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Correlation of leukocyte adhesiveness, adhesion molecule expression and leukocyte-induced contraction following balloon angioplasty

[1,3]Simon Kennedy, [2]Allan R. McPhaden, *,[1]Roger M. Wadsworth & [1]Cherry L. Wainwright

[1]Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G4 0NR and [2]Department of Pathology, Glasgow Royal Infirmary, Castle Street, Glasgow G4 0SF

1 The aim of this study was to examine the changes in leukocyte adhesion and leukocyte-induced contraction in balloon-injured rabbit subclavian artery and to correlate these changes with vessel morphology and expression of adhesion molecules on the injured arteries.

2 Rabbits were anaesthetized and their left subclavian arteries were injured by balloon inflation and withdrawal followed by sacrifice at 2, 24, 48 h or 8 days after injury. The left and right subclavian arteries were removed and leukocytes were isolated from autologous rabbit blood. Leukocyte-induced contraction was measured in 5-HT precontracted artery rings and leukocyte adhesion was measured using ^{51}Cr-labelled leukocytes. Immunocytochemistry using paraffin-embedded tissue was employed to detect changes in the expression of adhesion molecules on injured arteries.

3 Autologous leukocytes caused a contraction of rabbit subclavian artery rings, which was prevented by L-NAME (10^{-3} M). Balloon-induced injury abolished the contractile response to leukocytes, which correlated with loss of carbachol-induced relaxation

4 Balloon injury markedly enhanced the adhesiveness of the subclavian artery for leukocytes, most notably at 24 and 48 h after injury (1.7 and 1.8 fold respectively). Increased leukocyte adhesion at these two time points correlated with an upregulation of E-selectin, P-selectin and VCAM-1 expression on the remaining endothelium of the injured artery.

5 Vessel morphology revealed that balloon inflation had induced an infiltration of inflammatory cells into the vessel wall, the greatest increase being seen at 24 h after injury.

6 It is concluded that an increase in the expression of E-selectin, P-selectin and VCAM-1 following balloon-induced injury leads to enhanced leukocyte adhesion and migration into the injured vessel. *British Journal of Pharmacology* (2000) **130,** 95–103

Keywords: Angioplasty; subclavian artery; balloon injury; E-selectin; L-NAME; P-selectin; leukocyte adhesion; VCAM-1

Abbreviations: eNOS, endothelial nitric oxide synthase; HBSS, Hank's buffered salt solution; H&E, haematoxylin and eosin; 5-HT, 5-hydroxytryptamine; ICAM-1, intercellular adhesion molecule-1; i.m., intramuscular; L-NAME, L-nitroarginine methyl ester; TBS, tris-buffered saline; VCAM-1, vascular cell adhesion molecule-1

Introduction

There is a substantial body of evidence to implicate leukocytes in both the immediate and the long-term effects of vascular injury induced by balloon angioplasty. Angioplasty activates leukocytes to produce free radicals, proteolytic enzymes and growth factors (De Servi *et al*., 1990; Ikeda *et al*., 1994; Baj *et al*., 1994; Kowalski *et al*., 1995) and to express adhesion molecules on their surface (Baj *et al*., 1994; Neumann *et al*., 1996; Mickelson *et al*., 1996; Inoue *et al*., 1996). Consequently, following injury, leukocytes rapidly adhere to damaged endothelium and migrate into the subendothelial layers of the vessel (Pärsson *et al*., 1994; Gonschior *et al*., 1995; Wilensky *et al*., 1995) to an extent which is dependent on the severity of the injury (Merhi *et al*., 1995). Prevention of leukocyte adhesion by administration of antibody to leukocyte integrin receptors significantly reduced the growth of neointima following injury of the rabbit carotid artery (Golino *et al*., 1997). Moreover, endotoxin-induced accumulation of leukocytes in the artery wall, in the absence of injury, induced localized neointimal growth (Forney-Prescott *et al*., 1989). Thus, early interactions between leukocytes, platelets and endothelium and the presence of thrombus at the injury site may be pivotal in controlling vascular tone regulation and influencing the long-term outcome of angioplasty (Marcus & Saifer, 1993).

The localization and adhesion of leukocytes to injured areas of arteries requires interaction between adhesion molecules and their counter receptors on the leukocyte and in the vessel. Although many studies have demonstrated changes in adhesion molecule expression on leukocytes (Baj *et al*., 1994; Neumann *et al*., 1996; Mickelson *et al*., 1996; Inoue *et al*., 1996) or changes in soluble adhesion molecules in the blood (Kurz *et al*., 1994) after vascular injury, few studies have examined adhesion molecule expression on the injured vessel. One study has reported upregulation of VCAM-1 and ICAM-1 following angioplasty balloon injury to the rabbit aorta (Tanaka *et al*., 1993). However, there have been no reports of the effect of injury on E-selectin and P-selectin, responsible for the initial tethering interaction of leukocytes to the endothelium, and it has not been shown whether the increased expression of adhesion molecules is associated with altered adhesion of leukocytes.

Vasospasm and vascular recoil are common features following balloon angioplasty (Wilensky *et al*., 1995).

*Author for correspondence.
[3]Current address: Institute de Recherches Servier, Centre de Recherches de Suresres, 11 rue des Mailineaux, 92150 Suresres, France.

