CARDIAC AND PULMONARY REPLACEMENT

REDUCTION OF MONOCYTE ADHESION TO XENOGENEIC TISSUE BY ENDOTHELIALIZATION: AN ADHESION MOLECULE AND TIME-DEPENDENT MECHANISM

Great interest has been shown for the seeding of autologous endothelial cells on prosthetic materials. We investigated the inflammatory and immunogenic properties of xenogeneic tissue before and after seeding with cultured human great saphenous vein endothelial cells in vitro. Adhesion of monocytes to xenogeneic tissue with or without endothelium and the endothelial cell expression of E-selectin, intercellular adhesion molecule 1, vascular adhesion molecule 1, and major histocompatibility complex class II antigens were investigated 1, 3, and 7 days after seeding. Both monocyte adhesion and endothelial adhesion molecule expression were relatively high 1 day after seeding and were significantly lowered after 3 to 7 days. There was no difference between monocyte adhesion and adhesion molecule expression on viable or nonviable xenogeneic tissue. Monocyte adhesion and adhesion molecule expression increased after interleukin-1 β or interferon- γ stimulation of the endothelial cells. The results suggest that human endothelial cells exhibit an early proinflammatory and immunogenic activity immediately after seeding. Three and 7 days after seeding, the endothelialized surface is less adhesive for monocytes as compared with nonendothelialized tissue. These findings have implications when cultured or intraoperatively recruited endothelial cells are used clinically. (*J THORAC CARDIOVASC SURG* 1995;110:1583-9)

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The use of bioprosthetic heart valves is limited because of several problems related to the nature of the bioprosthetic tissue. It is likely that the absence of a protective barrier of endothelial cells (ECs) between the blood and the vessel wall not

only makes the wall more thrombogenic, but also might be a cause of degeneration. The calcification is partly believed to be caused by plasma protein insudation into the matrix of the cusps.¹ The degenerative process involves the loss of the donor endothelium with subsequent disorganization of the underlying collagen fibers, fibroblast necrosis, and leaflet thickening.^{2,3} Degeneration of biologic vascular tissue partly depends on exposure of the native xenogeneic or allogeneic cells and matrix components to circulating leukocytes. The nonendothelialized vessel wall may also be a nidus for bacterial infection and the origin for intrinsic or extrinsic calcification.⁴ Intrinsic calcification occurs as a result of biochemical properties of the material itself, for example, glutaraldehyde-dependent calcification. Extrinsic calcification is associated with the elements or tissue not initially implanted, for example, calcification within thrombus, infected vegetations, or other pseudointima.⁵ Furthermore, there is strong evidence that glutaraldehyde fixation, which is the predominating technique used for tissue tan-

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ning, itself promotes calcification and tissue degeneration.^{5,6} For these reasons the use of glutaraldehyde-fixated biologic valves is restricted mainly to elderly patients. Several different techniques to neutralize glutaraldehyde such as the use of L-glutamic acid⁷ have been presented. So far, no neutralizing technique has been described to extend durability or to allow EC ingrowth in vivo.

A decade ago, the vascular endothelium was considered a "nonsticking" lining of the vascular wall. Today, it is widely accepted that the endothelium is highly antithrombogenic and that especially the endothelium of the postcapillary venules plays a key role in the inflammatory response. Normally, the ECs form a barrier between the inflammatory cells and the vessel wall. Seeding of biologic grafts with autologous ECs has therefore been suggested as a way to decrease graft rejection, degeneration, and possibly calcification. ECs express adhesion molecules that bind circulating leukocytes, either constitutively or after induction by cytokines such as thrombin, interleukin-1 (IL-1), tumor necrosis factor- α , or interferon- γ (IFN- γ). At least six leukocyte-binding adhesion molecules expressed by human ECs have been identified: P-selectin, E-selectin, intercellular adhesion molecule 1 (ICAM-1), ICAM-2, vascular adhesion molecule 1 (VCAM-1), and platelet cellular adhesion molecule 1 (PCAM-1) (CD31).⁸⁻¹⁰

