Evaluation of endothelial cell integrins after *in vitro* contact with polyethylene terephthalate

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The aim of this research was to evaluate the effect of polyethylene terephthalate (Woven Dacron) on the expression of endothelial integrins. Human umbilical vein endothelial cells were cultured on the material for 24 h. The integrins VLA-2 ($\alpha_2\beta_1$-CD49b/CD29), receptor for laminin and collagen, VLA-5 ($\alpha_5\beta_1$-CD49e/CD29), receptor for fibronectin, VLA-6 ($\alpha_6\beta_1$-CD49f/CD29), receptor for laminin, and $\alpha V\beta_3$-CD51/CD61 (receptor for vitronectin) were evaluated by flow cytometry. After contact with polyethylene terephthalate, a slight but significant decrease in the percentage of both CD29 and CD49e positive cells was observed, which suggests a lower number of cells expressing the fibronectin receptor $\alpha_5\beta_1$. Moreover, a significant increase in the mean channel for CD49b and for the vitronectin receptor CD51/CD61 was observed. The reduction in the fibronectin receptor could account for the poor endothelialization observed *in vivo* on polyethylene terephthalate. The increased expression of the vitronectin receptor, favoring the migration of smooth muscle cells, could give some information about the pathogenesis of intimal hyperplasia, which is a complication of vascular grafts.

1. Introduction

Endothelial cell cultures are useful to assay the biocompatibility of materials for vascular grafts. In fact, the development of the neointima is an important step which positively influences graft patency [1–3]. The conditions to improve the efficacy of endothelial seeding on the prosthesis have long been established [4]. However, it is necessary to investigate the mechanisms regulating endothelial adhesion to the substratum in order to manufacture the most suitable materials to this technique.

Cell adhesion is a complex phenomenon, regulated by molecules occurring on the cell membrane, which determine both cell–cell and cell–matrix adhesion. Adhesion molecule expression on cell surface is not constant in time, and it is modified by various substances. Adhesion molecules are commonly divided into the superfamily of immunoglobulis and the families of integrins, selectins and caderins [5].

Integrins are integral glycoproteins of the cell membrane composed by a chain α and a chain β and are divided into eight [6] subfamilies which are distinguished by the different chains β. Endothelial cells express integrins belonging to the subfamilies β_1 and β_3.

Subfamily β_1 includes seven integrins, characterized by the same chain β (CD29) combined with different chains α, represented in six cases by a variable of CD49 and in one case by CD51. CD49x/CD29 have been named VLA (Very Late Activation), since the first two of the series (VLA-1 and VLA-2) appear on the lymphocytes 2–4 weeks after *in vitro* antigen stimulation. VLA-2 ($\alpha_2\beta_1$-CD49b/CD29), VLA-5 ($\alpha_5\beta_1$-CD49e/CD29) e VLA-6 ($\alpha_6\beta_1$-CD49f/CD29) are expressed by endothelial cells. The ligands of the sub-family β1 integrins are molecules of the extracellular matrix. VLA-2 is a receptor for laminin and, with less affinity, for collagen. It is expressed also on platelets and activated T lymphocytes. VLA-5 promotes cell adhesion to fibronectin and occurs also on monocytes, platelets and T lymphocytes. VLA-6 is a receptor for laminin and it is expressed also on fibroblasts, platelets and epithelial cells.

Subfamily β_3 is characterized by β chain (CD61) combined with different chains α. Endothelial cells express GPIIb/IIIa ($\alpha II\beta_3$; CD41/CD61) and the receptor for vitronectin ($\alpha V\beta_3$; CD51/CD61). The receptor for vitronectin is involved in cell–cell interaction, in conditions of cell confluence, and in the cell–substratum interaction, in conditions of lower cell density. It binds to

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fibrinogen, von Willebrand factor and thrombospondin, as well as to vitronectin, and is expressed on endothelial cells, platelets, megacaryocytes and activated lymphocytes B.

Integrins are involved in endothelial functions such as inhibition of coagulation and complement activation, maintenance of monolayer integrity and anchorage to subendothelial matrix [7].

In this study, the changes induced by polyethylene terephthalate, of integrin expression on HUVEC were evaluated.

2. Materials and Methods

2.1. Materials

Polyethylene terephthalate extra soft woven vascular prosthesis (Woven Dacron or WD) was tested (Vascutek, Inchinnan, UK). The material porosity, as declared by the manufacturer, was 150 ml/cm^2/min. The material, sterilized with ethylene oxide, was stored at room temperature.

