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Protecting the Endothelial Integrity of Internal Thoracic Arteries

Abstract

Background: Previous functional studies on human internal thoracic arteries, comparing the effect of the traditional harvesting method (occlusion with a clip) with a method leaving the artery perfused, revealed considerably impaired endothelial function associated with enhanced contractility after clipping. We have now investigated whether these observations could be correlated (1) with plasma markers of endothelial dysfunction, and (2) with structural changes in the endothelial layer. **Methods:** 32 patients were randomly distributed into groups of clipped and perfused arteries. Arterial blood samples were obtained from both the artery and extracorporeal circulation to determine sP-selectin, sE-selectin, sL-selectin, and thrombomodulin using enzyme-linked immunosorbent assay. Arteries from three patients were examined by scanning electron microscopy. **Results:** Concentrations of sP-selectin and thrombomodulin were significantly higher in plasma from clipped arteries compared to perfused arteries, whereas sE-selectin and sL-selectin concentrations were similar within the groups. Scanning electron microscopy revealed significant structural changes and loss of endothelial cells in clipped arteries. **Conclusion:** Biochemical and structural results support our findings that leaving the internal thoracic artery perfused preserves endothelial function in the arterial graft.

Key words

Internal thoracic arteries · coronary artery bypass surgery · endothelium · harvesting

Introduction

It is now well established that using the internal thoracic artery has a long-lasting impact on survival and function after coronary artery bypass grafting [1–3]. Nevertheless, early postoperative incidental vasospasm of the internal thoracic artery [4,5] and the development of intima proliferation or atherosclerosis years after coronary artery bypass grafting [6] have been reported. Both of these problems may lead to adverse outcomes for the patient.

We recently demonstrated the impact of internal thoracic artery preparation on endothelial function *in vitro* [7]. We compared the functional activity of the internal thoracic artery when prepared by the "traditional" harvesting technique with a slightly modified method. The "traditional" technique involves preparing the vessel in a pedicle, cutting it at the distal end, occluding it with a clip and storing it in a papaverine-soaked tissue until it is implanted (clipped artery). The modified technique differs from the "traditional" approach in leaving the artery in place distally (no cutting, no clipping), to allow continuous perfusion (perfused artery). Our experiments showed that occluding the inter-

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nal thoracic artery, even for an extremely short time, diminishes the endothelium-dependent relaxation of the artery induced by acetylcholine, and increases the force of its contractile response to 5-hydroxytryptamine and endothelin-1, suggesting loss of the basal vasodilating properties of the endothelium [7]. Disturbing the critical balance between endothelium-derived relaxing and contracting factors is believed to predispose the vascular smooth muscle to increased tone, decreased vasomotion, changes in structure or geometry and, eventually, to atherosclerosis with graft failure.

The endothelium plays the key role in vascular tone and homeostasis because it releases autocrine and paracrine substances. In addition to mediating vasodilation, the endothelium exerts antiatherogenic effects through potent inhibition of platelet aggregation, smooth-muscle proliferation, and leukocyte adhesion [8,9].

Adhesion molecules are involved in leukocyte rolling, adhesion and transmigration across endothelial cells. They play an important role in inflammatory disorders as well as in a variety of other pathophysiological processes. The soluble forms of P-, E-, and L-selectin belong to the selectin family of adhesion molecules. Increased levels of these molecules have been found at different sites of lesions in various diseases. Another reliable marker for endothelial cell damage is thrombomodulin, which is a cell-surface protein. It acts as a thrombin receptor and serves as a factor of anticoagulation.

The aim of the present experiments was to investigate whether the previously described functional changes in the internal thoracic artery following different preparation methods can be correlated (1) with plasma markers of endothelial dysfunction or damage, namely soluble selectins and thrombomodulin, and/or (2) with morphological changes assessed by scanning electron microscopy.

