visualize the antigen-antibody complex. The IgG fraction of goat anti-human factor VIII serum (Incstar, Stillwater, MN) was used at dilution of 1:1,000. As a control, the primary antibody was replaced with the IgG fraction of normal goat serum at the same dilution. For scanning electron (SE) microscopy, tissues were fixed in 3% glutaraldehyde-phosphate buffer, cut into longitudinal strips, and dehydrated in a series of alcohol baths; the final bath was 100% acetone. The tissue was dried at a critical point with liquid CO2 and then was spatter coated with gold for examination in a JEOL JEM100 electron microscope with a CFSIS D40 scanning attachment. Electron micrographs used in this study were representative of the tissue as a whole as well as of the experimental group.

To analyze endothelial regrowth, animals received an injection of 0.5% Evans blue dye solution (3 ml/kg iv) 1 h before death. This dye binds to proteins within the circulation, which subsequently binds to denuded areas of aorta, staining them blue. Areas of endothelial regrowth remain unstained. Harvested aortic segments, three collateral vessels in length, were cut longitudinally along the ventral surface and were mounted on glass microscope slides. Aortic segments were then digitized using an HP Scanjet 4 c, and endothelial regrowth was measured using IP Lab Spectrum software and reported as percent regrowth. All components of Krebs buffer solution were obtained from Mallinkrodt (St. Louis, MO); Evans blue and chemicals used in organ bath studies were from Sigma Chemical (St. Louis, MO).

The data obtained within an experimental group were averaged and reported as means ± SE. These data were then analyzed using the Statview SE+ statistics package using ANOVA and the Scheffé’s F-test to determine differences between the groups. P < 0.05 was considered significant.

**Fig. 1.** Typical Evans blue staining of sections of thoracic aortas from euglycemic (A) and diabetic (B) rabbits 2 wk after catheter injury. White areas indicate endothelial regrowth, whereas the dark portions of the vessel are denuded areas that stained blue.
RESULTS

Analysis of endothelial growth. Evans blue was used to analyze the degree and extent to which endothelial regrowth occurred in that it enables one to differentiate between denuded portions of the vessel and those covered by endothelial cells. Figure 1 shows vessels with typical staining for Evans blue from euglycemic (A) and diabetic (B) rabbits 2 wk after catheter injury. Endothelial regrowth appears white, and the denuded areas that stained blue appear dark. Before catheter injury, the endothelia of euglycemic and diabetic rabbits were completely intact, and Evans blue staining was absent (data not shown). This suggests that the diabetic state itself has no apparent effect on the endothelium before catheter injury. Two weeks after catheter injury, thoracic aorta from diabetic animals had a 2.2-fold increase in percent endothelial regrowth compared with vessels from euglycemic animals (P < 0.0001; Fig. 2).

To characterize the endothelial regrowth shown in the Evans blue analysis, factor VIII immunolocalization was performed. We have previously shown that the luminal surface of thoracic aorta from untreated catheterized rabbits stains positively for factor VIII-related antigen 2 and 4 wk after catheter injury (20). These data were interpreted to indicate that the cells lining the luminal surface after catheter injury were endothelial cells. Figure 3A is a light micrograph of thoracic aorta from a diabetic rabbit, which illustrates the junction where endothelial regrowth (appeared white in Fig. 1) meets an endothelial denuded portion of the vessel (appeared dark in Fig. 1) 2 wk after catheter injury. The section was stained for factor VIII, which was localized to the cells lining the luminal surface and are interpreted as endothelial cells. Segments of the vessel covered by endothelial regrowth, which stained for factor VIII (Fig. 3B), had much less neointimal thickening compared with portions of the vessel with no factor VIII staining (Fig. 3C). This supports the idea that endothelial regrowth is associated with the inhibition of neointimal thickening after injury. Similar results were observed in euglycemic rabbits as previously published (20).

