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Vascular Adhesion Protein-1, Intercellular Adhesion Molecule-1 and P-Selectin Mediate Leukocyte Binding to Ischemic Heart in Humans

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OBJECTIVES The expression of endothelial adhesion molecules and their functional significance in leukocyte adhesion to human myocardial blood vessels in acute myocardial infarction (AMI) were studied.

BACKGROUND Leukocyte extravasation, mediated by specific adhesion molecules, exacerbates tissue injury after restoration of blood supply to an ischemic tissue. Experimental myocardial reperfusion injury can be alleviated with antibodies that block the function of adhesion molecules involved in leukocyte emigration, but the relevant molecules remain poorly characterized in human AMI.

METHODS Semiquantitative immunohistochemistry and in vitro adhesion assays were used to study the expression and granulocyte binding abilities of different endothelial adhesion molecules in human AMI. Changes in the molecular nature of vascular adhesion protein-1 (VAP-1) were evaluated using immunoblotting.

RESULTS Certain endothelial adhesion molecules (intercellular adhesion molecule [ICAM-2], CD31 and CD73) were expressed in myocardial blood vessels homogeneously in normal and ischemic hearts, whereas others (E-selectin and peripheral lymph node addressin) were completely absent from all specimens. The synthesis of ICAM-1 was locally, and that of P-selectin regionally, upregulated in the infarcted hearts when compared with nonischemic controls. Vascular adhesion protein-1 showed ventricular preponderance in expression and alterations in posttranslational modifications during ischemia-reperfusion. Importantly, P-selectin, ICAM-1 and VAP-1 mediated granulocyte binding to blood vessels in the ischemic human heart.

CONCLUSIONS Human P-selectin, ICAM-1 and VAP-1 appear to be the most promising targets when antiadhesive interventions preventing leukocyte-mediated tissue destruction after myocardial ischemia are planned. (J Am Coll Cardiol 2000;36:122–9) © 2000 by the American College of Cardiology

Inappropriate inflammatory responses can cause severe tissue destruction. During ischemia-reperfusion, such as in acute myocardial infarction (AMI), the tissue damage may result not only from direct anoxic and hypoxic injury but also from other deleterious events occurring after the re-establishment of blood flow to the occluded vascular bed. The reperfusion injury is partly caused by oxygen radicals, proteolytic enzymes and cytokines released by adhered and activated leukocytes that infiltrate into the affected area (1,2), because neutrophil depletion or prevention of neutrophil accumulation significantly diminishes tissue damage and enhances the recovery of cardiac function (3–8).

Leukocytes gain entrance into the myocardium by binding to and passing through the endothelial layer of blood vessels. Multiple homing-associated leukocyte receptors interact in a concerted manner with vascular adhesion ligands during the process of extravasation (9,10). On endothelium, selectin-type adhesion molecules (E-selectin [CD62E] and P-selectin [CD62P]) can bind leukocytes under conditions of flow and mediate the initial tethering and rolling events. After activation of the leukocyte, other endothelial molecules such as intercellular adhesion molecule-1 (ICAM-1, CD54) and ICAM-2 (CD102) and vascular cell adhesion molecule-1 (VCAM-1, CD106) and CD31 are brought into play. These molecules allow stable adherence of the leukocyte and the subsequent extravasation between endothelial cells. Still other endothelial adhesion molecules, like vascular adhesion protein-1 (VAP-1) (11) and CD73 (12) are also involved in leukocyte-endothelial cell interactions.

An upregulation of adhesion molecules in myocardial ischemia and reperfusion in animal models has been reported (13–15). The success of reducing injury during reperfusion by anti-adhesive therapy in animals raises hopes for analogous intervention strategies in humans. An adhesion molecule that is absent from the normal myocardium but is induced after ischemia would be an ideal target for therapy. Here we analyzed the expression and granulocyte-

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Abbreviations and Acronyms

AMI	= acute myocardial infarction
EAM	= endothelial adhesion molecule
ICAM	= intercellular adhesion molecule
mAb	= monoclonal antibody
PNAd	= peripheral lymph node addressin
VAP-1	= vascular adhesion protein-1
VCAM-1	= vascular cell adhesion molecule-1

binding function of several endothelial adhesion molecules (EAMs) in normal and infarcted cardiac specimens in man.