Patients and Methods

A total of 32 male patients with a mean age of 63.6 ± 8.3 years undergoing elective coronary artery bypass grafting were randomised into two groups, with similar demographic data, risk factors, and preoperative chronic medications (Table **1**). In three patients, the endothelium of the internal thoracic artery was examined by scanning electron microscopy. In this series from each patient a first small sample of the internal thoracic artery was taken immediately before and a second sample after blood stasis had been induced for 57 ± 3 min (mean ± SEM) by clipping. Permission for the experimental use of remnant tissue and blood samples was obtained from the local ethics committee and each patient.

Preparation during surgery

Clipped arteries

The internal thoracic artery was dissected and prepared in a pedicle. No skeletonised internal thoracic arteries were used. Following systemic administration of heparin, the artery was clipped, wrapped in a cloth soaked in papaverine, and stored under the manubrium sterni until it was used. After institution of the extracorporeal circulation and cardioplegic arrest of the heart, peripheral vein anastomoses were carried out. Thereafter the redundant part of the internal thoracic artery lying proximally to the clip was dissected for the *in vitro* study. The first millilitre of blood running out of the internal thoracic artery was caught in a cryotube. Blood was simultaneously drawn from the arterial line of the extracorporeal circulation as a control. The samples were immediately transferred to the laboratory and centrifuged at 3000 g for 10 minutes. The platelet-poor plasma was frozen and stored in liquid nitrogen.

Table **1** Characteristics of patients

	Clipped arteries	*Perfused arteries*	*p-value*
Number of patients	16	16	
Mean age, years (SD)	65.7 (8.5)	61.5 (7.5)	0.138
Mean height, cm (SD)	173.0 (7.0)	173.3 (7.0)	0.838
Weight, kg (SD)	81.0 (14.3)	84.8 (13.1)	0.423
Time until implantation, min[a] (SD)	71.2 (9.2)	66.6 (13.8)	0.171
No. of smokers	6	8	0.722
No. of patients with			
– *hypertension*	12	14	0.653
– *IDDM*	3	3	1.0
– *hypercholesterolaemia*	10	12	0.70
No. of patients taking			
– *β-blocker*	14	14	1.0
– *ACE-inhibitor*	4	6	0.70
– *Ca-channel blocker*	4	5	1.0
– *HMG-CoA reductase-inhibitor*	14	13	1.0
– *acetylsalicylic acid*	15	16	1.0

SD: standard deviation; IDDM: insulin dependent diabetes mellitus; [a] time between end of preparation of the internal thoracic artery and performing the anastomosis to the left anterior descending artery.

Perfused arteries

In this group the arteries were also prepared and in a pedicle, but remained connected to the blood flow *in situ* without being detached from the remainder of the artery until implantation. Blood samples from the perfused internal thoracic artery and from the extracorporeal circulation of the same patient were taken as described above, immediately before implantation.

Biomarkers

Soluble thrombomodulin was determined using a quantitative enzyme-linked immunosorbent assay kit (Immubind® Soluble Thrombomodulin ELISA kit, American Diagnostica Inc., Greenwich, CT, USA). Human soluble leukocyte selectin (sL-selectin), human soluble endothelial selectin (sE-selectin), and human soluble platelet selectin (sP-selectin) were determined using ELISA kits purchased from R & D Systems Europe Ltd, Abingdon, Oxon, UK.