Angiographically measured vasoconstriction resulted in 45.5% reduction in luminal diameter immediately after deep injury to the pig carotid artery (Merhi *et al*., 1995). Moreover neutropenia reduced this vasoconstrictor response by 41% (Mehri *et al*., 1994). Angioplasty caused the release of leukotrienes and lipoxin, vasoconstrictor substances produced by leukocytes, from the artery wall (Brezinski *et al*., 1992). In addition, activated leukocytes have the ability to release a number of other potentially vasoactive substances such as free radicals, proteolytic enzymes and PAF which can act directly or alternatively may interact with mediators released from the endothelium and platelets (Siminiak *et al*., 1995). Leukocyte-derived vasoactive substances may impair endothelium-dependent relaxation to vasodilators such as acetylcholine, probably by destroying nitric oxide (Simniniak *et al*., 1995; Liu *et al*., 1993; De Kimpe *et al*., 1993). The importance of leukocyte-induced changes in vascular tone is related to the suggestion that chronic vasoconstriction may be partially responsible for the loss of luminal area after angioplasty (Shaw *et al*., 1995). However it is not known whether the contractile activity of leukocytes is modified following vascular injury.

In view of the importance of leukocytes in the processes occurring early after vascular injury, the aim of this study was to examine the adhesion and vascular effects of rabbit isolated leukocytes on balloon injured rabbit subclavian arteries at four time points early after injury. Using a model of arterial injury previously developed in our laboratory (Hadoke *et al*., 1995) we investigated how vascular injury modifies leukocyte adhesion and the response of the vessel to leukocyte-derived vasoactive substances. Furthermore, immunocytochemistry was employed to identify the presence of the adhesion molecules ICAM-1, VCAM-1, P-selectin and E-selectin on control and injured vessels in order to assess whether changes in these vascular adhesion molecules could account for alterations in leukocyte adhesion after injury.

Methods

Angioplasty procedure

A total of 33 male New Zealand White rabbits (2.5–3.0 kg) were studied. Twenty-seven animals underwent balloon angioplasty of the left subclavian artery and six were non-operated control animals. Prior to angioplasty, rabbits were premedicated with Hypnorm® (fluanisone/fentanyl citrate mixture; 1.2 ml i.m.), 100 mg (i.m.) of ampicillin suspension and 500 U of heparin (i.v.) *via* the marginal ear vein. The animals were anaesthetized and maintained on a mixture of 2% nitrous oxide and 1.5–2% halothane in oxygen. An incision was made on the inner aspect of the hind leg and the femoral artery was exposed. Two silk ligatures were placed round the extreme ends of the exposed artery and the distal ligature was tied. A 3.0 mm balloon catheter (Advanced Cardiovascular Systems Inc., Temecula, CA, U.S.A.) containing a 0.014 in. steerable guidewire was introduced into the artery through a small incision and advanced into the subclavian artery under fluoroscopic guidance. Once in position the balloon was inflated twice to a pressure of 10 atmospheres for a period of 30 s with 1 min between inflations. The balloon was inflated for a third time to a pressure of 8 atms and withdrawn half its length to ensure endothelial denudation. All inflations were monitored by a radio-opaque contrast medium, iopamidol (Niopam 370®) which filled the balloon during inflation. The balloon was deflated and withdrawn, the proximal ligature was tied off and the wound closed. All animals were given 1 ml of buprenorphine (Vetergesic®) i.m. as analgesic cover immediately after the procedure. All surgical procedures were performed under a Project Licence issued under the U.K. Home Office Animals (Scientific Procedures) Act 1986.

Leukocyte isolation

Angioplastied animals were euthanased 2 h ($n=8$), 24 h ($n=6$), 48 h ($n=7$) or 8 days ($n=6$) after angioplasty by slow injection of a mixture of sodium pentobarbitone (typically 60 mg kg^{-1}) and heparin sodium (1000 units kg^{-1}) into the marginal ear vein. The chest was opened, the pericardium was removed and blood (60 ml) was withdrawn from the pulmonary artery *via* a wide bore needle into sterile 20 ml syringes containing 2 ml of 3.8% sodium citrate. After removal, blood was immediately transferred into sterile 10 ml centrifuge tubes containing 2 ml of 6% dextran solution and left to sediment at room temperature for 2 h. The leukocyte-rich upper layer was removed and centrifuged at $300 \times g$ for 5 min. The resultant cell pellet was hypotonically lysed with distilled water to remove contaminating erythrocytes and layered onto 1 ml of Histopaque® (density 1.077 g ml^{-1}). A second centrifugation at $300 \times g$ for 20 min yielded a white cell pellet which was resuspended in Hank's balanced salt solution (HBSS). Isolated cells were counted using an automated cell counter (Medonic Cellanalyzer CA460, Sweden) and diluted with HBSS to yield a suspension containing 5×10^6 cells ml^{-1}. Leukocytes were kept at room temperature prior to use.

Functional study

Left and right subclavian arteries were rapidly dissected and placed in aerated Krebs solution. The first 2 cm of the left subclavian artery from the aortic arch to the bifurcation was cut into rings of 3–4 mm length. The first ring adjacent to the aortic arch was discarded, the next ring was used in the adhesion study, the next two in the functional study and the remaining ring was used for immunocytochemistry. The right subclavian artery was divided in a similar manner. For functional experiments the four rings of artery (two each from the left and right artery) were suspended between two parallel intra-luminal wires, one fixed and the other attached to an isometric transducer (FT03 Grass Instrument Division, RI, U.S.A.), in 5 ml organ baths filled with Krebs solution (37°C) of the following composition (mM): NaCl, 118.3; $NaHCO_3$, 25; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2 and glucose 11.1. The subclavian artery rings were placed under a previously determined optimum resting force of 3 g (Hadoke *et al*., 1995) to equilibrate for 1 h after which they were sensitized by three separate additions of 40 mM KCl. The artery rings were precontracted using 5-HT, since this is released from platelets during thrombus formation, and gives a stable contraction. Artery rings were precontracted with 5-HT 10^{-7} M, which gave $33 \pm 10\%$ of the maximum contraction to 5-HT. Autologous leukocytes (10^4–10^6 cells) were then added to observe any changes in force (contraction or relaxation). The total volume of leukocyte suspension added to the bath was 1120 μl. In control experiments, an equivalent volume of HBSS was found to have no effect on vessel tension. In each experiment one control and one injured artery ring were treated with 10^{-3} M L-NAME added 30 min before the start of the experiment. L-NAME 10^{-3} M had no significant effect on the magnitude of the 5-HT-induced contraction (left untreated 2.7 ± 0.3 g, left with L-NAME 2.6 ± 0.3 g, right untreated 2.3 ± 0.3 g, right with L-NAME 2.9 ± 0.3 g). At the end of