METHODS

Cardiac specimens. The ischemia-reperfusion material consists of 10 patients who died in the coronary care unit of Turku University Central Hospital due to AMI (Table 1). At autopsy, samples across the entire thickness of the ventricular wall were taken from the macroscopically infarcted area, remote left ventricle, borderzone between the infarcted area and the remote myocardium and from the ischemia-reperfusion area. Reperfusion specimens were obtained from six thrombolyzed patients (with rapidly resolving ST changes and resolution of the chest pain [16]) from the area supplied by a patent infarct-related artery.

Autopsy samples of nonischemic ventricles (8) and atria (3) were from patients who died of non-AMI causes. We also studied right atrial samples obtained from 12 patients undergoing heart surgery. All these tissues were macroscopically normal.

The mean age in the control group was 55 years and in the infarcted group 63 years. In the control group 3/8 and in the infarcted group 2/10 were women. To ensure optimal quality of these autopsy samples, special arrangements were made to shorten the time between death and autopsy to $<$48 h whenever possible (mean 2.4 days).

Human tonsil tissue was used as a positive control and as a standard for staining intensity in each experiment. In studies controlling possible effects of postmortem autolysis with tonsils intentionally exposed for autolysis for 0 to 8 days, hilar lymph nodes from cadavers stored up to six days and surgical samples from papillary muscle, we did not see any significant changes in the ability of our antibodies to react with EAMs. The only exception was VCAM-1, which in the tonsil showed slight but progressive decrease. All procedures for tissue collection were approved by the National Board of Medicolegal Affairs in Finland.

Table 1. The Descriptive Data of the AMI Patients

Patient Number	Age of AMI at Death	Reperfusion (+ = yes; − = no)
1	12 h	−
2	3 days	+
3	21/2 days	+
4	24 h	+
5	6 h	−
6	18 h	+
7	3 days	−
8	11 h	+
9	2 days	−
10	7 days	+

AMI = acute myocardial infarction.

Antibodies and immunohistochemical stainings. Mouse monoclonal antibodies (mAbs) against human CD31 (2C8, IgM), CD73 (4G4, IgG1), ICAM-1 (5C3, IgG1), ICAM-2 (CBR-1C2/2, IgG2a), E-selectin (P2H3, IgG1 and 1.2B6, IgG1), P-selectin (WAPS12.2, IgG1), VAP-1 (1B2, IgM), VCAM-1 (P8B1, IgG2b) and negative controls 3G6 (IgG1, against chicken T cells), 7C7 (IgM, against chicken bursal epithelium), kit2c75 (IgG2a, against chicken c-kit receptor), 3-298 (IgG2b, against chicken CD8 alpha) and MB40.5 (IgG1, against human HLA-ABC) were used. Peripheral lymph node addressin (PNAd) was detected with mAb MECA-79 (rat IgM). None of the mAb stains formalin-fixed, paraffin-embedded sections, and, therefore, use of frozen sections was mandatory.

Samples of the infarction hearts were processed to paraffin embedded histological specimens in a standard way. The adjacent tissue samples were immediately embedded in OCT-compound (Tissue-Tec, Miles Inc., Elkhart, Indiana), frozen in liquid nitrogen, and 5 to 8 μm thick serial sections were cut. One section stained with the van Gieson method was used to ascertain sample classification (infarction, borderzone, remote or reperfused myocardium) and to localize the inflammatory cell infiltrates. The remaining sections were stained for the EAMs using indirect immunoperoxidase technique as described (11).

Data analysis. All samples were immunostained at least twice. The interexperimental variation of the staining intensity was controlled by including samples from the infarction and control hearts in the same experiments and by including a few given specimens in each staining batch. All sections were first studied for positivity, and the staining intensity and the number of positive vessels were recorded in the whole sections.

In the case of ICAM-1, P-selectin and VAP-1, a semiquantitative scoring (17) was used. In this system the number and intensity of positive cardiac vessels was analyzed in comparison with tonsil vessels used as a positive control: score 0 was assigned to samples with no positive blood vessels and score 3 to samples with staining equal to tonsil. Scores 1 and 2 were adjusted to cover the staining patterns in between (see also examples in Fig. 1 and 2). In the case of VAP-1, score 4 was given to the samples that had more and brighter VAP-positive vessels than in the tonsil. The samples were analyzed in a blinded manner by two independent readers (VAP-1 sections twice by three independent readers). The score for each EAM plotted Figure 3 is the mean of all estimates given to vasculature in each sample. The mean ($\pm$SEM) of each sample group is also shown. The data were statistically analyzed with Kruskal-Wallis test, analysis of variance with Bonferroni correction and Fisher's exact test when applicable, and the