2.2. Medium and solutions

The buffer (HUVEC buffer) used to wash the endothelial cells consisted of 0.14 M NaCl (Carlo Erba), 0.004 M KCl (Merck), 0.001 M glucose (Riedel de Haen), 0.001 M K monobasic phosphate (Carlo Erba), 0.01 M Na bibasic phosphate (Carlo Erba).

The culture medium consisted of complete medium, an equal mixture of Medium RPMI 1640 and Medium 199 (Gibco Lim., Paisley, UK), supplemented with 25 mM Hepes buffer (Imperial), 2 mM L-glutamine (Eurobio, Les Ulis, France), 100 IU/ml penicillin (Pool Industries), 100 µg/ml streptomycin (Pool Industries) and 20% human serum [8].

2.3. Endothelial cell cultures

Human endothelial cells were isolated from the umbilical vein by enzyme treatment with collagenase according to Jaffe *et al*. (9).

The umbilical vein was washed with saline, to eliminate blood, then it was perfused with a solution of collagenase IV-S (Sigma) in phosphate buffered saline (PBS). After incubation at 37 °C for 15 min, the vein was washed with HUVEC buffer and the eluate containing cells detached by collagenase was collected. The suspension was washed with medium, then the cells were seeded in a 25 cm^2 tissue culture treated polystyrene flask. The culture was incubated at 37 ± 1 °C with 5% CO_2 and 95% air.

2.4. Contact between endothelial cells and material

The experiments were performed on cultures at the second passage. Cells were detached with a prewarmed solution of 0.05% trypsin-0.02% EDTA for 1 min at 37 °C. The proteolytic action of the solution was blocked by the addition of complete medium (v/v). In some experiments HUVEC were harvested by a ''cell scraper'' in order to determinate the method of detachment more suitable for the immunofluorescence reaction.

Cells were counted in a Bürker chamber and resuspended in medium at the concentration of 1×10^5 cells/ml.

For the experiments, 6 cm diameter tissue culture treated polystyrene plates were used. The material, put on the bottom of the plates, was shaped to cover the whole surface. Endothelial cells were seeded onto the material in direct contact with it. Endothelial cells cultured on polystyrene plates of the same diameter, but in the absence of the test material, were used as negative control. Other cultures were incubated with endotoxin (LPS) at a concentration of 10 µg/ml (from *E.coli* 055 : B5) (Sigma).

For each experiment the following subcultures were prepared: 1 plate for negative control, 1 plate for WD, and 1 plate for LPS. The cultures were incubated for 24 h at 37 °C, with 5% CO_2 and 95% air.

2.5. Integrin assay

After 24 h the cultures were trypsinized and resuspended in HUVEC buffer. The cells were incubated with each monoclonal antibody directed against VLA-2 ($\alpha_2\beta_1$-CD49b/CD29), VLA-5 ($\alpha_5\beta_1$-CD49e/CD29), VLA-6 ($\alpha_6\beta_1$-CD49f/CD29) and vitronectin receptor ($\alpha V\beta_3$-CD51/CD61). After 30 min of incubation at 4 °C, the samples were washed with HUVEC buffer in order to remove the exceeding antiserum. As secondary antibody a goat polyclonal antibody F(ab′)2 was used, directed against mouse Ig, conjugated with fluorescein isothiocyanate (GAM-FITC). The samples were incubated with the conjugate, previously titrated to establish the optimal concentration, for 30 min at 4 °C. After incubation, the samples were washed to remove the exceeding fluorescent antibody. For every experiment, the non-specific bond of GAM-FITC was evaluated, by incubating an aliquot of cells only with the conjugate.

The fluorescence of the samples was evaluated by flow cytometry (Epics-Elite, Coulter, Hialeah, FL). The HUVEC gate was determined both by evaluating cell size (forward scatter) and by evaluating granularity (side scatter). Within this area 5000 events were collected, on which the proportion of positive cells and the fluorescence intensity were determined. The latter was measured as the mean channel of positive cells peak on logarithmic scale.

2.6. Statistical evaluation

Statistical evaluation was perfomed using the software StatView 4.5^{TM} for Macintosh (Abacus Concept Inc.). The results were expressed as mean ± standard error (mean ± SE). The statistical analysis of the effect of the test material on the expression of integrins was performed with a non-parametric test for paired data (Wilcoxon signed rank test). The differences were considered significant for values of $p < 0.05$.