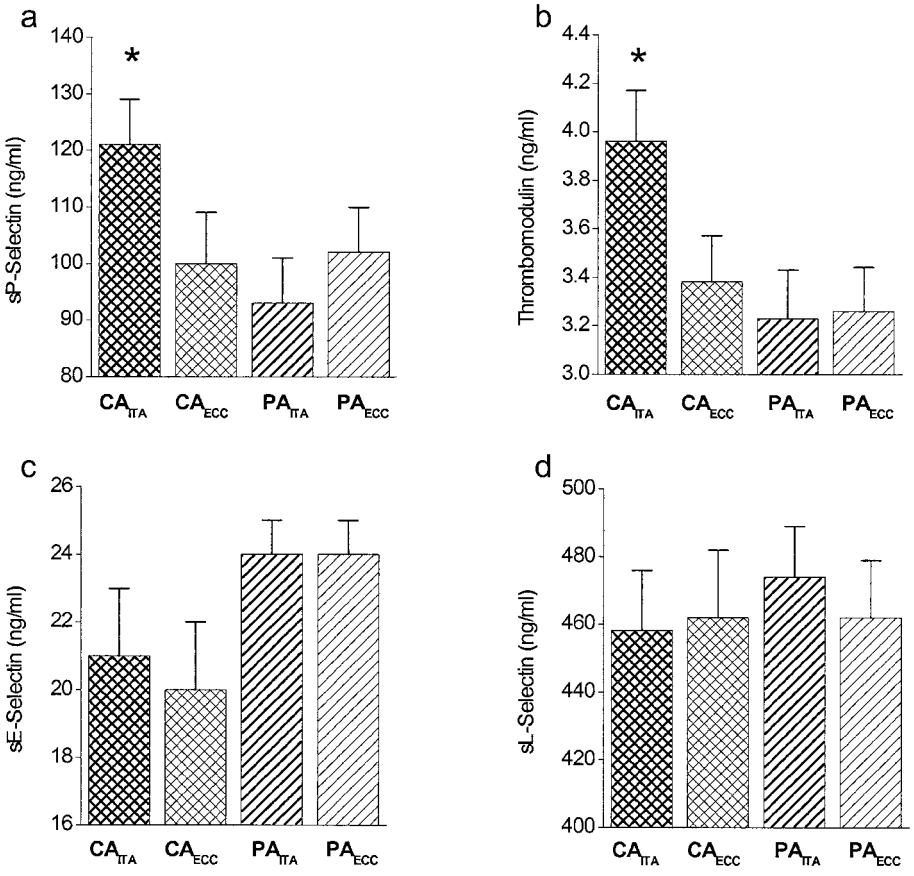

Fig. **1 a** to **d** Levels of **a** sP-selectin, **b** thrombomodulin, **c** sE-selectin, and **d** sL-selectin in plasma from clipped internal thoracic arteries (CA_{ITA}), perfused internal thoracic arteries (PA_{ITA}), and corresponding control samples from the extracorporeal circulation (CA_{ECC} and PA_{ECC}, respectively). * significant difference compared to the other group; for each column n = 16 patients.

Scanning electron microscopy

Two samples of each vessel were taken, the first directly after preparation of the vessel, serving as a perfused artery sample. The second sample was harvested after the artery was occluded with a clip and stored, shortly before the anastomosis to the left anterior descending artery was done. The second specimen was allocated to the clipped artery group. Thus both samples represented the same region of the vessel. The samples were immediately fixed at 4 °C in 2.5 % PBS-buffered glutaraldehyde (pH 7.20, 300 mOsmol) for 24 h, cut longitudinally with a razor blade, dehydrated, and dried to the critical point with liquid CO_2. The region of the clipped artery sample that was occluded and bruised by the clip was removed. The specimens were mounted on stubs with conductive silver paste, sputtered with gold in an argon atmosphere, and examined by two blinded independent observers using a Philips ESEM XL 30 scanning electron microscope at 20 kV.

Statistical analysis

Results are presented as mean ± SEM (standard error of the mean), and demographic data as mean ± SD (standard deviation). We used the Wilcoxon matched-pair test for *intra*individual comparisons (in blood samples from the internal thoracic artery and the extracorporeal circulation of the same patients), and the Mann-Whitney U-test for *inter*individual comparisons. For a comparison of dichotomous baseline patient characteristics Fisher's exact test was used. Although histograms indicated that the outcomes were normally distributed, we used non-parametric tests, as the sample size was small and normality could not be assumed based on the Central Limit Theorem. This is the more stringent approach leading to larger (less significant) *p*-values compared with analyses based on parametric tests. *P*-values < 0.05 were considered to indicate statistical significance. All tests were two-sided. Statistical analysis was performed using the SPSS (Version 11.0, SPSS Inc., 2001, Chicago, IL, USA).