most experiments, endothelial function was tested by the addition of 10^{-6} M carbachol. In rings which had been treated with L-NAME, relaxation to 10^{-5} M sin-1 was measured. All experiments were performed in the presence of 10^{-6} M indomethacin to abrogate any effect of prostanoids. Responses were calculated as the percentage increase in the tone induced by 5-HT at the point of addition of the first aliquot of leukocyte suspension.

Leukocyte adhesion assay

Leukocyte adhesion to the endothelial surface of segments of subclavian artery was measured using ^{51}Cr-labelled leukocytes employing a modification of the method described by Tsao *et al*. (1994). Briefly, an aliquot of 10^6 leukocytes in HBSS was labelled for 1 h at 37°C with 5 μCi of ^{51}Cr (specific activity 1 mCi ml^{-1}, $Na_2{}^{51}CrO_4$ in 0.9% saline). The leukocytes were then washed twice and resuspended in HBSS at a concentration of 10^7 leukocytes ml^{-1} of HBSS. Five-microlitre samples of supernatant from each of the two washes and from the cell suspension were taken and counted to assess the efficiency of the labelling procedure. After the second wash the activity of each 5-μl aliquot of the final leukocyte suspension was 2500–3500 c.p.m. This degree of chromium uptake agrees with that reported in the literature (Gallin *et al*., 1973; Kinoshita *et al*., 1989). Three-to-four-millimetre rings of both the left and right subclavian arteries removed from the animals were opened longitudinally and pinned out, luminal-side upwards using small dissecting pins onto Sylgard blocks in a humidified chamber (37°C). An aliquot containing 5×10^4 labelled leukocytes (in 5 μl) was added gently to the luminal side of each artery and adhesion was allowed to proceed for 30 min. Tissues were then washed with HBSS to remove non-adherent cells and adhesion was quantified by duplicate counts of the vessel segments in a gamma counter. Leukocyte adhesion was expressed as the percentage of the cells added that remained adherent after washing. When required, the endothelium was removed by rubbing gently with a wooden probe.

Histology and immunocytochemistry

Artery rings were fixed in neutral buffered formalin (100 ml of 40% formaldehyde mixed with 900 ml of phosphate buffer (4 g of $NaH_2PO_4{\cdot}2H_2O$ and 6.5 g of Na_2HPO_4 per litre) for morphological and immunocytochemistry studies. The rings were embedded in paraffin, 4 μm sections were cut, and mounted on polysilane coated glass slides. The number of experiments in which there was sufficient tissue for histology and immunocytochemistry was: 2 h group $n=5$, 24 h group $n=6$, 48 h group $n=7$, 8 day group $n=5$.

The endothelium was examined in sections from all animals both by immunocytochemistry for eNOS and in haematoxylin and eosin (H & E) stained sections. The immunocytochemical protocol was as described below for the adhesion molecules, except that the primary antibody was mouse monoclonal anti-eNOS (Transduction Laboratories, Lexington, KY, U.S.A.). The percentage of the inner circumference that was covered with endothelial cells was determined for each section stained for eNOS. Other features that were graded on H & E sections were the presence of thrombus and infiltration by leukocytes. A three-point scale was used where 0 = no thrombus or leukocyte infiltration, 1 = focal thrombus or infiltration and 2 = subtotal or total thrombus or infiltration.

Inflammatory cells present in the artery wall were identified by the presence of naphthol AS-D chloroacetate esterase or of α-naphthyl acetate esterase (specific for granulocytic and monocytic leukocytes respectively; Yam *et al*., 1971). Slides were deparaffinized in xylene and taken through graded alcohols to water. They were incubated for 24 h at 37°C with either (1) napthol AS-D chloroacetate plus fast Corinth V or (2) with α-naphthyl acetate plus fast blue RR. Slides were then washed, counterstained in haematoxylin and mounted in aqueous medium. Cells staining for naphthol AS-D chloroacetate esterase and for α-naphthyl acetate esterase always corresponded to cells that were morphologically determined to be neutrophils or macrophages respectively when stained with haematoxylin and eosin. Both cell types were counted in four high power fields; one at each of the four compass points (north, south, east and west) by an observer blinded to the time point at which each vessel had been removed after injury. To examine the adhesion of leukocytes, sections of artery used in the adhesion assay were fixed with formal saline for 15 min while still pinned to the Sylgard block. Arteries were then embedded end-on in paraffin, allowing transverse sections to be cut and stained for naphthol AS-D chloroacetate esterase.