3. Results

Thirteen experiments were carried out. Each experiment was performed on a different primary culture.

Some preliminary tests were performed to establish the most efficient method to detach cells, without altering

the integrin expression. The detachment with the cell-scraper determined a remarkable loss of cells, the presence of cell aggregates and high values of non-specific fluorescence ($> 30\%$). When the cells were detached with trypsin-EDTA, a monocellular suspension was obtained, the retrieval of the cells was almost complete, and the non-specific bond was $< 5\%$. Therefore the latter method was chosen for the experiments.

The integrin expression after contact with the test material is shown in Figs 1 and 2. Isolated endothelial cells expressed integrins constitutively, as demonstrated by the negative control. Antigen CD29, common to all integrins β1, was expressed on all cells ($98.7 \pm 0.6\%$), as well as CD49e ($99.1 \pm 0.3\%$). CD49b occurred on $78.9 \pm 7.9\%$ of cells, CD49f on $72.5 \pm 8.0\%$ and CD51/CD61 on $84.9 \pm 7.8\%$.

The incubation with LPS did not modify the percentage of positive cells (CD29: $97.8 \pm 1.0\%$, CD49b: $87.8 \pm 5.8\%$; CD49e: $98.8 \pm 0.3\%$; CD49f: $66.1 \pm 6.5\%$; CD51/CD61: $87.5 \pm 6.7\%$).

The contact with WD determined a decrease in the number of CD29 and CD49e, which was respectively $97.6 \pm 0.9\%$ and $98.2 \pm 0.5\%$. The small decrease in percentage was significant compared to the negative control cultures ($p = 0.018$ and $p = 0.027$, respectively), due to the scarce variability in the expression of these integrins. The other integrins were not modified by the contact with WD and were expressed with the following percentages: CD49b: $85.6 \pm 8.0\%$; CD49f: $76.2 \pm 6.3\%$; CD51/CD61: $89.8 \pm 4.5\%$.

The amount of antigen expressed on the single cells, i.e. the mean channel, was different according to the type of molecule analyzed. CD29 was the antigen with the highest fluorescence intensity (40.7 ± 12.8). Also the receptor for vitronectin CD51/CD61 presented a remarkable expression (27.1 ± 7.4), whereas other molecules had a lower fluorescence intensity (CD49b: 4.3 ± 0.5; CD49e: 18.0 ± 2.8; CD49f: 3.1 ± 0.6).

The incubation with LPS determined a significant increase in the intensity of expression of CD49b (6.5 ± 1.0, $p = 0.04$), while no significant variation was observed for other molecules which had the following fluorescence intensities: CD29: 32.3 ± 4.2; CD49e: 17.5 ± 2.3; CD49f: 2.9 ± 0.8; CD51/CD61: 25.5 ± 7.5.

The contact with WD determined a significant increase in the expression of CD49b and CD51/CD61, whose mean channel values resulted respectively 9.4 ± 2.4 ($p = 0.042$) and 42.3 ± 10.2 ($p = 0.046$). The other molecules did not show any significant variation in their expression and had the following mean channel values: CD29: 42.6 ± 8.4; CD49e: 18.9 ± 3.7; CD49f: 5.4 ± 1.6.

4. Discussion

Adhesion between the endothelial cells and an acellular substratum is mediated by integrins, which are also involved in the adhesion process between endothelium and biomaterials [10].

The absence of a complete endothelial lining is an important cause of the thrombogenicity of a material [11] and greatly hampers the manufacturing of small-diameter grafts. Dacron, which is a material widely used for the substitution of large arteries, cannot be used to substitute small-diameter arteries. The search for materials suitable to substitute small diameter vessels is one of the greatest challenges in vascular surgery.

Some materials put in contact with endothelial cells can favor the release of pro-coagulant factors [12], and modify the expression of some adhesion molecules involved in inflammatory processes and responsible for the adhesion between endothelium and leukocytes [13].