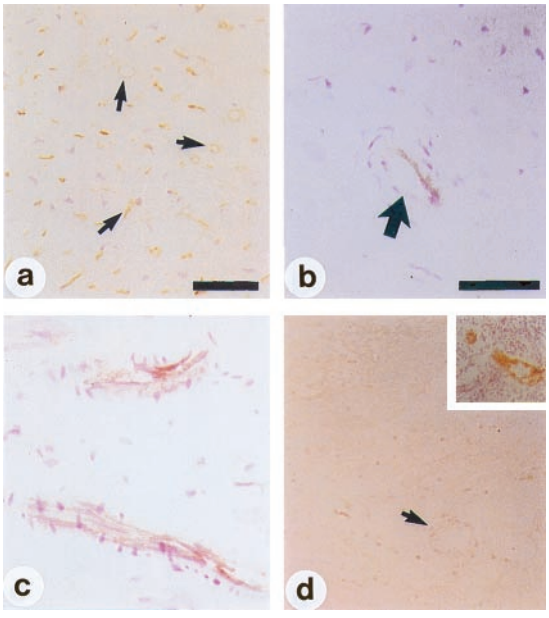

Figure 1. Cardiac expression of endothelial adhesion molecules. **a)** Blood vessels in normal myocardium are CD31 positive. **b)** A VCAM-1 positive blood vessel in normal atrium. **c)** ICAM-2 positive vessels in infarction heart. **d)** Negative staining of infarcted heart with anti-E-selectin mAb (similar absence of reactivity was also seen with all negative control mAbs, data not shown). In the inset, E-selectin positive vessels in the tonsil are shown. Representative vessels are indicated by **arrows.** Bar shown in **(a)** is 100 μm. The bar shown in **(b)** is 50 μm in all other figures. ICAM = intercellular adhesion molecule; mAb = monoclonal antibody; VCAM = vascular cell adhesion molecule-1.

statistical significance was set at $p < 0.05$. In addition, VAP-1, ICAM-1 and P-selectin positivity of endothelia in distinct vessel types (capillary, arterial and venous endothelia) were studied in 17 samples.

Functional in vitro tests. The adhesive function of P-selectin, ICAM-1 and VAP-1 in the heart samples was evaluated using an in vitro Stamper-Woodruff binding assay (18). In brief, saturating concentrations of function-blocking mAbs against VAP-1, P-selectin, ICAM-1, a pool of isotype-matched negative controls or medium alone were incubated on the frozen heart sections. Thereafter, granulocytes freshly isolated from healthy volunteers (3×10^6 cells in 50 μl RPMI1640 medium containing 10% FCS) were applied onto each tissue section under constant rotation. After a 30 min rotation at +7°C, the unattached granulocytes were tilted off, and the sections with adherent cells were fixed with 1% glutaraldehyde. The granulocytes attached to the blood vessels of the sections were counted under a dark field microscope.

Biochemistry of VAP-1. The molecular weights of VAP-1 in remote myocardium, borderzone and infarcted areas and in the tonsil were compared in immunoblotting using a previously described method (19). In brief, tissue samples were lysed, digested with Vibrio cholerae neuraminidase, run under nonreducing conditions in SDS-PAGE and analyzed using enhanced chemiluminescence detection system (ECL, Amersham Int.; Buckinghamshire, United Kingdom).

RESULTS

Induction of ICAM-1, P-selectin and VAP-1 in post-ischemic hearts. Frozen sections from hearts were immunohistochemically stained for nine different EAMs. CD31 (Fig. 1a), ICAM-2 and CD73 were constitutively present in all samples, and the reactivity was confined to the endothelial cells. E-selectin and the PNAd were completely absent from the normal hearts in every specimen. Vascular cell adhesion molecule-1 was weakly positive in the vessels of 12/15 atrial samples but only in 1/8 of normal ventricles (Fig. 1b). Intercellular adhesion molecule-1 showed mainly weak staining in normal heart (Fig. 2a and 3a). Faint P-selectin expression was observed in approximately half of the atrial samples. In normal ventricles, only one sample out of eight showed a few P-selectin positive vessels (Fig. 3b). Vascular adhesion protein-1 was expressed in all specimens in the small vessels of the myocardium and in the muscular layer of larger blood vessels. The ventricular expression of VAP-1 was statistically significantly higher than that in the atria (Fig. 3c and Table 2). Thus, cardiac vessels in normal ventricles practically lack E-selectin, P-selectin and VCAM-1, synthesize low levels of ICAM-1 and ICAM-2 and are clearly positive for VAP-1.

