Nitric Oxide Modulation of Neutrophil-Endothelium Interaction: Difference Between Arterial and Venous Coronary Bypass Grafts

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Objectives. This study sought to evaluate the relation between the pattern of neutrophil-endothelial adhesion in saphenous vein (SV) and internal mammary artery (IMA) grafts and the endothelial production of nitric oxide (NO).

Background. Autologous IMA and SV grafts (SVGs) are increasingly used as conduits for coronary bypass grafting. Previous studies have demonstrated a greater production of endothelial-derived relaxing factor (NO) from IMA than from SVGs. Because of the well known role of NO in modulating the adhesion of polymorphonuclear leukocytes to the endothelium, we studied the pattern of neutrophil adhesion to the endothelium of IMA and SVs under basal conditions and after inhibition of NO synthesis.

Methods. Segments of IMA and SVs were obtained from 20 patients undergoing coronary artery bypass graft surgery. We evaluated the adhesion of both unstimulated and activated neutrophils to the endothelial surface of IMA and SVs in both basal conditions and after inhibition of NO synthesis with Nω-nitro-L-arginine methyl ester.

Results. Under basal conditions, no difference in unstimulated neutrophil adhesion to endothelium was observed between the two vessel conduits. After neutrophil activation, a significantly (p < 0.05) greater adhesion of neutrophils was observed in the SV than in the IMA. After inhibition of NO release, the adhesion of activated neutrophils increased in both vessels, and no significant difference between them was observed. The increased adhesion was attenuated by both L-arginine and sodium nitroprusside.

Conclusions. The lesser neutrophil adhesion to the endothelium of the IMA is a consequence of enhanced release of NO at this level; this effect could be responsible for the better early and long-term patency of this conduit over the SVG in coronary bypass grafting.

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pattern of PMN adhesion to the endothelium of both IMA and SVGs and to relate basal endothelium release of NO to alterations of PMN adherence to vascular endothelium.

**Methods**

**Material.** The material for this study was obtained from 20 patients undergoing coronary artery bypass surgery, in whom the IMA and the saphenous vein (SV) were used as bypass grafts. There were 15 men and 5 women with a mean age of 54.5 years (range 41 to 68). Written informed consent was obtained from each patient. The study was approved by the Ethical Committee of the Medical School of Catanzaro.

**Neutrophil isolation.** Human neutrophils were purified from patient blood on Ficoll-sodium diatrizoate (Hypaque) gradients. Isolated PMNs were >99% pure as assessed by Wright’s stained cytocentrifuge preparation and >99% viable as assessed by exclusion of trypan blue.

**PMN activation.** PMN activation was performed according to the method of Lo and co-workers (16). Labeled PMNs were treated with phorbol dibutyrate (PDB, 300 ng/ml) for 15 min at 37°C, washed three times with HAP buffer (Dulbecco’s phosphate buffered saline containing human serum albumin, 0.5 mg/ml; glucose, 3 mmol/liter; and aprotinin, 0.3 U/ml), and suspended in medium 199 before the adhesion assay. Treatments of PMNs with this agonist did not alter PMN viability, as assessed by exclusion of trypan blue.

**PMN adherence assay.** PMNs were fluorescently labeled with a hydrophobic fluorescent compound (3,3’-dioctadecylorocarbocyanine perchlorate (DiI) (Fluka, Sigma-Aldrich, Milan, Italy) as described by Lo et al. (16). Cells at 4 to 8 × 10⁶ cells/ml were incubated with 50 µg/ml DiI in HAP buffer for 10 min at 0°C, unbound dye was removed by three washes with HAP buffer and labeled PMNs were resuspended in medium 199 for the adhesion assay.