In order to obtain a good endothelialization, the substratum should have some prerequisites, i.e. (i) to be strongly adhesive for cells, so that blood flow cannot detach them, (ii) not to induce thrombosis, (iii) to favor the growth of endothelial cells, and (iv) not to alter the endothelial modulation of hemostasis and thrombosis (10). Since synthetic surfaces are rarely lined by endothelium spontaneously [14], some attempts were made at coating prosthetic materials with peptidic sequences specifically recognized by endothelial cell adhesins [15, 16], or with monoclonal antibodies directed against endothelial antigens [17]. Therefore the study of adhesins expressed by endothelium is useful both to assay the biocompatibility of materials for vascular prostheses, and to improve the capability of favoring endothelialization.

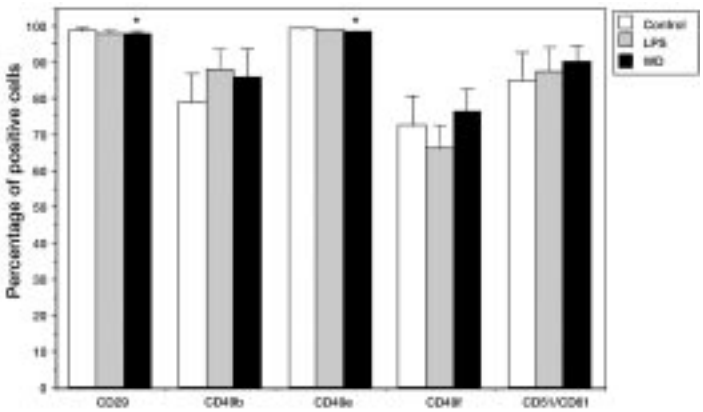

Figure 1 Expression of integrins on endothelial cells: percentage of positive cells.

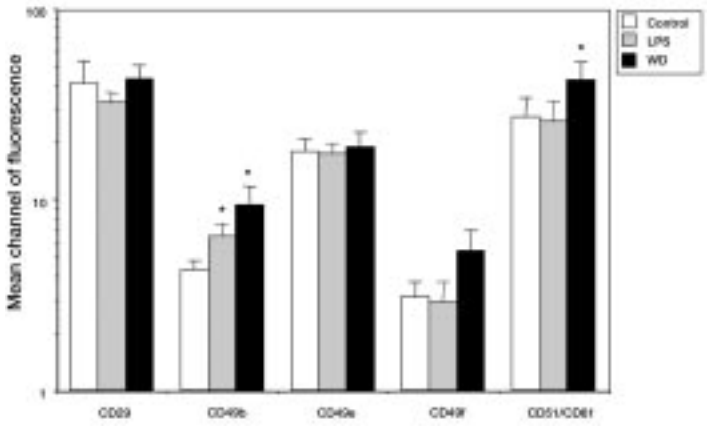

Figure 2 Expression of integrins on endothelial cells: fluorescence intensity.

This study evaluates the modification induced by Woven Dacron on the integrins expressed by endothelial cells cultured *in vitro*. Trypsin-EDTA was used to detach the cells, because it determined a negligible effect on integrins expression, as already determined in preliminary experiments and reported previously (18).

By flow cytometry, not only the percentage of positive cells but also the amount expressed by the cells are evaluated. Every cell is singularly tested and an arbitrary value is attributed to the fluorescence it emits, which corresponds to a ''channel''. The quantity of expressed molecule is not equal in all cells but it presents a gaussian distribution and the mean channel represents the mode, i.e. the value measured more frequently in the cells. Therefore the mean channel of the fluorescence peak of positive cells yields an indication of the amount of antigen occurring in every cell.

Almost all cells of the negative controls, i.e. the cultures in contact only with tissue culture treated polystyrene, expressed CD29 and CD49e, whereas CD49b, CD49f and CD51/CD61 were not expressed on the whole of the cells. The amount of antigen occurring in every cell was expressed with a different intensity:

$$\mathrm{CD29} > \mathrm{CD51/CD61} > \mathrm{CD49e} > \mathrm{CD49b} > \mathrm{CD49f}.$$

The maximum expression intensity observed with CD29 can be explained by the fact that the chain β1 is common to many integrins expressed on endothelial cells. Variability in the integrin expression could be justified with variations in culture conditions, such as the number of passages, serum and confluence state which can influence the antigen expression [7]. Since in our experiments, the characteristics of the medium and the number of passages were standardized, it can be presumed that a certain variability in the integrin expression depends on intrinsic differences between the 13 different isolates.