Immunocytochemistry was used to detect the presence of adhesion molecules on sections of rabbit subclavian arteries using four antibodies (directed against ICAM-1, E-selectin, P-selectin and VCAM-1) followed by a streptavidin-biotin-peroxidase method (Hsu *et al*., 1981). Briefly, sections were deparaffinized in xylene and taken down to water. Endogenous peroxidase activity in the tissue was destroyed by immersing the slides in a 3% aqueous solution of hydrogen peroxide for 10 min. All slides were then washed in tris-buffered saline (TBS) and blocked with a 1 : 5 dilution (in TBS) of rabbit serum for 15 min. Excess serum was drained off and primary antibody was applied. For all four antibodies, the primary antibody was incubated with the tissue section for 24 h at 4°C at a pre-determined optimum dilution of 1 : 1000 (or 1 : 500 in the case of the antibody to ICAM-1) containing 10% normal rabbit serum. Slides were then washed in TBS and biotinylated anti-goat IgG secondary antibody was added for 30 min (1 : 200 dilution with secondary antibody diluent containing 5% normal rabbit serum). After washing in TBS, peroxidase-labelled streptavidin diluted 1 : 200 in TBS was added to the slides for a further 30 min. After washing, slides were immersed for 10 min in 0.05% 3,3′-diaminobenzidine tetrahydrochloride solution followed by 0.5% copper sulphate solution for a further 10 min to enhance the colour of the brown reaction product. The slides were then placed in haematoxylin for 15 s to counterstain the nuclei, differentiated in 0.5% acid alcohol, placed in Scot's tap water ($NaHCO_3$ 0.35% plus $MgSO_4$ 2% in water) to 'blue', dehydrated in xylene and mounted in DPX.

For all four antibodies, staining was quantified using a five-point grading scale where 0 = no staining, 0.5 = focal staining of the endothelium but with areas of endothelium which were unstained, 1 = mild staining of the endothelium with all cells being positive, 1.5 = uniform, moderate staining of the endothelial layer and 2 = intense staining of the whole endothelium. The slides were given a code and all slides graded blind by one investigator (A.R. McPhaden) at one time.

Materials

Hypnorm was obtained from Janssen Pharmaceuticals, Beerse, Belgium; Amfipen (100 mg ml^{-1}) was from Mycofarms Ltd., Cambridge, U.K.; Niopam 370 (iopamidol) was purchased from E. Merck Pharmaceuticals Ltd., Middlesex, U.K. and Vetergesic from Reckitt & Colman Pharmaceuticals Ltd. Sodium citrate, dextran (mw approx. 500,000), Histopaque

1077, Hank's balanced salt solution, 5-hydroxytryptamine, indomethacin, carbachol, L-NAME, DAB and Harris haematoxylin and eosin stains were all obtained from Sigma, Poole, Dorset, U.K. Sin-1 was generously provided by Hoechst-Marion-Roussel, Frankfurt, Germany; Sagatal was obtained from Rhône Mérieux Ltd., Harlow, Essex, U.K. and heparin was from Leo Laboratories Ltd., Buckinghamshire, U.K. ^{51}Cr radioisotope was obtained from Amersham International Plc., Little Chalfont, Buckinghamshire, U.K. Polyclonal anti-human antibodies to ICAM-1, E-selectin, VCAM-1 and P-selectin were purchased from R&D Systems (Europe) Ltd., Abingdon, Oxfordshire, U.K.; rabbit serum was supplied by Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, U.K.; biotinylated anti-goat IgG antibody was obtained from Vector Laboratories, Burlinghame, CA, U.S.A. and streptavidin-POD was obtained from Boehringer Mannheim, GmbH, Germany.

Statistics

All results are shown as mean ± s.e.mean where n = the number of rabbits used. For the leukocyte-vessel function studies, a two-way ANOVA was employed to determine the significance of changes in vascular tone between control groups and groups treated with L-NAME and between angioplastied arteries and the relevant non-injured control vessel. Changes in leukocyte adhesion were determined using a Student's paired t-test comparing uninjured and injured arteries at each time point. Alterations in adhesion molecule expression were determined using a non-parametric Mann-Whitney test to compare the staining grades in the uninjured with the injured arteries at each time point. In all cases a P value of <0.05 was taken to be indicative of statistical significance.

Results

Leukocyte-induced artery contraction

Cumulative addition of 10^4 to 10^6 rabbit leukocytes caused a concentration-dependent contraction of 5-HT precontracted rabbit right (non-injured) subclavian arteries from all animals (Table 1). Incubating the ring with 10^{-3} M L-NAME 30 min prior to the addition of leukocytes significantly reduced but did not abolish the contractile response. Figure 1 shows the cumulative dose-response curve to leukocytes, in the absence and presence of L-NAME, in non-injured right subclavian arteries removed 2 h after surgery. The magnitude of the response was consistent with previous studies using vessels from non-operated rabbits.

The leukocyte-induced contraction was abolished or very strongly inhibited in left subclavian arteries taken from animals which were killed 2, 24 or 48 h after angioplasty (Figure 1, Table 1). The small residual contraction seen in injured arteries at 2 h after injury was abolished by incubating the ring with L-NAME (Table 1). Both 24 and 48 h after angioplasty, the injured artery failed to contract in response to leukocytes. At the 8 day time point injured arteries showed a partial restoration of contraction in response to leukocytes, although the magnitude of the contraction was still significantly reduced compared to that induced by leukocytes in the non-injured right subclavian artery (Table 1). At this time point, L-NAME did not attenuate the leukocyte-induced contraction in the injured arteries (Table 1). The abolition of leukocyte-induced contraction in balloon-injured arteries was not related to reduced arterial contractility since the response to 5-HT was not significantly different at any of the four time points studied (Table 2).

Right subclavian artery rings which were not treated with L-NAME consistently relaxed in response to 10^{-6} M carbachol ($69 \pm 4\%$ relaxation of combined 5-HT and leukocyte induced tone, $n=22$). L-NAME treated rings relaxed fully in response to 10^{-5} M sin-1. In contrast, carbachol was unable to induce any significant relaxation of angioplastied arteries taken at 2, 24 and 48 h ($8 \pm 5\%$, $n=16$) and only a modest relaxation ($19 \pm 10\%$, $n=6$) of injured arteries at the 8 day time point. This shows that the angioplasty procedure produced marked impairment of endothelial function that lasted to the 48 h time point, and that there was partial restoration of endothelial function at 8 days after injury.