Segments of both IMA and SV harvested at the time of bypass surgery were opened carefully and placed endothelial side up in separate 5-ml round cell-culture dishes containing 3 ml of Krebs-Henseleit (K-H) solution, as described by Ma and coworkers (17). After 10 min of preincubation of the vessel segments, autologous unstimulated DiI-labeled PMNs (10 µl of 10⁶ cells/ml) were added and incubated for 20 min. Vessel segments were then removed from culture dishes and dipped three or four times in fresh K-H solution. These vessel segments were then placed on a glass slide with the endothelial side up. The number of PMNs adhering to the endothelium surface in five separate microscopic fields was counted manually on an inverted microscope equipped for fluorescence using the filter IF355-550. Values of five replicates were averaged, and variations between replicates were <10%.

In a second series of experiments, PDB-stimulated autologous PMNs were incubated for 20 min with both SV and IMA vessel segments. PMN adhesion to the endothelium was evaluated as previously described.

**Inhibition of basal NO release from IMA and SV endothelial cells.** In the present study, we observed the effect of inhibiting basal NO release from both the IMA and the SV on PMN adherence to endothelium. Nω-nitro-L-arginine methyl ester (L-NAME, 1 mmol/liter) was incubated with vessel segments for 30 min. These vessel segments were then transferred to fresh K-H solution that did not contain L-NAME. Stimulated autologous PMNs were then incubated for another 20 min with these vessel segments. PMN adhesion to the endothelium was evaluated as described before.

In other experiments, the L-NAME vessel segments were coincubated with L-arginine (10⁻² mol/liter) or nitroprusside (1 mmol/liter) to determine whether substrate or exogenous NO inhibits neutrophil adhesion to L-NAME–treated vascular grafts.

**Statistics.** All values are expressed as mean value ± SEM. Comparisons between groups were made by using two-way analysis of variance followed by the Bonferroni correction for t test comparison. Statistical significance was set at p < 0.05.

**Results**

**PMN adhesion.** Figure 1 shows the percent of neutrophil adhesion to the endothelium of SVG and IMA segments. Without stimulation, very few PMNs bound to endothelium,
and no significant difference was observed between the two vessels. In contrast, when PMNs were stimulated by PDB treatment, the adhesion and ruffle formation of neutrophils on endothelium were markedly enhanced in both vessel segments. However, on the basis of counts made before and after washing, the percent of PMNs adhering to the IMA endothelium was significantly lower than that in the SVG endothelium (p < 0.05).

In an attempt to relate the reduced PMN adherence to the IMA endothelium to the greater basal NO production, we studied the effects of L-NAME added directly to the bath. Figure 1 summarizes the results. Addition of L-NAME directly to the bath significantly increased PMN adherence in both vessel segments, with a greater increment observed in the IMA. Although the percent of neutrophils adhering to the IMA endothelium was lower than that observed in the SVG endothelium, this difference failed to reach statistical significance. Moreover, Figure 1 shows that both the addition of L-arginine and the supplementation of exogenous NO by nitroprusside significantly reduced L-NAME–induced neutrophil adhesion to vascular endothelium. These results suggest that endogenous NO generated from IMA endothelium acts as an inhibitor of neutrophil adherence.

Discussion

The aortocoronary bypass operation has been widely applied to the treatment of ischemic heart disease. There is uniform agreement that, if technical problems are excluded (18,19), early graft failure is mainly linked to acute thrombosis, whereas late graft failure is mostly due to atherosclerotic changes within the vessel wall (20).

Neutrophils and graft occlusion. Although the pathogenetic mechanisms that lead to early and late graft occlusion are not completely understood, evidence continues to grow for the important role of neutrophil-endothelial interactions, regulated by both humoral and local mediators. Although the exact pathophysiology of the damage remains unclear, the presence of leukocytes in the vessel wall through the release of proteolytic enzymes and the generation of oxygen free radicals may aggravate the endothelial damage and further stimulate platelets, thus having a potential bearing on the subsequent development of restenosis (21). Conversely, activated granulocytes have been shown (22) to increase the response of platelets to stimulation and directly to cause platelet aggregation. Platelets can also adhere directly to granulocytes, so that deposition or activation of platelets in ischemic tissue could promote local neutrophil accumulation. Boogaerts et al. (23) reported that increased PMN aggregation and adherence induced by platelet-derived products augmented endothelial cell damage, probably by increasing expression of complement receptors on human PMNs. Angelini et al. (24) using a pig model of autologous SV to common carotid artery bypass grafting found an extensive leukocyte and platelet adhesion in denuded area of vein grafts, especially in those made with distended veins. Likewise, platelet and leukocyte adhesion has been observed in human SVs examined shortly after grafting in the coronary circulation (18,19).