A transmission electron micrograph (TEM) of a representative transverse section of aorta from a euglycemic animal 2 wk after catheter injury further illustrates what was shown in Fig. 3. Figure 4A is a TEM from an area of endothelial regrowth and shows the luminal
surface lined with cells that stained positively for factor VIII-related antigen. The arrows indicate a section of the vessel completely covered by endothelial cells. The intimal area between these endothelial cells and the internal elastic membrane is negligible. Figure 4 shows a transverse section TEM from an area of impaired endothelial regrowth (stained with Evans blue), which highlights the gap between endothelial cells on the luminal surface and the significantly greater area of the neointima.

SE microscopy was used to further characterize the morphology of the luminal surface of the vessel. We compared segments of the vessel having undergone significant endothelial regrowth with segments of impaired regrowth from euglycemic (Fig. 5) and diabetic (Fig. 6) rabbits 2 wk after catheter injury. Figures 5A and 6A are low-magnification (×230) SE micrographs of the junction between endothelial regrowth and denuded areas of vessels from euglycemic and diabetic rabbits, respectively. The areas of regrowth are characterized by parallel folds that are aligned along the longitudinal axis of the vessel in the direction of blood flow. Figures 5B and 6B are micrographs of higher magnification (×1,200) focused on the longitudinal folds of the endothelial regrowth of vessels from euglycemic and diabetic rabbits, respectively. Figure 5C is a higher magnification (×1,200) of an area of impaired regrowth. In these areas, there exist numerous microvillose-like projections of the surface of the regenerating endothelial cells, and many rough areas or naked gaps are present.

Analysis of endothelial function. ACh-induced changes in blood flow compared with the effects of an endothelium-independent vasodilator, such as NTG, are used clinically to evaluate endothelial function (9). Similarly, ACh-induced relaxation of a vascular ring is used as a bioassay to measure the ability of the endothelium to generate NO (2). Because endothelial regrowth was found to be enhanced in the diabetic rabbit and because inhibitory endothelium-derived substances, such as NO, have been shown to inhibit neointimal thickening, the response of the vessels to endothelium-dependent vasoactive agonists was tested. Before catheter injury, thoracic aortas from diabetic and euglycemic animals had similar ACh-induced relaxation, suggesting that the diabetic state itself had no significant effect on endothelial function (Fig. 7). Two weeks after catheter injury, relaxation in response to ACh was significantly attenuated in both diabetic and euglycemic vessels, with no significant difference between them. This relaxation response demonstrated a trend toward control by 4 and 8 wk, yet remained significantly attenuated compared with control (data not shown). These responses were found to be consistent throughout the length of the aorta.

To show that the measured ACh-induced relaxation was via a cGMP-dependent pathway rather than due to the production of prostanoids, the relaxant responses to increasing doses of ACh were measured in the presence of either the nonselective COX inhibitor indomethacin (10⁻⁵ M) or the TXA₂-receptor antagonist SQ-29,548 (3 µM). It was found that neither indomethacin (Fig. 8A) nor SQ-29,548 (Fig. 8B) had an effect on the relaxant response to ACh. This suggests that arachidonate metabolites were not involved in modulating the relaxation response to ACh.

It was also found that neither catheter injury nor the diabetic state affected the responses of the underlying
VSMC layer to increasing doses of NTG (Fig. 9A) or 120 mM KCl (Fig. 9B). Thus vasoactive capabilities of the VSMC were not affected by catheter injury or the diabetic state.

DISCUSSION

It was hypothesized in this study that the attenuated neointimal thickening present in the diabetic rabbit 2 wk after endothelial denudation by catheter injury may be due in part to the rate and degree of endothelial regrowth, as well as the restoration of endothelial cell phenotype and function. This study showed that vessels from diabetic animals had greater endothelial regrowth, whereas endothelium-dependent ACh-induced relaxation remained impaired 2 wk after catheter injury. The response to NTG was unaltered. Thus the generation of NO or possibly alterations in ACh-induced muscarinic receptor activation, rather than the relaxant response to NO, were attenuated, suggesting that the presence of a more prolific endothelial layer inhibits the formation of the neointima after catheter injury.

NO and cGMP have been reported to inhibit VSMC proliferation and migration, primary determinants of neointimal thickening in response to vascular injury (11). When administered before catheter injury, precursors of NO suppress neointimal thickening and increase endothelial function in the rat (29) and rabbit (13, 21, 32), presumably subsequent to the synthesis of inducible NO synthase (NOS). Furthermore, it has been shown that endothelial NOS gene transfer restores NO production in injured coronary arteries, reduces luminal narrowing (18, 33), and inhibits VSMC proliferation and migration (5).