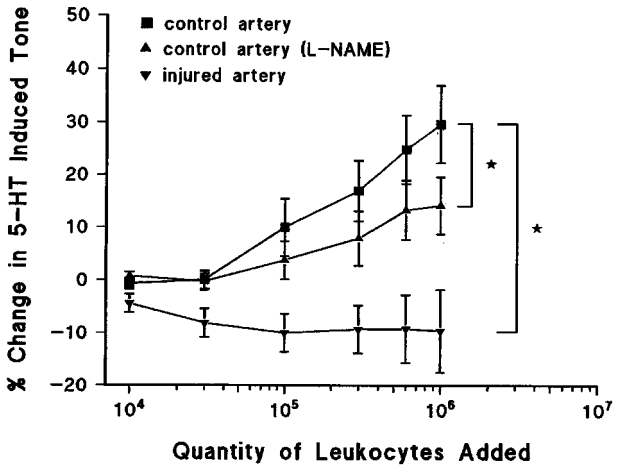

Figure 1 The effect of addition of unstimulated rabbit leukocytes to control (right) and injured (left) rabbit subclavian artery rings precontracted with 10^{-7} M 5-HT. ■, control; ▲, L-NAME (10^{-3} M) treated artery; ▼, injured artery removed 24 h after injury. Values are expressed as means ± s.e.mean, $n=6$. ★$P<0.05$ (entire curve compared with two-way ANOVA).

Table 1 Contraction of rabbit subclavian arteries in response to 10^6 leukocytes

	% increase in force in 5-HT contracted arteries			
	Time after angioplasty			
	2 h	*24 h*	*48 h*	*8 days*
Right subclavian artery (control)	34 ± 11	29 ± 7	64 ± 12	36 ± 11
Control artery + L-NAME (10^{-3} M)	1 ± 8*	10 ± 10	26 ± 4*	15 ± 3
Left subclavian artery (injured)	8 ± 8*	−10 ± 8*	3 ± 5*	14 ± 2
Injured artery + L-NAME (10^{-3} M)	0.4 ± 7*	−9 ± 7*	5 ± 3*	11 ± 10*

Data are shown as mean ± s.e.mean of 6–7 experiments. *$P<0.05$ with respect to corresponding right artery without L-NAME (one-way ANOVA with Tukey's test).

Leukocyte adhesion to the endothelium of intact arteries

Leukocyte adhesion to left (17±2%) and right (19±3%) subclavian arteries was not significantly different in a group of non-operated control animals. In non-injured right subclavian arteries taken from angioplastied animals, adhesion was not significantly different at any of the four time points studied (Figure 2). Two hours after balloon-induced injury to the left subclavian artery, adhesion was significantly increased with respect to the right subclavian artery. At both 24 and 48 h after angioplasty, adhesion to injured arteries was markedly increased (1.7 fold at 24 h and 1.8 fold at 48 h) with respect to the non-injured artery. Eight days after injury, adhesion to the injured artery was still elevated with respect to the control artery but this did not reach statistical significance (Figure 2). Enhanced leukocyte adhesion to injured arteries could not be accounted for by an absence of endothelium since denudation of control arteries was insufficient to induce an increase in leukocyte adhesion (adhesion was 23±5% in denuded arteries and 30±6% in control arteries, $n=6$). Morphologically, adherent leukocytes appeared to be predominantly tethered to the endothelium with no evidence of transendothelial migration.

Presence of leukocytes in the artery wall following injury

All of the 23 non-injured right subclavian arteries appeared morphologically normal with no sign of endothelial denudation, mural thrombus, medial or adventitial disruption or inflammatory cell infiltration into the vessel wall. In contrast, injured left subclavian arteries displayed definite areas of damage, which often extended the full width of the vessel wall. The greatest level of damage at all time points was found in the vascular endothelium and, in individual vessels with more extensive denudation, this was often associated with the presence of adherent, overlying thrombus. However, circumferential denudation was rare and the majority of angioplastied vessels retained some endothelium. The endothelial layer had 65±11% loss at 2 h, 56±9% loss at 24 h, 32±6% loss at 48 h and 10±3% loss at 8 days after angioplasty. Of the five injured vessels taken from rabbits killed 8 days after angioplasty, two had started to develop fibrocellular neointima while vessels from the earlier time points showed no signs of neointimal growth. Polymorphonuclear leukocytes were detected in vessels removed at all four time points. Cell morphology and enzyme cytochemistry revealed that these were granulocytic leukocytes, and were predominantly present in the media and adventitia of injured vessels. The number of granulocytes counted per artery cross-section was 2 h – 5.6±2.8 $n=5$; 24 h – 18.0±11.3 $n=6$; 48 h – 5.3±1.3 $n=7$; 8 days – 1.8±1.2 $n=5$. Macrophages were not observed in injured vessels removed at the three earlier time points but were detected at low levels in the adventitia in some vessels removed 8 days after injury.

Table 2 5-HT-induced contraction of control and balloon-injured rabbit subclavian arteries

Contraction (g) induced by the addition of 10^{-7} M 5-HT		
Time point after injury	*Control artery*	*Injured artery*
2 h	2.3±0.5	2.0±0.5
24 h	1.9±0.5	2.1±0.6
48 h	1.2±0.2	1.7±0.3
8 days	2.3±0.2	1.8±0.6

Data are shown as mean±s.e.mean, $n=6-7$.