The ECs also express the ABO antigen system. They constitutively carry major histocompatibility complex class I (MHC class I) antigens and they have inducible MHC class II antigens (HLA-DR).¹¹ IFN- γ stimulation for 48 hours induces the expression of MHC class II antigens¹² and thus enables the ECs to act as antigen-presenting cells to T lymphocytes.¹¹

A potential hazard to the clinical outcome when freshly recruited or cultured ECs are used for endothelialization of cardiovascular prosthesis is a high expression of EC-leukocyte adhesion molecules. Recruitment of leukocytes would possibly enhance the degenerative process. The aim of this study was to investigate the interaction between monocytes, ECs, and EC adhesion molecule expression with a xenogeneic vascular wall. We used cultured adult human great saphenous vein ECs, the human monocytic cell line U 937, and porcine aorta for these investigations. The monocyte adhesion to deendothelialized versus reendothelialized xenogeneic tissue was studied. Cellular interactions with viable and nonviable xenogeneic tissues were compared. Time dependence of monocyte adhesion was

studied and compared with the expression of E-selectin, ICAM-1, VCAM-1, and MHC class II antigens.

Material and methods

Isolation, cultivation, and characterization of human great saphenous vein ECs. The use of human great saphenous veins was approved by the Ethics Committee at the Karolinska Hospital. ECs were isolated and cultured as described previously.¹³ In brief, 3 to 5 cm long residual segments of the great saphenous vein received from patients undergoing coronary bypass operations were used. The ECs were isolated by collagenase treatment (0.1%, Worthington, Freehold, N.J.). Harvested cells were routinely cultured in minimal essential medium (MEM) with the addition of 40% pooled heat-inactivated (56° C, 30 minutes) human serum, antibiotics, and cyclic adenosine monophosphate-elevating compounds. Two days before the experiments began, the ECs were detached with a 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid (1:1) solution and seeded in MEM containing only 30% human serum and antibiotics. The ECs were characterized as endothelial by immunohistochemical staining of von Willebrand factor-related antigen, PCAM-1, prostacyclin production, and through their typical cobblestone appearance.¹³ In each experiment, cells from a single donor in the fifth to the seventh passage were used.

Matrix preparation. Pig aorta specimens were procured under sterile conditions. The aortas were deendothelialized by firm but gentle scraping of the intimal surface with a scalpel blade as previously described.¹⁴ For devitalization, the specimens were put in deionized water and stored for 48 hours at 4° C. The viable pieces were stored in MEM containing 20% human serum (48 hours, 4° C). Before seeding, the pig aorta was allowed to soak overnight in seeding medium. The aorta was cut into pieces approximately 3 cm² and seeded as described in the following section. The viability of the pig aorta was secured through enzymatic digestion of minced pieces of the tissue by overnight incubation in 0.1% collagenase and 0.16% dispase (Boehringer Mannheim, GmbH, Mannheim, Germany) in MEM supplemented with 5% human serum and antibiotics. This was done on nonendothelialized tissue at 1, 3, and 7 days under culture conditions. Digested tissue was triturated, washed twice, and plated in MEM containing 30% human serum and antibiotics.

Cell seeding procedures and stimulation. The ECs were seeded at a density corresponding to 150,000 cells/cm² on pieces of viable and nonviable porcine aorta and on gelatin-coated tissue culture plastic as controls. The ECs were cultured in MEM containing 30% human serum and antibiotics for 1, 3, and 7 days on the respective matrix. Human recombinant IL-1 β (Sigma Chemical Co., St. Louis, Mo.; 100 U/ml for 12 hours in MEM containing 5% human serum) was used for stimulation of E-selectin, VCAM-1, and ICAM-1 expression. Human recombinant interferon (IFN- γ , Boehringer Mannheim, 1000 U/ml for 48 hours in MEM containing 5% human serum) was used for stimulation of MHC class II antigen expression. Immediate confluency on day 1 was confirmed with a modified hematoxylin and eosin staining (see Fig. 3, d)¹⁵ followed by