Results

Adhesion molecules

The mean plasma concentration of sP-selectin in blood samples from the internal thoracic artery of clipped artery was 121 ± 6 ng/ml compared with 101 ± 8 ng/ml ($p = 0.019$) in blood taken from the extracorporeal circulation of the same patient at the same time. Plasma concentrations of sP-selectin from clipped arteries were also significantly higher compared with both the perfused internal thoracic artery samples (93 ± 7 ng/ml, $p = 0.019$) and the control extracorporeal circulation samples from patients whose arteries were perfused (103 ± 7 ng/ml, $p = 0.046$, Fig. **1 a**). Similar results were obtained for thrombomodulin concentrations, which were significantly higher in samples taken from clipped internal thoracic arteries (3.96 ± 0.21 ng/ml) compared to samples from the extracorporeal circulation (3.38 ± 0.19 ng/ml, $p = 0.003$). Moreover, in the perfused artery group thrombomodulin levels in both the internal thoracic artery (3.23 ± 0.20 ng/ml; $p = 0.006$) and the extracorporeal circulation (3.26 ± 0.18 ng/ml; $p = 0.01$) were similar to that in the extracorporeal circulation of patients with clipped arteries but sig-

nificantly ($p < 0.05$) lower than in the clipped arteries (Fig. **1 b**). Plasma levels of sE-selectin (Fig. **1 c**) and sL-selectin (Fig. **1 d**) did not differ between the groups.

Scanning electron microscopy

In a blinded analysis by two independent observers all specimens were matched correctly to the clipped or perfused artery groups. The endothelial cells were generally elongated with a typical orientation characterised by the physiological orientation of the blood (Fig. **2**). In some specimens of both groups shrinkage as a result of fixation led to more pronounced protrusions of individual cells. The perfused artery group showed, in general, a more flatly extended endothelium with clearly recognisable cell borders and no signs of intimal fracture or endothelial cell loss (Fig. **2 a**). Cellular and non-cellular deposits were seen only rarely in the perfused artery specimens (Fig. **2 b**). Spike and bleb formation, indicative of cell-membrane and cytoskeleton alterations, appeared only focally in the perfused artery (Fig. **2 c**). Significant structural changes at the endothelial cell surface were found only in the clipped arteries (Fig. **2 d – f**). Furthermore, exposure of basement membrane and loss of individual endothelial cells were detected only in the clipped arteries (Fig. **2 d, f**).

Discussion

Recently published organ-bath studies have shown that a slight modification of the surgical technique of internal thoracic artery harvesting, avoiding blood stasis and alteration in wall tension, may significantly improve the preservation of endothelial function of the arterial graft [7]. In the present study plasma concentrations of soluble adhesion molecules of the selectin class, thrombomodulin and the morphological structures of the endothelial cell layer after the same surgical procedures were investigated.

It was found that the mean plasma sP-selectin concentration in blood from the clipped internal thoracic artery was significantly higher than in blood from the extracorporeal circulation of either group and in that from the perfused internal thoracic artery. sP-selectin is found in a pre-formed state in the Weibel-Palade bodies of endothelial cells and in alpha granules of platelets. The major function of sP-selectin is the mediation of inflammation by promoting the rolling movement of neutrophils, monocytes, lymphocytes, and platelets along the vascular wall [10,11], and by stimulating the adherence of leukocytes to the activated endothelium. P-selectin is thus a key adhesion receptor that mediates the recruitment of leukocytes into a lesion [12]. In response to a variety of inflammatory and thrombogenic agents the stored P-selectin is mobilized to the cell surface within minutes. Against this background the present findings support the contention that blood stasis and altered wall tension may damage the endothelium and its function.