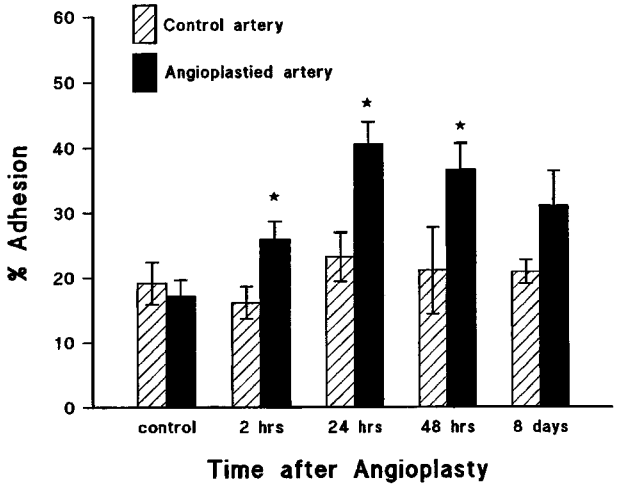

Figure 2 Effect on leukocyte adhesion of balloon-induced injury to the left subclavian artery compared with the non-injured right subclavian artery from the same animals. Leukocyte adhesion is expressed as the percentage of the 5×10^4 leukocytes added that remained adherent after washing (means±s.e.mean, $n=6-8$). ★$P<0.05$ with respect to the non-injured artery at the same time point.

Adhesion molecule expression

In non-injured arteries, low levels of immunoreactivity was detected to all four adhesion molecules in the endothelium, but no specific staining occurred in the medial or adventitial layers. Immunoreactivity in uninjured arteries remained unaltered at each time point after operation. In the injured arteries, cells lining the intima were identified as endothelial cells by their morphology and by positive immunoreactivity to eNOS. Enhanced immunoreactivity was detected in endothelial cells at certain time points for E-selectin, P-selectin and VCAM-1, but no immunoreactivity appeared in any other cell type present (Figure 3). E-selectin staining in endothelial cells was significantly increased at the 24 and 48 h time points (Figure 4). P-selectin staining was significantly enhanced in the endothelial cells of the injured arteries at 24 h, with a tendency to be increased at 2 and at 48 h (Figure 5). The staining pattern of VCAM-1, resembling that of E-selectin, was significantly increased in vascular endothelial cells after 24 and 48 h (Figure 6). After 8 days the level of staining had returned to normal for each of the adhesion molecules. For ICAM-1 no differences were apparent in the degree of staining between control arteries and injured arteries at any of the four time points.

Discussion

Immunocytochemistry

Balloon angioplasty of the rabbit subclavian artery has been previously characterized to involve growth of neointima, detectable at 7 days (Hadoke *et al*., 1995) following rupture of the internal elastic lamina with damage to the endothelium and medial layer. In the present study a low level of immunoreactivity for the adhesion molecules E-selectin, P-selectin, VCAM-1 and ICAM-1 was found on endothelial cells in healthy arteries. The first three adhesion molecules were upregulated in those endothelial cells remaining after angioplasty at 24 and 48 h (i.e. damaged endothelial cells) but