Fig. 1. Diagram showing time-dependent variations of expression of adhesion molecules E-selectin (*E-sel*), VCAM-1, and ICAM-1 on days 1, 3, and 7, as well as MHC class II antigen expression. Expression after stimulation with IL-1 β and IFN- γ is also shown. Human ECs were seeded on porcine aortic tissue and adhesion molecule expression was measured by FACS analysis. Expression was shown to be highest on day 1, and it declined on day 3 and remained low on day 7. Statistical analysis was made with means of mean fluorescence index from four to six experiments as single data points in a paired Student's *t* test. Asterisks denote *p* < 0.001.

examination in an epifluorescence microscope (Fluovort; Leica AB, Instruments GmbH, Nussloch, Germany.).

Monocyte adhesion procedures. Nonviable pig aorta specimens were cut into 3 cm² pieces and used deendothelialized or reendothelialized as already described. Monocytes from the cell line U 937 were used (250,000 cells/cm²) and allowed to attach for 30 minutes on specimens kept under culture conditions for 1, 3, and 7 days. Before day 7, specimens were stimulated with IL-1 β as described. The pieces were rinsed in phosphate-buffered saline solution followed by hematoxylin and eosin staining, inspection, and evaluation in a fluorescence microscope. On three or four micrographs of randomly chosen areas of each type of tissue from three or four different experiments, the number of adhered monocytes was counted in six squares per micrograph measuring 200 $\times$ 200 μ m at $\times$ 100 magnification. For statistical analysis, a paired Student's *t* test was applied on mean counts of

adhered monocytes on triplicate tissue samples in three or four experiments.

Fluorescence-activated cell sorting (FACS) analysis preparation. The ECs were detached with trypsin and ethylenediaminetetraacetic acid as described (6 minutes), followed by washing and centrifugation at 250 g in a solution of phosphate-buffered saline solution containing 0.5% bovine serum albumin. The cells were incubated on ice for 1 hour with the primary antibodies. Monoclonal antibodies raised in mice were used as follows: E-selectin diluted 1:200; ICAM-1 1:50; VCAM-1 1:100 (British Bio Technology, Abingdon, Great Britain), and MHC class II antigen 1:50 (Dakopatts A/S, Copenhagen, Denmark). As a negative control, a nonspecific polyclonal mouse immunoglobulin G1 antibody (Dakopatts) diluted 1:20 was used. After incubation the cells were washed in phosphate-buffered saline and bovine serum albumin solution and incubated with the secondary antibody, a fluorescein-

Fig. 2. Diagram showing monocyte adhesion to endothelialized and deendothelialized xenogeneic tissue. Variation in time-dependent manner depending on time after seeding was shown. There was decline in adhesion after 3 days and adhesion remained low on day 7. Adhesion to deendothelialized surface was significantly higher as compared with adhesion to endothelialized surface. When ECs were stimulated (*stim.*) with IL-1 β , there was significant increase in adhesion as compared with that on nonstimulated tissue. Statistical analysis was made with means of adhered monocytes of triplicate cultures from three or four experiments as single data points in paired Student's *t* test. Asterisks denote *p* < 0.001. w/o, without.

conjugated F(ab')₂ fragment of rabbit antimouse antibody (Dakopatts) at room temperature for 1 hour. The samples were fixed in paraformaldehyde (0.25% in phosphate-buffered saline solution) for up to 7 days before the FACS analysis.

FACS analysis. The samples were flushed before analysis to guarantee a single cell suspension. With a Becton Dickinson FACS-can instrument (Becton Dickinson & Co., Rutherford, N.J.) with a 488 nm laser and Consort 30 software used according to the manufacturer's instructions, 10,000 events (cells) were counted in each sample. A bead control was used to make comparative analysis possible among the different experiments.

Statistical analysis. On average 90% of the events were used for determination of the mean fluorescence index of each variable in each experiment. The number of experiments was four to six. The mean fluorescence index in each experiment was used as a single data point in a paired Student's *t* test. The standard error of the mean did not exceed 15% of the mean values.