In our experimental model the modification of integrins was also analyzed by incubating the cells with endotoxin, to evaluate whether the quantitative modifications induced by the material are comparable to the ones induced by a powerful inflammatory stimulus. Other agents such as IL-1β or TNFα induced significant modifications in the integrin expression by monocytes or macrophages [19], but not by endothelial cells [7].

After incubation with endotoxin we observed no significant modifications in the percentage of cells expressing integrins, but a significant increase in the CD49b was demostrated.

The contact with WD induces a slight but significant decrease in the percentage of positive cells for CD29 and CD49e, and a significant increase in the mean channel both of CD49b and CD51/CD61. CD29 and CD49e is $\alpha_5\beta_1$, which interacts with fibronectin. Fibronectin is important for the adhesion of endothelial cells to the substratum. In fact, endothelial cells adhere to hydrophobic polymers such as Dacron and Teflon, once they are coated with fibronectin, whereas they adhere to a lower extent to the same polymers uncoated or coated with albumin or immunoglobulins [20]. Fibronectin, bound to a solid substrate, provides a biochemical signal necessary for the proliferation of endothelial cells [21]. Fibronectin-coated Dacron after 9 days *in vitro* presents a better cell lining than the uncoated Dacron, but an incomplete lining compared to fibronectin-coated PTFE [22].

Hydrophobic polymers like polyethylene terephthalate and fluoroethylene-propylene copolymer adsorb strongly serum proteins which are not displaced by fibronectin produced by endothelial cells [21]. The fact that polyethylene terephthalate induces a decrease in the number of cells expressing the fibronectin receptor, could be the consequence of the lower amount of fibronectin deposited on the material. Such a phenomenon could explain the scarce endothelialization of Dacron *in vivo* [23, 24].

CD49b/CD29 corresponds to the integrin VLA-2/$\alpha_2\beta_1$, which is the receptor for collagen and laminin. Antibodies to CD49b and CD29 caused a marked inhibition of capillary formation, demonstrating the role of this integrin in the differentiation of endothelial cells during angiogenesis [25]. It is well-known that neoangiogenesis accompanies tissue regeneration and healing which occurs 2–3 days after damage; neoangiogenesis intervenes also when there is a persistent inflammatory stimulus or there are ample areas of necrosis. The increased expression of CD49b leads to the hypothesis that both endotoxin and WD produce such an inflammatory stimulus as to induce, after 24 h culture, the structural modifications preceding the differentiation of endothelial cells.

The increase in $\alpha V\beta_3$, i.e. the receptor for vitronectin, was observed only after contact with WD. By means of immunohistochemical analysis and *in situ* hybridization, a temporary increase in the expression of $\alpha V\beta_3$ and osteopontin was demonstrated after a damage in the coronary arteries [26]. The authors concluded that antigen $\alpha V\beta_3$, which intervenes in adhesive and migratory cell-matrix events, seemed to play a very important role in the pathogenesis of the restenosis process after a damage to the arterious wall. In fact, selective blockade of $\alpha V\beta_3$ limited neointimal growth and lumen stenosis following deep arterial injury. The increased expression of $\alpha V\beta_3$ after contact with WD could be one possible mechanism of intimal hyperplasia in vascular grafts.

5. Conclusion

The reduction in the expression of the fibronectin receptor induced by Woven Dacron, could contribute to explain the poor endothelialization of this material *in vivo*. The increased expression of CD49b, induced both by endotoxin and Woven Dacron, suggests that the latter produces such an inflammatory stimulus as to induce the structural modifications preceding endothelial cell differentiation. The increased expression of the vitronectin receptor, which favors smooth muscle cells migration, could give a pathogenic contribution to the intimal hyperplasia, which represents a complication of implants in polyethylene terephthalate.