In the reperfused infarction hearts up to 90% of the leukocytes seen in the myocardium in patients dying within two days were polymorphonuclears, whereas in the older lesions the proportion of lymphocytes increased to 75%. Expression of CD31, CD73, ICAM-2 (Fig. 1c), PNAd and E-selectin (Fig. 1d) was similar in the AMI ventricles and in the ventricles of controls. The absence of E-selectin in the area of infarction was confirmed by another anti-E-selectin mAb against a different epitope. Only samples from one AMI heart were weakly positive for VCAM-1. In contrast, the number of ICAM-1 positive vessels and their intensity of staining in the AMI ventricles was higher than in the ventricles of the control hearts (Figs. 2a, b, 3a and Table 2). There were no statistically significant differences in ICAM-1 expression between remote, borderzone, infarcted and reperfused areas or between capillary and venous endothelia. Also P-selectin showed induction during AMI (Fig. 2c to 2e and Fig. 3b). When compared with normal ventricles, the upregulation of P-selectin was statistically significant in the remote parts and in the reperfused areas of the AMI hearts (Fig. 3b and Table 2). The induction of P-selectin was mainly seen in capillaries or in endothelia of larger (venous) blood vessels. The slightly lower P-selectin expression in the infarcted areas was due to less intense expression of P-selectin in the capillary size blood vessels. The overall ventricular expression of VAP-1 in capillaries and venous endothelial cells was not upregulated during AMI (Fig. 3c and Table 2). However, the intensity of VAP-1 expression varied in the infarction hearts (Fig. 2, f to h). Vessels within the areas of dense leukocytic infiltration often displayed strong VAP-1 expression (Fig. 2h).