With respect to the mechanism by which NO produces relaxation of rings prepared from the rabbit thoracic aorta, we have shown in previous studies that the relaxant response to ACh was inhibited and with increasing doses converted to a contractile response by the NOS inhibitor Nω-nitro-L-arginine (2). Furthermore, it was found that ATP-sensitive potassium (K_{ATP}) channels were not involved in mediating the response to ACh-induced relaxation in that glibenclamide, a K_{ATP}-channel antagonist, had no effect on the relaxant response (2). As an extension of that study, we have preliminary data that suggest that the response to ACh is indeed endothelium dependent and exerts its effects through a cGMP-dependent pathway rather than through hyperpolarization. After manual denudation of the endothelium of vessels from euglycemic and diabetic animals, the relaxation response to ACh was found to be inhibited. Charybdotoxin and tetraethylammonium, inhibitors of large-conductance calcium-activated potassium channels, also had no effect on
ACh-induced relaxation of rings with an intact endothelial cell layer (data not shown). These observations further support our previous observation that the response of rabbit thoracic aortic rings to ACh is NO dependent via a cGMP mechanism and is not due to hyperpolarization. These data suggested that ACh induces relaxation of the vessel by a cGMP-dependent mechanism subsequent to NO generation.

We and others have previously shown instances in which pharmacological interventions that similarly attenuate intimal thickening have differing effects on ACh-induced relaxation of vascular rings (7). Angiopeptin has been shown to significantly attenuate intimal thickening and is associated with a significant increase in ACh-induced relaxation of vascular rings (9). The calcium-channel antagonist felodipine has been shown to attenuate neointimal thickening after catheter injury but does not improve ACh-induced relaxation of aortic rings. Conversely, the calcium-channel antagonist isradipine had no effect on neointimal thickening.
although it did improve ACh-induced relaxation. Interestingly, all of the beneficial effects of the calcium-channel antagonists were inhibited when coadministered with L-arginine (1).

The SE microscopy and TEM data in this study suggest that areas of the injured vessel with significant endothelial cell regrowth are morphologically more normal compared with damaged areas. Because diabetic rabbits had enhanced endothelial cell regrowth after injury, the proportion of the vessel covered by a more normal endothelium would also be significantly greater than the euglycemic counterpart. The changes present in the electron micrographs illustrate what is typical of the endothelium as it grows back after injury and allows for the comparison between those areas and denuded portions of the vessel. Thus the enhanced endothelial regrowth is reflective of the changes in the electron micrographs and vice versa. The attenuated neointimal thickening present in vessels from diabetic animals may be a function of the overall area of regrowth and the inhibitory effect of the endothelium on neointimal thickening rather than alterations in NO generation. The endothelial regrowth in vessels from diabetic rabbits may produce endothelium-derived inhibitory factors that modify the pathophysiological events involved in the responses to vascular injury, namely the inhibition of VSMC proliferation, migration, or matrix production. We have previously shown that VSMC proliferation is significantly decreased by 2 wk after catheter injury in diabetic compared with euglycemic rabbits (27). Endothelium-derived substances, such as NO, have been suggested to inhibit VSMC proliferation and the formation of the neointima after catheter injury. The NO-generating capacity of the vessel 2 wk after injury, however, was found to be similarly impaired in diabetic and euglycemic rabbits. Therefore, NO-dependent inhibition of the proliferative response to injury is probably not the mechanism behind which neointimal thickening in diabetic animals is attenuated. There exist other endothelium-derived inhibitory factors that may have a similar effect and that may be lost upon catheter injury yet upregulated by the diabetic state. Furthermore, it may also be suggested that endothelial dysfunction is not a good predictor of neointimal thickening that ensues upon vascular injury.