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References

1. V. S. SOTTIURAI, J. S. T. YAO, W. R. FLINN and R. C. BATSON, *Surgery* **93** (1983) 809.
2. M. PASIC, W. MULLER-GLAUSER, B. ODERMATT, M. LACHAT, B. SEIFERT and M. TURINA, *Circulation* **92** (1995) 2605.
3. M. PASIC, W. MULLER-GLAUSER, L. VON SEGESSER, B. ODERMATT, M. LACHAT and M. TURINA, *Eur. J. Cardiothorac. Surg* **10** (1996) 372.
4. J. S. ANDERSON, T. M. PRICE, S. R. HANSON and L. A. HARKER, *Surgery* **101** (1987) 577.
5. T. M. CARLOS and J. M. HARLAN, *Immunol. Reviews* **114** (1990) 5.
6. R. O. HYNES, *Cell* **69** (1992) 11.
7. M. MUTIN, F. DIGNAT-GEORGE and J. SAMPOL, *Tissue Antigens* **50** (1997) 449.
8. P. B. VAN WACHEM, B. W. L. MALLENS, A. DEKKER, T. BEUGELING, J. FEIJEN, A. BANTJES, J. P. DETMERS and W. G. VAN AKEN, *J. Biomed. Mater. Res.* **21** (1987) 1317.
9. E. A. JAFFE, R. L. NACHMAN, C. G. BECKER and C. R. MINICK, *J. Clin. Invest.* **52** (1973) 2745.
10. R. SBARBATI DEL GUERRA and M. G. CASCONE, *Biomateriali* **1/2** (1993) 29.
11. K. M. MULLER and G. DASBACH, *Curr. Top. Pathol.* **86** (1994) 273.
12. E. CENNI, D. GRANCHI, M. CERVELLATI, A. DI LEO and A. PIZZOFERRATO, *Minerva Cardioangiol.* **44** (1996) 49.
13. E. CENNI, D. GRANCHI, G. CIAPETTI, E. VERRI, D. CAVEDAGNA, S. GAMBERINI, M. CERVELLATI, A. DI LEO and A. PIZZOFERRATO, *Biomaterials* **18** (1997) 489.
14. S. K. WILLIAMS, B. E. JARREL, L. FRIEND, J. S. RADOMSKI, R. A. CARABASI, E. KOOLPE, S. N. MUELLER, S. C. THORNTON, T. MARINUCCI and E. LEVINE, *J. Surg. Res.* **38** (1985) 618.
15. H. B. LIN, C. GARCÌA-ECHEVERRIA, S. ASAKURA, W. SUN, D. F. MOSHER and S. L. COOPER, *Biomaterials* **13** (1992) 905.
16. K. P. WALLUSCHECK, G. STEINHOFF, S. KELM and A. HAVERICH, *Eur. J. Vasc. Endovasc. Surg.* **12** (1996) 321.
17. A. DEKKER, A. A. POOT, J. A. VAN MOURIK, M. P. A. WORKEL, T. BEUGELING, A. BANTJES, J. FEIJEN and W. G. VAN AKEN, *Thromb. Haemost.* **66** (1991) 715.
18. M. MUTIN, F. GEORGE, G. LESAULE and S. SAMPOL, *Endothelium* **4** (1996) 289.
19. Y. H. KANG, C. H. LEE, S. E. BRUMMEL, H. H. NEWBALL, and J. FORRESTER, *J. Leukoc. Biol.* **57** (1995) 624.
20. P. B. VAN WACHEM, C. M. VRERIKS, T. BEUGELING, J. FEIJEN, A. BANTJES, J. P. DETMERS and W. G. VAN AKEM, *J. Biomed. Mater. Res.* **21** (1987) 701.
21. A. POOT, T. BEUGELING, A. DEKKER, J. SPIJKERS, J. A. VAN MOURIK, J. FEIJEN, A. BANTJES and W. G. VAN AKEN, in ''Biomaterial-tissue interfaces'', edited by P. J. Doherty, R. L. Williams, D. F. Williams and A. J. C. Lee (Elsevier, Amsterdam, 1992) p. 253–262.
22. T. L. FOXALL, K. R. AUGER, A. D. CALLOW and P. LIBBY, *J. Surg. Res.* **41** (1986) 158.
23. G. PASQUINELLI, A. FREYRIE, P. PREDA, T. CURTI, M. D'ADDATO and R. LASCHI, *Scanning Microsc.* **4** (1990) 351.
24. Y. NOISHIKI, Y. TOMIZAWA, Y. YAMANE and A. MATSUMOTO, *Artif. Organs* **19** (1995) 7.
25. Y. KUBOTA, Y. KAWA and M. MIZOGUCHI, *J. Dermatol. Sci.* **12** (1996) 36.
26. S. S. SRIVATSA, L. A. FITZPATRICK, P. W. TSAO, T. M. REILLY, D. R. HOLMES JR, R. S. SCHVARTZ and S. A. MOUSA, *Cardiovasc. Res.* **36** (1997) 408.

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