Results

It was found that adhesion to the nonendothelialized porcine aorta was significantly more common than adhesion to the endothelialized specimens on days 3 and 7 (Fig. 2). The spontaneous adhesion of

monocytes to EC-seeded porcine aorta was highest on day 1, declined on day 3, and was still low 7 days after seeding (Fig. 2 and Fig 3, *a* and *c*). Stimulation of the endothelialized tissue with IL-1 β significantly increased the adhesion of monocytes as shown in Fig. 2 and Fig. 3, *b*.

A spontaneous time-dependent cellular adhesion molecule and MHC class II antigen expression by unstimulated human saphenous vein ECs seeded on xenogeneic vascular tissue was found. E-selectin, ICAM-1, VCAM-1, and MHC class II antigen expression varied as indicated in Fig. 1. This corresponded to the adhesion of monocytes. The adhesion molecule expression was similar on viable and nonviable xenogeneic tissue (Fig. 1). The adhesion molecule expression showed a similar pattern after seeding on gelatin-coated tissue culture wells (Fig. 1). However, it was also shown that the unspecific mouse immunoglobulin G binding to the human saphenous vein ECs was higher 1 day after seeding, although not as high as the specific adhesion molecule expression ($\leq 80\%$ of the lowest specific binding). The seeded human saphenous vein ECs responded with a significant increase in cellular adhesion molecule expression after IL-1 β or IFN- γ stimulation (Fig. 1). The unspecific binding was not increased after cytokine stimulation (not shown).

The confluency of the endothelium 1 day after seeding was confirmed by hematoxylin and eosin staining followed by inspection in a fluorescence microscope (Fig. 3, *d*).

The tissue viability was ensured by the establishment of fibroblast-like cells (vigorously migrating, non-colony forming, spindle-shaped cells with a length >2 times cell width) after enzymatic digestion of unseeded aortic tissue kept 48 hours at 4° C as described and thereafter under culture conditions for 1, 3, or 7 days. The proportion of viable cells versus nonviable cells in the tissue was not determined nor was the exact nature determined through specific staining. No cells could be isolated from nonviable tissue stored in sterile water as previously described.

Discussion

Because ECs play an important role in preventing thrombus formation and leukocyte adhesion and may also diminish plasma insudation and calcification, endothelialization of biologic and synthetic prosthetic materials appears feasible. In this study we showed that monocyte adhesion to xenogeneic tissue can be reduced by endothelialization of the

Fig. 3. **a**, Scanning electron micrograph showing spontaneous adhesion of monocytes to a piece of deendothelialized xenogeneic tissue. **b**, Scanning electron micrograph showing adhesion of monocytes to endothelialized tissue after stimulation with IL-1 β . There was significant increase in adhesion as compared with that on nonstimulated tissue ($p \leq 0.001$). **c**, Scanning electron micrograph showing adhesion of monocytes to an endothelialized piece of porcine aorta. **d**, Micrograph showing confluent monolayer of human ECs established 1 day after seeding. Modified hematoxylin and eosin staining was routinely used for this purpose.

tissue. Monocyte adhesion, however, was relatively high 1 day after seeding despite a confluent endothelium. Three and 7 days after seeding, monocyte adhesion was significantly lower as compared with that on the nonendothelialized tissue. Interestingly, the expression of adhesion molecules was also highest on day 1, decreased on day 3, and remained low on day 7. This suggests a time-dependent difference in the properties of the endothelium after seeding and that the cells are less proinflammatory and immunogenic after 3 to 7 days. The data also clearly show that the seeded ECs respond adequately after IL-1 β or IFN- γ stimulation as investigated 7 days after seeding. The presence of xenogeneic cells in the viable porcine aorta did not influence the seeded ECs to increase their expression of adhesion mole-

cules. This supports the theory that an endothelialized surface is less proinflammatory than a deendothelialized surface¹⁶ on both viable and nonviable tissue. The nonspecific immunoglobulin G binding to the human saphenous vein ECs was also relatively high 1 day after seeding on viable and nonviable xenogeneic tissue, as well as on gelatin-coated tissue culture plastic. However, the expression was lower as compared with the binding of specific antibodies. One possibility for the nonspecific binding is a transient up-regulation of EC receptors. This may reflect a hyperactive state of the ECs immediately after seeding. The reason for this is unclear but may be a result of manipulative steps during the preparative process, trypsinization, centrifugation, and trituration.