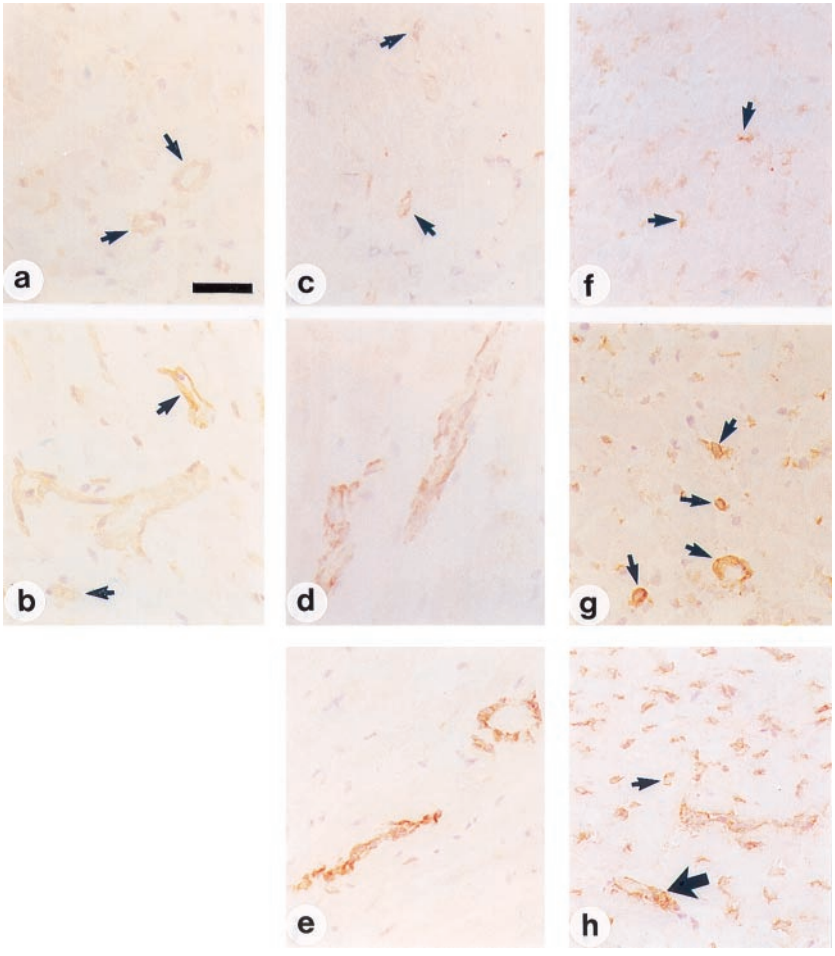

Figure 2. Inducibility and scoring of ICAM-1, P-selectin and VAP-1. **a)** Moderate (score 2) and **b)** strong (score 3) ICAM-1 positive samples. **c)** Weak (score 1), **(d)** moderate (score 2) and **(e)** strong (score 3) P-selectin positive vessels in heart specimens. **f)** Moderate (score 2), **(g)** strong (score 3) and **(h)** extremely strong (score 4) VAP-1 positive vessels in infarction heart. Representative vessels are indicated by **arrows.** Bar shown in **(a)** is 50 μm. ICAM = intercellular adhesion molecule; VAP-1 = vascular adhesion protein-1.

We did not observe any systematic trend of increasing or decreasing expression of EAMs when the scores of semiquantitatively analyzed molecules were correlated to the time elapsed from the first symptoms of AMI to the death of the patients. We conclude that the expression of ICAM-1 and P-selectin is induced in all parts of the ischemic heart, and the most intense VAP-1 expression is found at areas of leukocytic infiltration. In contrast, the expression of other adhesion molecules does not change during ischemia-reperfusion.

VAP-1 undergoes structural changes during ischemia. Since the function of an adhesion molecule is determined not only by its expression but by appropriate posttranslational modifications as well, we studied VAP-1 as an example of possible effects of ischemia on the processing of a protein. In the normal heart (lane 6 in Fig. 4) VAP-1 was exactly of the same molecular weight as in lymphoid organs (lane 9 in Fig. 4). In the ischemic heart, the apparent molecular weight of VAP-1 was slightly higher. The apparent molecular weight of cardiac VAP-1 increased after a sialidase treatment, the effect being more pronounced in ischemic than in normal tissue. The electrophoretic mobility of the sialidase-treated VAP-1 from normal myocardium remained faster than that from the ischemic sites. The retardation of electrophoretic mobility after sialidase treatment is consistent with the removal of large numbers of negatively charged sialic acids from this sialoglycoprotein molecule. The biochemical analyses were repeated with three other patients with the same results. These findings indicate that VAP-1 in all specimens carries these sialic acid modifications but that the normal heart had a smaller protein core or differences in other oligosaccharide modifications.

P-selectin, ICAM-1 and VAP-1 mediate granulocyte binding to myocardium. Based on the expression data, the adhesive function of ICAM-1, P-selectin and VAP-1 in mediating granulocyte binding to the blood vessels of the infarcted myocardial specimens was studied using an in vitro adhesion assay. In this binding assay only occasional bound granulocytes (1–3/100 vessels) were seen in the normal control heart. In remote areas of the infarcted heart, only a few adherent leukocytes (1–8/100 vessels) were seen. In