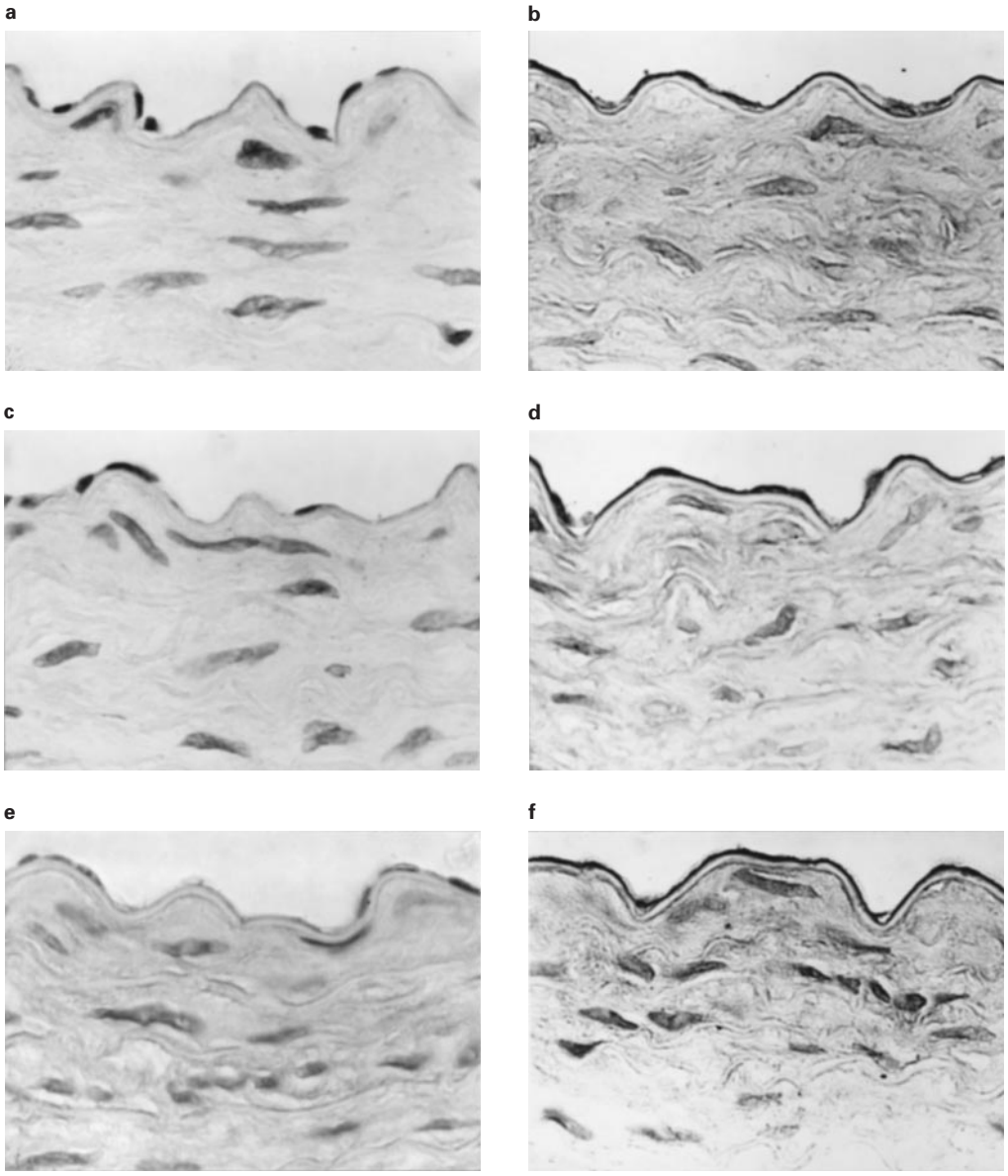

Figure 3 Immunocytochemical staining of left and right subclavian arteries with the endothelial surface upwards to the top of each photograph. The control right subclavian arteries stained for E-selectin, P-selectin and VCAM-1 are shown in (a), (c) and (e) respectively. The left subclavian arteries subjected to balloon angioplasty and then stained for E-selectin, P-selectin and VCAM-1 are shown in (b), (d) and (f) respectively. Enhanced endothelial staining intensity is seen in (b), (d) and (f) compared with the matched controls. E-selectin and P-selectin staining is shown at the 24 h timepoint after angioplasty whereas VCAM-1 staining is shown at 48 h after angioplasty. Magnification $\times 400$.

expression was normal at 8 days after injury (including regenerated endothelium). The smooth muscle did not express adhesion molecules before or after injury. The level of E-selectin was significantly raised at 24 and 48 h after injury and the level of P-selectin was significantly raised at 24 h after injury. E-selectin is normally expressed 2–8 h after endothelial activation (Hsu *et al*., 1981) and its persistence at 48 h in the present study may indicate continuous activation of the remaining endothelium, possibly induced by leukocyte-derived free radicals or cytokines. E-selectin controls leukocyte rolling by an interaction with leukocyte-expressed counter-receptors and therefore could be responsible for the enhanced adhesion observed in injured vessels in the present study. This would cause leukocyte activation, leading to firm adhesion *via* ICAM-1. *In vivo* this would allow adherence to the injured area without the necessity for direct activation of circulating leukocytes by the angioplasty procedure. ICAM-1 expression remained constant at all time points in this study. Expression of VCAM-1, like E-selectin, was significantly increased at 24 and 48 h after injury. VCAM-1 interacts with the adhesion molecule VLA-4 which is expressed on monocytes and lymphocytes (Gerszten *et al*., 1996) but not neutrophils. It has been reported that VCAM-1 expression is sensitive to oxidant stress (Marui *et al*., 1993) and therefore the presence of activated leukocytes at the site of injury may ultimately enhance the expression of this molecule. Although it is likely that the inflammatory cell infiltrate in the media of injured vessels consisted predominantly of polymorphonuclear leukocytes, the upregulation of VCAM-1 after injury may initiate the adhesion and transmigration of monocytes/macrophages which would be required to phagocytose necrotic tissue and promote healing and scar formation. Thus the present results suggest that, following balloon angioplasty injury, the endothelium undergoes changes that could facilitate the adhesion of neutrophils and macrophages *via* E-selectin, P-selectin, ICAM-1 and VCAM-1. Although no change in the

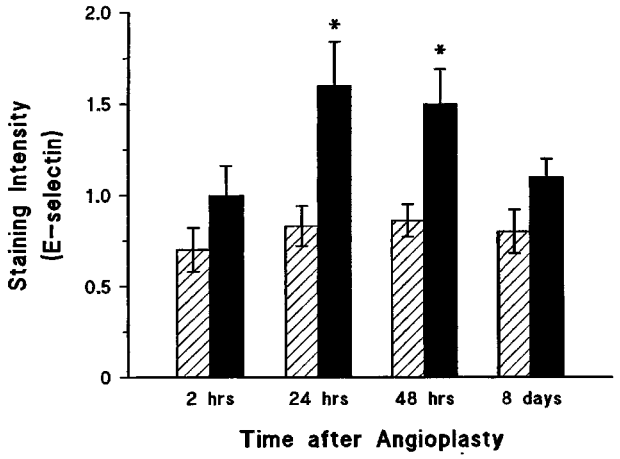

Figure 4 Quantification of E-selectin staining in endothelial cells of injured (left) and control (right) rabbit subclavian arteries removed at various time points after balloon-induced injury. Number of vessels analysed: 2 h ($n=5$), 24 h ($n=6$), 48 h ($n=7$), 8 days ($n=5$). Values are expressed as means ± s.e.mean. ★$P<0.05$ with respect to the non-injured artery at the same time point.

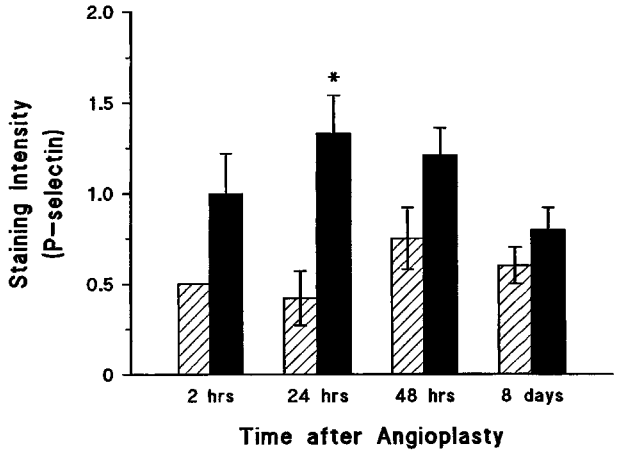

Figure 5 Quantification of P-selectin staining in endothelial cells of injured (left) and control (right) rabbit subclavian arteries removed at various time points after balloon-induced injury. Number of vessels analysed: 2 h ($n=5$), 24 h ($n=6$), 48 h ($n=7$), 8 days ($n=5$). Values are expressed as means ± s.e.mean. ★$P<0.05$ with respect to the non-injured artery at the same time point.