For procedures of in vitro endothelialization, the ECs are cultivated under quiescent circumstances in a culture dish and the cells are not adapted to the blood flow and shear forces they will be exposed to when implanted. It has been speculated that the maturation of the cytoskeleton takes 7 to 9 days and that this plays an important role in cell retention on expanded polytetrafluoroethylene grafts.¹⁷ Our results also indicate that implantation of seeded biologic materials should be done 3 to 7 days after seeding because the ECs are less activated at that time.

It has previously been shown that in cardiac transplant rejection, a lymphocyte-mediated process, there is excellent correlation of high VCAM-1 expression on venular ECs and T-cell infiltrates.¹⁸ Furthermore, one of the first single observations during development of experimental atherosclerosis is an endothelial VCAM expression.¹⁹ From our results, one may speculate that when seeding of biologic materials such as heart valves is done, the timing of implantation after seeding will be important because a low adhesion of monocytes to the implanted tissue is likely to reduce leukocyte adherence to the tissue. From previous findings, however, it is possible that the expression of ICAM-1, for example, may increase and VCAM may decrease in response to shear stress after implantation.^{20, 21}

The VCAM ligand VLA-4 is present on both monocytes and T lymphocytes and a high VCAM expression would possibly enhance the interaction of T lymphocytes with antigens presented by MHC class II molecules. The low expression of VCAM and MHC class II molecules by the seeded ECs suggests that ECs alone, as expected, were not immunocompetent to present a different expression pattern on viable and nonviable tissue. The establishment of an autologous endothelium on a graft surface may therefore mask potentially antigenic cells within the tissue.

Today, the vast majority of nonviable grafts comprise glutaraldehyde-preserved xenogeneic tissue in which the reason for the use of glutaraldehyde is to stabilize, sterilize, and reduce antigenicity of the biomaterial.²² However, recent data indicate that glutaraldehyde preservation may promote calcification and deterioration of transplanted biologic heart valves and that it impairs spontaneous reendothelialization.^{23, 24} New alternative techniques to create a durable tissue for biologic heart valve prostheses have been presented, such as dye-mediated photooxidation of pericardium.²⁵ In this study, the

porcine aorta was used as model tissue for a xenogeneic tissue, but there is a possibility that different prosthetic materials may have unique properties as regards monocyte adhesion and activation of the endothelium. This will need further investigation.

In conclusion, the findings in this study show that endothelialization of a xenogeneic tissue with cultured adult human ECs reduces monocyte adhesion and that this appears to be a mechanism dependent on time and adhesion molecule expression. The endothelium appears insensitive to viable xenogenic cells and the data further support the feasibility of creating an autologous endothelium on biologic prosthetic materials.