Elevated plasma levels of thrombomodulin indicate *in vivo* endothelial cell damage [17,18]. Thrombomodulin is a transmembrane proteoglycan, mainly located on the luminal surface of vascular and lymphatic endothelial cells and to a lesser extent on platelets and neutrophils. Besides the transmembrane form, thrombomodulin also exists in a soluble form in plasma. It has a high affinity for thrombin, forming a 1 : 1 complex [17]. When occupied, thrombomodulin converts thrombin from a pro-coagulant protein into the activator of protein C. Once activated thrombomodulin acts as a major anticoagulant through its ability to inactivate various blood factors (Va, VIIIa, Xa, and XIIIa). In competing for thrombin binding, thrombomodulin inhibits the proteolytic effect of thrombin in its clotting of fibrinogen, the inactivation of protein S and the induction of platelet aggregation [19,20]. The ELISA kit used in the present study measured whole and truncated forms of thrombomodulin as well as thrombomodulin/thrombin complexes. Nevertheless, the significantly higher thrombomodulin concentration in the clipped internal thoracic artery compared to the extracorporeal circulation as well as to the perfused artery and its control suggested activation of cellular adhesion and endothelial cell damage in the clipped artery.

In contrast to that observed with sP-selectin and thrombomodulin, concentrations of both sE-selectin and of sL-selectin were unchanged. Both sE-selectin [13] and sL-selectin [14] are also believed to play important roles as proinflammatory agents. However, while sP-selectin is mobilized within minutes, the release of sE-selectin and sL-selectin requires hours. Wyble et al. investigated E-selectin up-regulation after exposure to tumour necrosis factor α (TNF-alpha) and interleukin 1 in cultured human umbilical veins. In those studies up-regulation required 2 h (TNF-alpha) and 4 h (interleukin 1), the maximum being 6 h after exposure. Assays of soluble E-selectin demonstrated an even later increase starting after 12 h of exposure [15]. In patients with atherothrombotic cerebral infarction, Kozuka et al. demonstrated that from the onset until the subacute phase sP-selectin levels were elevated, while sE-selectin levels increased only during the subacute phase [16]. In the present experiments the average time of vessel occlusion in the clipped artery was 71.2 ± 9.2 min. It is possible, therefore, that the time of stimulation in our real-world setting of bypass surgery was too short to evoke significant changes in either sE-selectin or sL-selectin.

It remains to be elucidated whether the enhanced levels of thrombomodulin and sP-selectin in the clipped artery group are signs of endothelial cell damage due to an altered pressure-pulse curve with possible retrograde endothelial cell shedding or whether they are due to the stasis of the blood column with consecutive communication between blood cells and endothelium. Nevertheless, the extent and nature of the morphological alterations found by scanning electron microscopy supported the suggestion of functional impairment of endothelial cells in the clipped artery group. The alterations observed in the clipped artery group did not necessarily imply endothelial cell death with subsequent complete denudation of the basement membrane and possible thrombosis. However, even if the described severe alterations of the cytoskeleton were reversible the endothelial integrity would have been at least temporarily reduced. Although arterial samples from only 3 patients were examined, the qualitative morphological differences seen when comparing the endothelial cell surface before and after clipping were so evident that an arbitrary result was extremely unlikely, even with the limited numbers of samples examined in the present study.

In conclusion, plasma concentrations of adhesion molecules and scanning electron microscopy of the endothelial cell surface