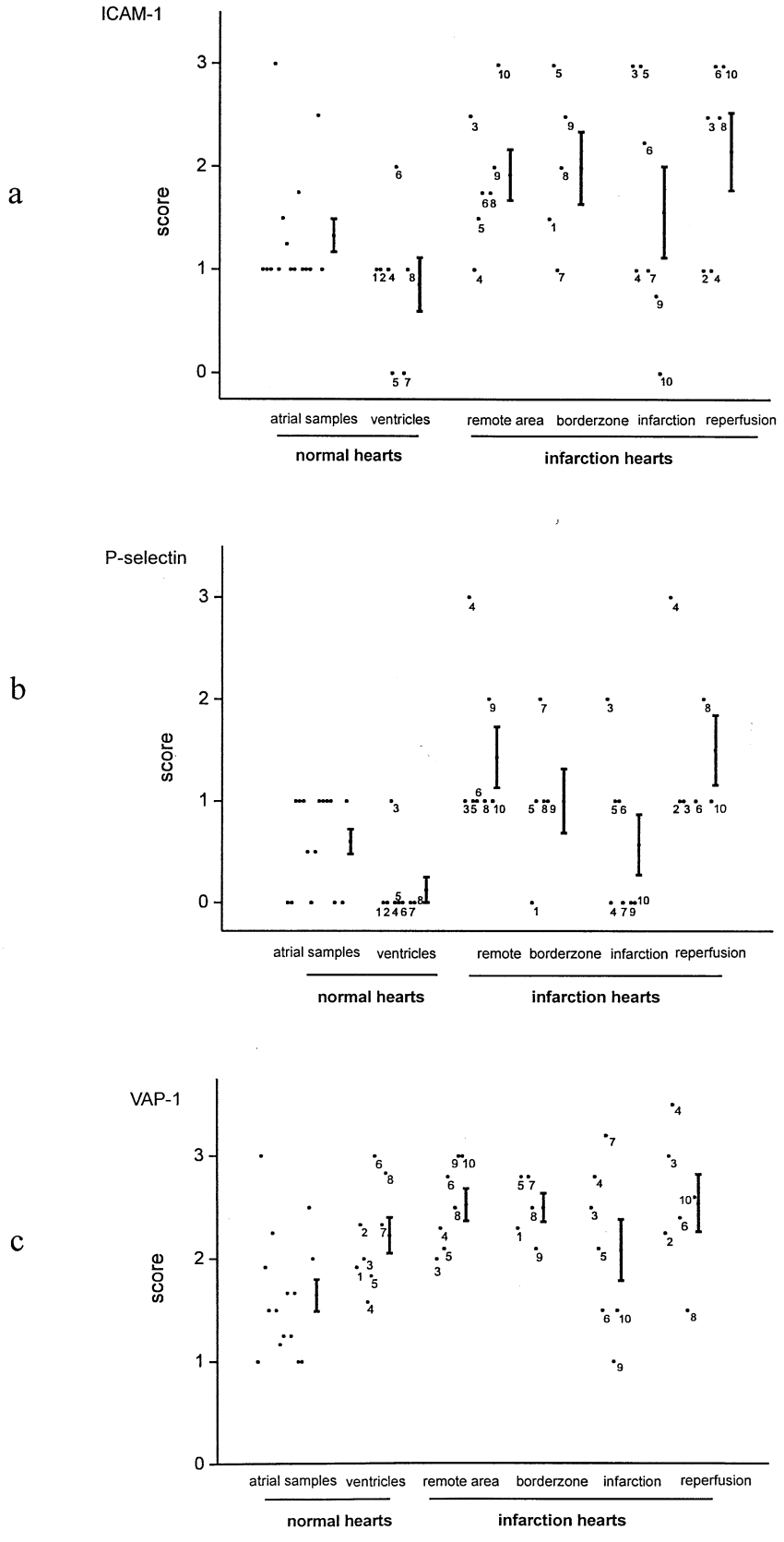

Figure 3. Intercellular adhesion molecule-1 and P-selectin are induced in ischemic heart, and VAP-1 expression shows ventricular dominance. The expression of **(a)** ICAM-1, **(b)** P-selectin and **(c)** VAP-1 in normal and infarction heart specimens were scored as described in the Methods section. The numbers refer to the numbers of individual acute myocardial infarction and control patients. ICAM-1 = intercellular adhesion molecule-1; VAP-1 = vascular adhesion protein-1.

Table 2. Summary of ICAM-1, P-Selectin and VAP-1 Expression in the Heart

Tissue	Sample	ICAM-1	P-Selectin	VAP-1
Normal	atrial	1.3 ± 0.16*	0.60 ± 0.12	1.6 ± 0.15]†
	ventricle	0.86 ± 0.26]]]†	0.13 ± 0.13]‡	2.2 ± 0.17]
Infarcted	remote	1.9 ± 0.25]	1.4 ± 0.30]	2.5 ± 0.16
	borderzone	2.0 ± 0.35]	1.0 ± 0.32	2.5 ± 0.14
	infarction	1.6 ± 0.45]	0.57 ± 0.30	2.1 ± 0.30
	reperfused	2.2 ± 0.38]	1.5 ± 0.34]	2.5 ± 0.28

*The semiquantitative scores of each sample group are given as mean ± SEM. Sample groups that differ statistically significantly are connected.
†$p < 0.05$; ‡$p < 0.01$.
ICAM-1 = intercellular adhesion molecule-1; VAP-1 = vascular adhesion protein-1.

contrast, in the reperfused areas many granulocytes specifically interacted with cardiac vessels (37–208 granulocytes/100 vessels; Fig. 5a). Granulocyte binding revealed a strong preference to certain vessels since even in the reperfused hearts many individual vessels lacked and others were filled with adherent granulocytes.