Boerboom et al. (25) evaluated the histologic and morphometric evolution of vein grafts in a nonhuman primate model. They found a significant increase in the prevalence of PMNs on the lumen surface and in both the intima and the media during the first 14 days after grafting. A leukocyte invasion into grafts during the first week after implantation has also been described, and the selective invasion of these cells has been regarded (26) as a response triggered by wall ischemia, fragmentation or necrosis of myocytes, and hemorrhagic foci.

Neutrophils could also play a role in the formation of the atheromatous plaque. In experimental models of atherosclerosis (27), neutrophils infiltrate damaged endothelium, and these cells have the potential to modulate endothelial permeability and cause local tissue damage and proliferation. As suggested by Nash and Shearman (28), it might be postulated that increased circulating levels of chemoattractants or cytokines would cause chronic or periodic neutrophil stimulation, thus promoting formation of atheroma.

Although passive neutrophils circulate in a nonadhesive state, they can at any time become adhesive. Ricevuti (29) demonstrated that PMN aggregability in the coronary sinus is greater in patients with angiographically documented coronary disease than in control subjects with normal coronary vessels. An important first step in the process of neutrophil-mediated endothelial damage involves the binding of neutrophils to endothelial cells. This process is largely regulated by complementary adhesion molecules; some of these are present essentially on the cell surface and others can be up-regulated in response to chemotactic and proinflammatory stimuli.

NO modulation of PMN-endothelium interaction. Recently NO has been shown to be an endogenous inhibitor of leukocyte chemotaxis adherence (14) and activation (30). Inhibition of NO synthesis resulted in increased leukocyte adherence in postcapillary venules, a response that could be prevented by adding exogenous NO (15) or a high concentration of L-arginine (14).

The results of the present study confirm the important role that NO plays in the modulation of neutrophil-endothelium interactions. The addition of L-NAME, a potent inhibitor of endothelial NO synthesis, to the endothelium of both SV and IMA grafts significantly increased neutrophil adherence to endothelial cells, with an almost similar percent of adherent PMNs observed in SV and IMA grafts. Moreover, this increased adhesion was prevented by both L-arginine and the NO donor nitroprusside. Thus, it is possible to speculate that the significantly lower PMN adherence to the endothelium of the IMA observed under basal conditions is a consequence of a greater production of endothelium-derived NO in the IMA than in the SVG. These results are in agreement with those of recent studies (31,32) using human models. The hypothesis that the superior patency of the IMA graft could be due to enhanced production of endothelium-derived relaxing factor (EDRF) was first made by Luscher and co-workers (33), who demonstrated that endothelium-dependent relaxation in re-
sponse to acetylcholine and platelet-derived products was greater in the IMA than in the SV, both obtained from patients undergoing coronary bypass surgery. In a similar fashion, Pearson and co-workers (34), using an experimental model of canine IMA perfusion, demonstrated that the right and the left IMA exhibited a significant intraluminal and extraluminal release of EDRF. Conversely, Chua et al. (35) observed a small basal release of EDRF from human SVs removed from patients undergoing elective coronary bypass surgery. They showed that the endothelium-dependent relaxation elicited by acetylcholine was impaired markedly in SVs grafted into the arterial circulation.

Conclusions. The results of our study demonstrate that the reduced production of endothelium-derived NO in the SVG compared with that in the IMA results in a greater neutrophil-endothelial adhesion. These findings suggest one possible mechanism for the observed net difference between the two types of conduits in early and long-term patency rates.

References