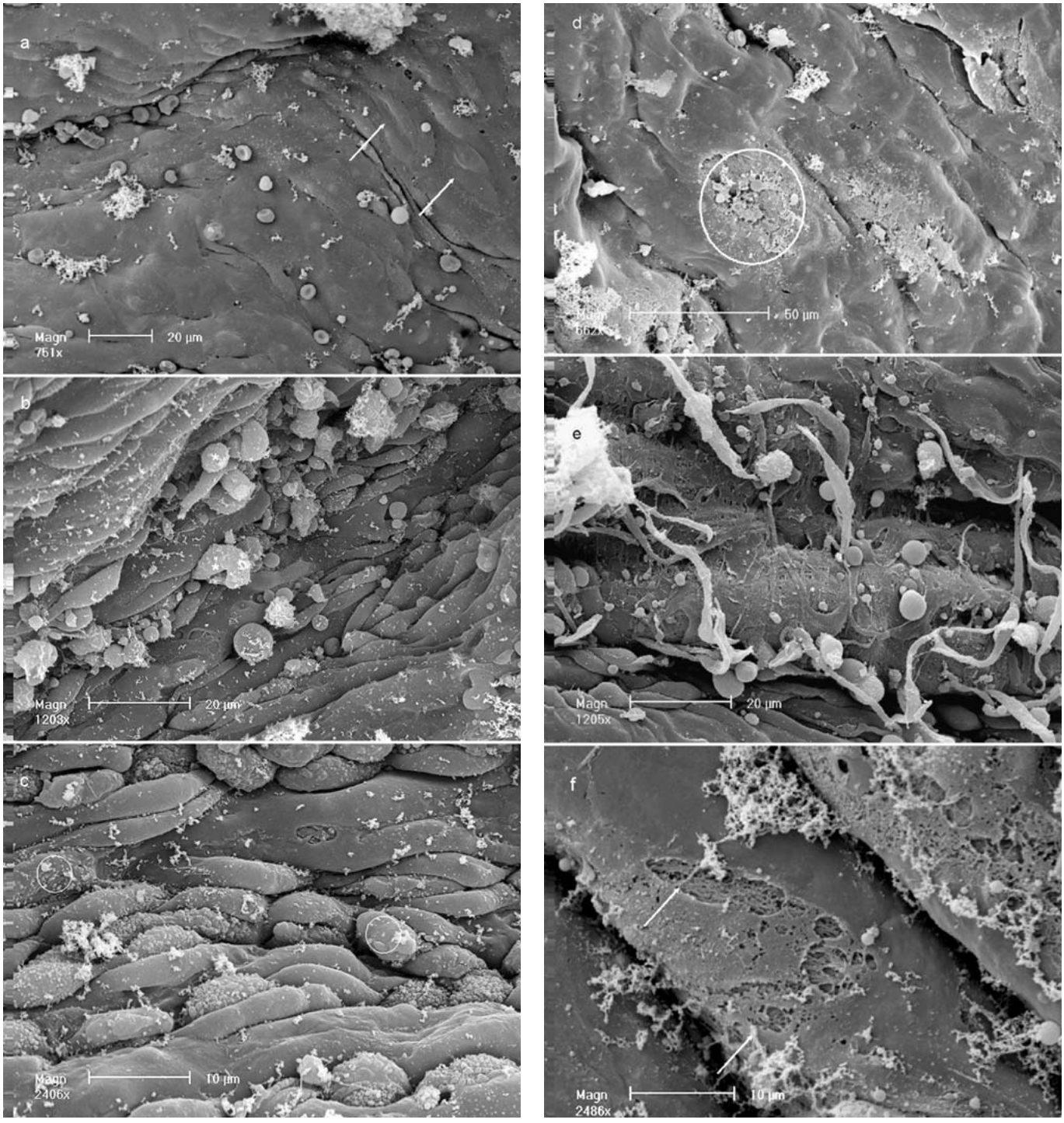

Fig. **2 a** to **f** Scanning electron micrographs of the endothelium of a perfused internal thoracic artery (**a – c**) and a clipped internal thoracic artery (**d – f**) at different magnifications (see bars). Perfused arteries show flatly extended endothelial cells (**a**) with prominent nuclei (arrows). In places, the endothelium appears more prominent (**b, c**) with depositions of blood cells (* in **b**) and non-cellular material. Cytoskeleton damage is occasionally visible on individual cells as spike and bleb formations (o in **c**). In contrast, clipped arteries show severe endothelial cell damage (o in **d**) and cell loss with exposure of basal lamina (arrows in **f**). Depositions of cellular remnants and fibres, as shown in **e**, are more frequently seen.

structure suggest that endothelial impairment and cell damage result from the "traditional" method of harvesting the internal thoracic artery by cutting the distal end and occluding the vessel with a clip. These biochemical and structural results, therefore, support the findings of our earlier functional studies that maintaining the perfusion of the internal thoracic artery during grafting of other coronary target vessels preserves the endothelial function of the arterial graft.