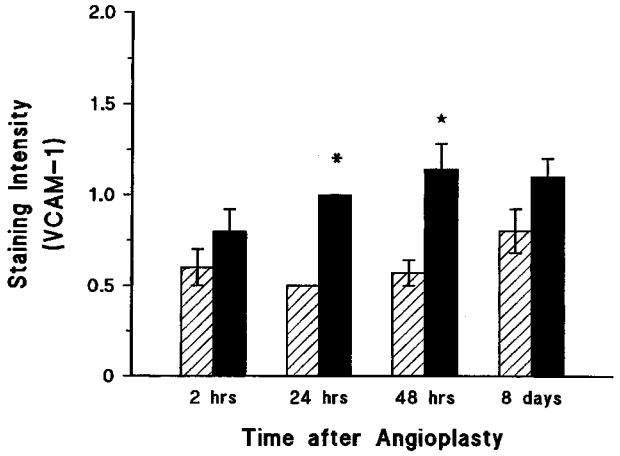

Figure 6 Quantification of VCAM-1 staining in endothelial cells of injured (left) and control (right) rabbit subclavian arteries removed at various time points after balloon-induced injury. Number of vessels analysed: 2 h ($n=5$), 24 h ($n=6$), 48 h ($n=7$), 8 days ($n=5$). Values are expressed as means ± s.e.mean. ★$P<0.05$ with respect to the non-injured artery at the same time point.

expression of ICAM-1 was found, it is possible that there may be changes in the avidity of this molecule that could further increase the adhesion of leukocytes following injury (Sellak *et al.*, 1994).

Leukocyte infiltration

Morphological analysis of the injured vessels showed significant numbers of leukocytes, mainly neutrophils, present within the artery wall at all time points. The entry of leukocytes into the artery wall occurs following adhesion, and in response to chemoattractants and cytokines (Nilsson, 1993). Moreover, the presence of leukocytes within the artery wall may further upregulate adhesion molecule expression in the endothelium (Wang *et al.*, 1994) through secretion of cytokines.

The effects of vascular balloon injury on leukocyte adhesion

In the present study, the injured artery had markedly increased adhesiveness to leukocytes 2, 24 and 48 h after angioplasty. This increased adhesiveness is a property of the injured vessel and not due to a change in the leukocytes, since adhesion to the undamaged right subclavian artery was unaltered. Thus any changes that the angioplasty procedure had induced in the circulating leukocytes (Baj *et al.*, 1994; Neumann *et al.*, 1996; Mickelson *et al.*, 1996; Inoue *et al.*, 1996) were not by themselves sufficient to induce a measurable increase in adhesion. The most likely explanation for the increased adhesiveness of leukocytes was due to upregulation of E-selectin, P-selectin and VCAM-1 that was observed at 24 and/or 48 h. In addition, we found that adhesion of leukocytes was unaltered by removal of the endothelium, demonstrating the importance of interaction of leukocyte integrins with components of the extracellular matrix (Walzog *et al.*, 1995). Thus the increased adhesion that we observed 2 h after injury may be mediated partly by the interaction of leukocytes with exposed sub-endothelial matrix and small areas of thrombus.

The effect of balloon injury on vascular responses to leukocytes

In the present study it has been shown that leukocyte-induced contraction of the rabbit subclavian artery rings was abolished at 2, 24 and 48 h after artery injury. The artery rings retained normal contractile responsiveness to 5-HT and thus the injury procedure did not alter overall contractile ability of the artery. Since leukocytes always gave normal contractions of uninjured (right) subclavian arteries, the loss of contractile ability following injury must be due to a change in the artery wall and not in the leukocytes. These results show that the vasoconstriction which occurs after angioplasty (Merhi *et al.*, 1995) is not caused by an enhanced response to leukocyte-induced vasoconstriction, despite increased leukocyte adhesion. The loss of vasoconstriction on addition of leukocytes is probably due to denudation of the endothelium by the angioplasty procedure. In agreement with this, we found that leukocyte-induced contraction of the rabbit subclavian artery in the present study was reduced by L-NAME.

Leukocytes release both vasoconstrictor substances (e.g. leukotrienes, enzymes) and vasodilator substances (e.g. superoxide, hypochlorite) and the vascular contraction that they produce depends on the balance between them. There is evidence that endothelial NO can diminish the release of oxygen-derived radicals, and thus inhibition of NO can prevent leukocyte-induced contraction (Kennedy *et al.*,

1998). Thus at the 8 day time point, when some endothelial function had returned, as demonstrated by a small carbachol-induced relaxation, the leukocytes were able to produce a small contraction. Thus at the time when leukocyte adhesion and infiltration was greatest, leukocyte-induced contraction was absent.

Conclusions

The present study has been the first to correlate adhesion molecule expression, adhesiveness of the artery wall and contractile response to leukocytes following vascular injury. It is concluded that angioplasty increases expression of E-selectin, P-selectin and VCAM-1 leading to enhanced adhesiveness of the damaged artery for leukocytes, and increased migration of leukocytes into the artery wall. Leukocyte-induced contraction was abolished following artery injury. Thus the present study provides evidence that the role of leukocytes in restenosis following vascular injury could be at the time point when cell proliferation occurs but is unlikely to be important at the stage of vascular spasm.

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