Alternatively, VSMC have been shown to inhibit endothelial cell growth in vitro (26). VSMC proliferation and the total number of VSMC within the neointima were found to be significantly decreased in diabetic compared with euglycemic rabbits (27). Thus

Fig. 8. A: effect of indomethacin (10^{-5} M) on the relaxant response to ACh of vascular rings prepared from thoracic aorta of euglycemic (n = 3) and diabetic (n = 3) rabbits. ■, Euglycemic control; ★, diabetic control; ○, euglycemic with indomethacin; △, diabetic with indomethacin. B: effect of SQ-29,548 (3 µM) on the relaxant response to ACh of vascular rings prepared from thoracic aorta of euglycemic (n = 3) and diabetic (n = 3) rabbits. ■, Euglycemic control; ★, diabetic control; ○, euglycemic with SQ-29,548; △, diabetic with SQ-29,548.

Fig. 9. A: effect of catheter injury and the diabetic state on relaxation in response to increasing doses of nitroglycerin (n = 5–7). ■, Euglycemic control; ★, diabetic control; ○, euglycemic 2 wk; △, diabetic 2 wk. B: contraction in response to 120 mM KCl of vascular rings from euglycemic (filled bars) and diabetic (open bars) rabbits before and 2 wk after catheter injury (n = 4–7).
vessels in which the extent of VSMC proliferation and migration into the neointima was greater and occurred before the arrival of distal endothelial cells, endothelial cell regrowth may have been attenuated compared with regrowth in vessels with less VSMC mitotic activity. Therefore, it could be suggested that decreased VSMC within the neointima allows in part for greater endothelial regrowth in the diabetic rabbits compared with vessels from euglycemic rabbits, which had greater neointimal hyperplasia.

The enhanced endothelial regrowth may also be due in part to the hyperglycemic state. High glucose has been shown to stimulate vascular endothelial cell migration, proliferation, and tube formation in vitro (15). It could be suggested that the hyperglycemia present in the diabetic rabbits may contribute to their enhanced endothelial regrowth compared with the euglycemic rabbits. However, high glucose has been shown in vitro to have toxic effects on endothelial cell growth and is implicated as the origin of vascular dysfunction in diabetic patients (29). The vascular dysfunction in diabetes has been reported primarily in regard to altered endothelium-dependent vasodilation. The possible mechanisms behind the vasodilatory dysfunction include the reduction in NO synthesis and release, high levels of oxygen-derived free radicals, the generation and release of vasoconstrictor prostanoids that counteract the effects of NO, and abnormalities in signal transduction caused by decreased expression of inhibitory G proteins and phosphoinositol metabolism and increased protein kinase C activation (8). In the present study, no impairment of endothelial function before catheter injury due to the diabetic state was present, as determined by ACh-induced relaxation of vascular rings. In addition, the presence of the nonselective COX inhibitor indomethacin or the TXA2-receptor antagonist SQ-29,548 did not affect the relaxant responses to ACh in vitro. This indicates that generation and release of vasoconstrictor and vasorelaxant COX metabolites were not involved in modulating the ACh-induced relaxant response in vessels from diabetic rabbits.

To further investigate the role of insulin in the responses to balloon catheter injury, it would be of interest to add to the experimental groups an insulin-treated, alloxan-induced diabetic rabbit. A decreased serum insulin concentration characteristic of chemically induced diabetic animals potentially plays a part in the formation of the neointima and VSMC proliferation and migration. In addition, insulin and the fasting blood glucose concentration have been shown to have regulatory effects on other mitogenic factors, such as endothelin-1, which could in turn contribute to alterations in the proliferative response to injury present in the diabetic rabbit (16, 13).

These data support the suggestion that severe hyperglycemia or possibly hypoinsulinemia may contribute to the attenuation in neointimal thickening through the stimulation of endothelial cell regrowth and the accompanying alteration in VSMC proliferation and migration. In addition, these data suggest that restoration of the NO-generating capacity of the endothelial cell regrowth is not a good candidate in the modulation of neointimal thickening present in the diabetic rabbit. This illustrates the suggestion that restoration of the endothelial anatomic integrity does not always coincide with the recovery of endothelium-dependent relaxation in the diabetic rabbit. Furthermore, the facilitation of endothelial cell regeneration, even in the presence of impaired physiological function, would help to reduce neointimal thickening after vascular injury.

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REFERENCES