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References

[1] Grondin CM, Campeau L, Lesperance J, Enjalbert M, Bourassa MG. Comparison of late changes in internal mammary artery and saphenous vein grafts in two consecutive series of patients 10 years after operation. Circulation 1984; 70: 1208 – 1212
[2] Loop FD, Lytle BW, Cosgrove DM et al. Influence of the internal-mammary-artery graft on 10-year survival and other cardiac events. N Engl J Med 1986; 314: 1 – 6
[3] Grapow MTR, Preiss M, Bernet F, Zerkowski HR. Surgical treatment in end-stage coronary artery disease. Kardiovask Med 2002; 5: 190 – 197
[4] Mills NL, Bringaze WL 3rd. Preparation of the internal mammary artery graft. Which is the best method? J Thorac Cardiovasc Surg 1989; 98: 73 – 77
[5] Sarabu MR, McClung JA, Fass A, Reed GE. Early postoperative spasm in left internal mammary artery bypass grafts. Ann Thorac Surg 1987; 44: 199 – 200
[6] Shelton ME, Forman MB, Virmani R, Bajaj A, Stoney WS, Atkinson JB. A comparison of morphologic and angiographic findings in long-term internal mammary artery and saphenous vein bypass grafts. J Am Coll Cardiol 1988; 11: 297 – 307
[7] Grapow MT, Kern T, Reineke DC et al. Improved endothelial function after a modified harvesting technique of the internal thoracic artery. Eur J Cardiothorac Surg 2003; 23: 956 – 960
[8] Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993; 329: 2002 – 2012
[9] Luscher TF, Noll G. The pathogenesis of cardiovascular disease: role of the endothelium as a target and mediator. Atherosclerosis 1995; 118: S81 – 90
[10] Frenette PS, Johnson RC, Hynes RO, Wagner DD. Platelets roll on stimulated endothelium *in vivo:* an interaction mediated by endothelial P-selectin. Proc Natl Acad Sci USA 1995; 92: 7450 – 7454
[11] Frenette PS, Wagner DD. Insights into selectin function from knockout mice. Thromb Haemost 1997; 78: 60 – 64
[12] Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. Circ 2001; 103: 491 – 495
[13] Ruchaud-Sparagano MH, Drost EM, Donnelly SC, Bird MI, Haslett C, Dransfield I. Potential pro-inflammatory effects of soluble E-selectin upon neutrophil function. Eur J Immunol 1998; 28: 80 – 89
[14] Bevilacqua MP, Nelson RM. Endothelial-leukocyte adhesion molecules in inflammation and metastasis. Thromb Haemost 1993; 70: 152 – 154
[15] Wyble CW, Hynes KL, Kuchibhotla J, Marcus BC, Hallahan D, Gewertz BL. TNF-alpha and IL-1 upregulate membrane-bound and soluble E-selectin through a common pathway. J Surg Res 1997; 73: 107 – 112
[16] Kozuka K, Kohriyama T, Nomura E, Ikeda J, Kajikawa H, Nakamura S. Endothelial markers and adhesion molecules in acute ischemic stroke – sequential change and differences in stroke subtype. Atherosclerosis 2002; 161: 161 – 168
[17] Chong AY, Blann AD, Lip GY. Assessment of endothelial damage and dysfunction: observations in relation to heart failure. Qjm 2003; 96: 253 – 267
[18] Boehme MW, Deng Y, Raeth U et al. Release of thrombomodulin from endothelial cells by concerted action of TNF-alpha and neutrophils: *in vivo* and *in vitro* studies. Immunology 1996; 87: 134 – 140
[19] Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. Proc Natl Acad Sci USA 1981; 78: 2249 – 2252
[20] Esmon NL, Owen WG, Esmon CT. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J Biol Chem 1982; 257: 859 – 864