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REFERENCES

1. Goffin Y, Bactik M. Porcine aortic vs. bovine pericardial valves: a comparative study of unimplanted and from patient explanted bioprostheses. *Life Support Syst* 1987;5:127-43.
2. Eberl T, Siedler S, Schumacher B, et al. Experimental in vitro endothelialization of cardiac valve leaflets. *Ann Thorac Surg* 1992;53:487-92.
3. Gavin JB, Barratt-Boyes BG, Hitchcock GC, Herdson PB. Histopathology of "fresh" human aortic valve allografts. *Thorax* 1973;28:482-7.
4. Frater RM, Gong G, Hoffman D, Liao K. Endothelial covering of biological artificial heart valves. *Ann Thorac Surg* 1992;53:371-2.
5. Schoen FJ, Harasaki H, Kim KM, Andersson HC, Levy RJ. Biomaterial-associated calcification: pathology, mechanisms and strategies for prevention [Abstract]. *J Biomed Mater Res* 1988;22:11.
6. Golomb G, Schoen FJ, Smith MS, et al. The role of glutaraldehyde-induced cross-links in calcification of bovine pericardium used in cardiac valve bioprostheses. *Am J Pathol* 1987;127:122.
7. Grimm M, Eybl E, Grabenwöger M, et al. Biocompatibility of aldehyde-fixed bovine pericardium: an in vitro and in vivo approach toward improvement of bioprosthetic heart valves. *J THORAC CARDIOVASC SURG* 1991;102:195-201.
8. Bevilacqua MP, Stengelin S, Gimbrone MA Jr, Seed B. Endothelial leukocyte molecule-1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science* 1989;243:1160-5.
9. Hsu-Lin SC, Berman CL, Furie BC, August D,

- Furie B. A platelet membrane protein expressed during platelet activation and secretion: studies using a monoclonal antibody specific for thrombin-activated platelets. *J Biol Chem* 1984;259:9121-6.
10. Bevilacqua MP. Endothelial-leucocyte adhesion molecules. *Annu Rev Immunol* 1993;11:767-804.
11. Pober J, Collins T, Gimbrone M, Libby P, Reiss C. Inducible expression of class II major histocompatibility complex antigens and the immunogenicity of vascular endothelium. *Transplantation* 1986;41:141-6.
12. Basham T, Merigan T. Recombinant interferon- γ increases HLA-DR synthesis and expression. *J Immunol* 1983;130:1492-4.
13. Hægerstrand A, Gillis C, Bengtsson L. Serial cultivation of adult human endothelium from the great saphenous vein. *J Vasc Surg* 1992;16:280-5.
14. Bengtsson L, Ragnarson B, Hægerstrand H. Lining of viable and nonviable allogeneic and xenogeneic cardiovascular tissue with cultured adult human venous endothelium. *J THORAC CARDIOVASC SURG* 1993;106:434-43.
15. Gillis C, Hægerstrand A, Ragnarsson B, Bengtsson L. Rapid visualization of viable and nonviable endothelium on cardiovascular prosthetic surfaces by means of fluorescent dyes. *J THORAC CARDIOVASC SURG* 1994;108:1043-8.
16. Jaffe E. Cell biology of endothelial cells. *Hum Pathol* 1987;18:234-9.
17. Zilla P, Fasol R, Preiss P, et al. Use of fibrin glue as a substrate for in vitro endothelialization of PTFE vascular grafts. *Surgery* 1989;105:515-22.
18. Briscoe D, Shoen F, Rice G, et al. Induced expression of endothelial-leucocyte adhesion molecules in human cardiac allografts. *Transplantation* 1991;51:537-9.
19. Cybulsky MI, Gimbrone M Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* 1991;251:788-91.
20. Ando J, Tsuboi H, Korenaga R, et al. Shear stress inhibits adhesion of cultured mouse endothelial cells to lymphocytes by downregulating VCAM-1 expression. *Am J Physiol* 1994;267:679-87.
21. Nagel T, Resnick N, Atkinson WJ, Dewey CF, Gimbrone MA. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. *J Clin Invest* 1994;94:885-91.
22. Woodroof A. Use of glutaraldehyde and formaldehyde to process tissue heart valves. *J Bioeng* 1978;2:1-9.
23. Eybl E, Griesmachher A, Grimm M, Wolner E. Toxic effects of aldehydes released from fixed pericardium on bovine aortic endothelial cells. *J Biomed Res* 1989;23:1355-65.
24. Bengtsson L, Rådegran K, Hægerstrand A. In vitro endothelialization of commercially available heart valve bioprostheses with cultured human cells. *Eur J Vasc Surg* 1993;7:393-8.
25. Moore MA, Bohashevsky IK, Cheung DT, et al. Stabilization of pericardial tissue by dye-mediated photooxidation. *J Biomed Mater Res* 1994;28:611-8.

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