Blocking of VAP-1 on the endothelial cells of the myocardial samples reduced the number of the adherent granulocytes by 60% when compared with control antibodies (Fig. 5b). Pretreatment of the cardiac sections with function-blocking mAbs against ICAM-1 and P-selectin also abrogated about half of the granulocyte binding to cardiac vessels. When all three adhesion molecules were rendered nonfunctional at the same time, an additive inhibitory effect was seen. Thus, in the vessels of infarcted human heart ICAM-1, P-selectin and VAP-1 mediate granulocyte binding in vitro.

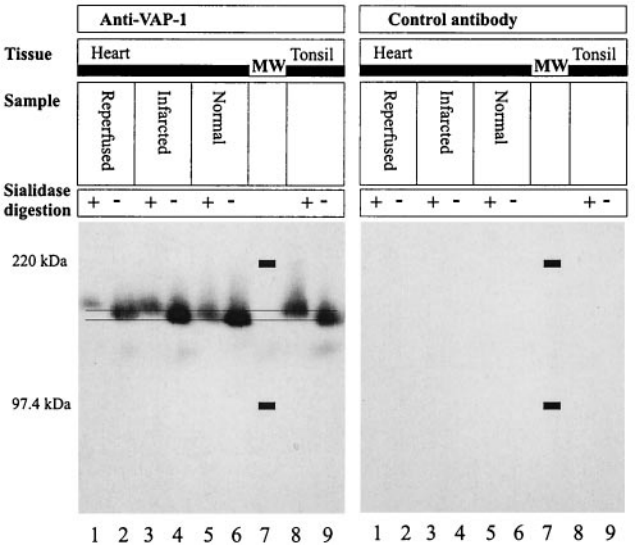

Figure 4. Molecular weight alterations of VAP-1 in the heart. After SDS-PAGE and immunoblotting, VAP-1 was immunodetected from untreated and sialidase treated lysates from normal, reperfused and infarcted heart and tonsil as control tissue. To assist size comparisons, all samples were analyzed in parallel lanes of the same gel, and after the scanning two horizontal lines were drawn. In the molecular weight (MW) lane the positions of the markers otherwise not visible in enhanced chemiluminescense are shown. VAP-1 = vascular adhesion protein-1.

DISCUSSION

Inducibility and localization of EAM in AMI hearts. Our material of ischemically injured human myocardium covers samples in which the tissue has been exposed to ischemia and reperfusion for various periods of time and, thus, provides an informative cross-sectional analysis of EAMs in human AMI. We showed that the normal heart expresses constitutively several endothelial adhesion molecules (CD31, CD73, ICAM-1, ICAM-2 and VAP-1) whereas E-selectin and PNAd are not present. Endothelial adhesion molecules are, in general, equally expressed in the atrial and ventricular myocardium of the normal heart (except VAP-1, see following text).

We found ICAM-1 and P-selectin to be upregulated during AMI. However, the induction was not limited to areas of ischemia-reperfusion. In fact, increased expression of ICAM-1 and leukocytic infiltration have also been found in the nonischemic areas of the infarcted heart (20–22). There may be several systemic factors contributing to the induction of these molecules in the whole malfunctioning heart. Cytokines, including IL-6 and TNF-alpha, which can be found in blood during hypoxia-ischemia of the heart, are potent inducers of synthesis of ICAM-1 and P-selectin (15,23). Hemodynamic changes, known to affect the synthesis of adhesion molecules (24), also take place during infarction.

P-selectin can be upregulated transiently within minutes in animal models. In addition to this release from storage granules, a slower, protein synthesis dependent modulation of P-selectin is also known (25), which probably accounts for our findings of P-selectin induction in AMI. E-selectin is also upregulated in an animal ischemia-reperfusion model (14) and in the human heart during allograft rejection (26,27). In contrast, in our series E-selectin was absent from all human heart specimens. The induction of E-selectin requires new protein synthesis and is maximal at 2 to 8 h (28). If E-selectin was expressed in ischemic human myocardium even transiently, it should have been seen in the patient who died 6 h after the beginning of the symptoms. Thus, we believe that genuine differences do exist in the extent and kinetics of P- and E-selectin expression between the in vivo models and in vivo situations in man, further emphasizing the importance of human studies.

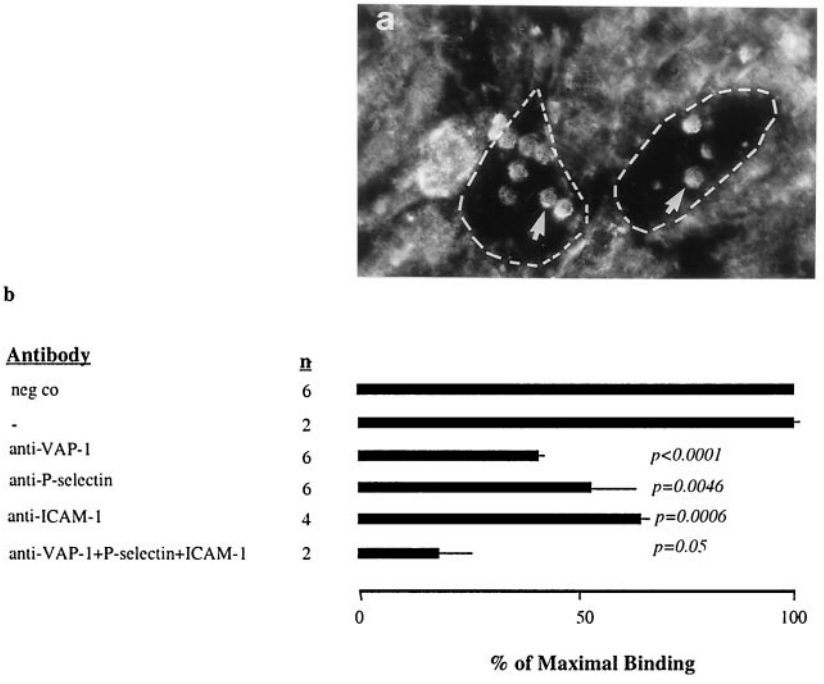

Figure 5. Intercellular adhesion molecule-1, P-selectin and VAP-1 mediate granulocyte adhesion to vessels in infarcted heart. **a)** In this dark field picture two cardiac vessels **(dashed lines)** are shown, one with seven bound granulocytes and the other supporting the binding of two granulocytes **(arrows). b)** Anti-VAP-1, P-selectin and anti-ICAM-antibodies separately or in combination decrease the number of granulocytes adhering to myocardial blood vessels when compared with the specimens treated with nonbinding (set as 100%) control antibodies or without any antibodies (−). ICAM = intercellular adhesion molecule; VAP-1 = vascular adhesion protein-1.

We observed a clear quantitative difference in the expression of VAP-1 between the atrial and ventricular myocardium. The finding was consistent in samples taken during surgery and autopsies. In an animal model the expression of VAP-1 expression in normal tissue is solely intracellular, whereas in the inflamed tissue luminal VAP-1 expression is found (29). Since definitive discrimination of VAP-1 reactivity in cytoplasmic granules (30) and at luminal (functional) position on the endothelial surface is not possible with immunohistochemistry in postmortem specimens, it is possible that induction of functionally relevant expression of VAP-1 in ischemically injured tissue is more selective than observed in this study. Also, in AMI material, prominent leukocyte infiltration was often observed around brightly VAP-1 positive vessels, suggesting that in these areas VAP-1 expression may be functionally more significant than appears from the pooled data of the whole section (Fig. 3c). The unique observation of the structural changes in VAP-1 during AMI can be considered as an example of an additional control mechanism to regulate the function of the EAMs. It may apply to other adhesion molecules as well.

Functional relevance and clinical implications of EAM in AMI. In animal models of myocardial damage, anti-P-selectin antibodies (4) and anti-ICAM-1 antibodies (5,7) have reduced the amount of tissue necrosis by 20% to 50%. The expression pattern of VAP-1, ICAM-1 and P-selectin together with the reduction of leukocyte interaction with vessels in infarcted hearts by 37% to 78% after pretreatment with function blocking antibodies strongly suggest that these molecules mediate leukocyte immigration into ischemically injured heart in man. The ex vivo binding assay used reflects the actual migration patterns in vivo (31). Notably, granulocyte binding to inflamed tonsils is only marginally affected by anti-VAP-1 mAbs (11), and, hence, the VAP-1 dependence of binding of polymorphonuclear cells to cardiac vessels is a notable extension in the biological role of this molecule.

Conclusions. Our results show that the dynamic events in human AMI result in altered expression of functionally active endothelial molecules mediating leukocyte-endothelium interactions. Our results indicate that potential target molecules for studies to prevent leukocyte mediated cardiomyocyte damage and endothelial dysfunction in man are found among ICAM-1, P-selectin and VAP-1.

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