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COMPARATIVE ASSESSMENT OF THE CYTOTOXICITY OF VARIOUS SUBSTRATES IN ORGAN CULTURE AND CELL CULTURE: A SCANNING ELECTRON MICROSCOPY STUDY

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Abstract

A comparative study of the behavior of chick embryo endothelial cells grown on various substrates was performed in order to establish the reliability and the limitation of both cell and organ culture methods. Following substrates were analyzed to compare these two different culture techniques: bovine serum albumin, pigskin gelatin and albumin + gelatin cross-linked by glutaraldehyde or carbodiimide, fibrin glue and negative control (Thermanox®). Parameters of cell growth and adhesion were calculated and compared with electron microscopic observations of cell morphology and of the extracellular matrix. Both culture methods provided complementary results and led to a similar classification of the biomaterials. However, the cell culture method exhibited a higher sensitivity to the surface properties of biomaterials which limited further experimentation. This was well illustrated by glutaraldehyde cross-linked protein membranes which did not support the growth of dispersed cells but enabled the formation of a cellular tissue in organ culture, thus allowing a cytocompatibility assessment. Endothelial cell morphology and extracellular matrix elaborated on biomaterials were compared to chicken blood vessels. Both methods showed that cells grown on fibrin glue and on gelatin or albumin + gelatin cross-linked by carbodiimide had a microscopic morphology similar to that of vessel wall. Organ and dispersed cell cultures provide complementary information relative to the cell behavior towards vascular prosthesis materials.

Key Words: Organ culture, cell culture, biomaterials, endothelial cells, extracellular matrix, scanning electron microscopy.

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Introduction

The cytocompatibility of biomaterials can be assessed by *in vitro* cell culture according to alternative techniques based on the growth either of dispersed cells or of organ fragments (Rae, 1986). Embryonic and adult type specific cells, enzymatically obtained from biopsies, are cultured in direct contact with biomaterials in order to test their surface cytocompatibility, whereas toxicity of the leached products of these materials is measured after diluting them in the incubation medium, under defined conditions. Both kinetics of cell attachment and cell proliferation are established using the trypsin sensitivity of radiolabelled cells (Taylor *et al.*, 1973; Ratner *et al.*, 1975; McAuslan and Johnson, 1987). Fibroblast cell lines such as L929 or MRC5 have been previously grown in routine tests and the validity of their response discussed (Warocquier-Clérout *et al.*, 1990). Currently, organ culture is widely used in cytocompatibility assessments (Rae, 1976; Hegyeli, 1973; Duval *et al.*, 1990). Each technique has its own advantages and limitations. Besides the easiness to set up a dispersed cell culture, this method is rapid, sensitive and offers the possibility of adding labelled precursors during the incubation period. In comparison, organ culture deals with smaller amounts of cells which behave as whole tissue and are less sensitive to the surface properties of the biomaterials (Sigot-Luizard *et al.*, 1980), but are able to maintain and even to re-express their differentiation properties (Adolphe *et al.*, 1991).

The limitations of dispersed cell culture are mainly due to its high susceptibility to bacterial infection compared to organ culture in which semi-solid agar medium does not favor the propagation of micro-organisms. The high sensitivity of dispersed cell culture for the surface properties of biomaterials restricts its application. Moreover, cell behavior may be substantially altered by enzymatic treatment for seeding on materials, and serial passages of cells induce a de-differentiation process.

The limitations of the organ culture technique lie in the variability of the results, statistically requiring a large number of samples (4 x 12 explants per determination).

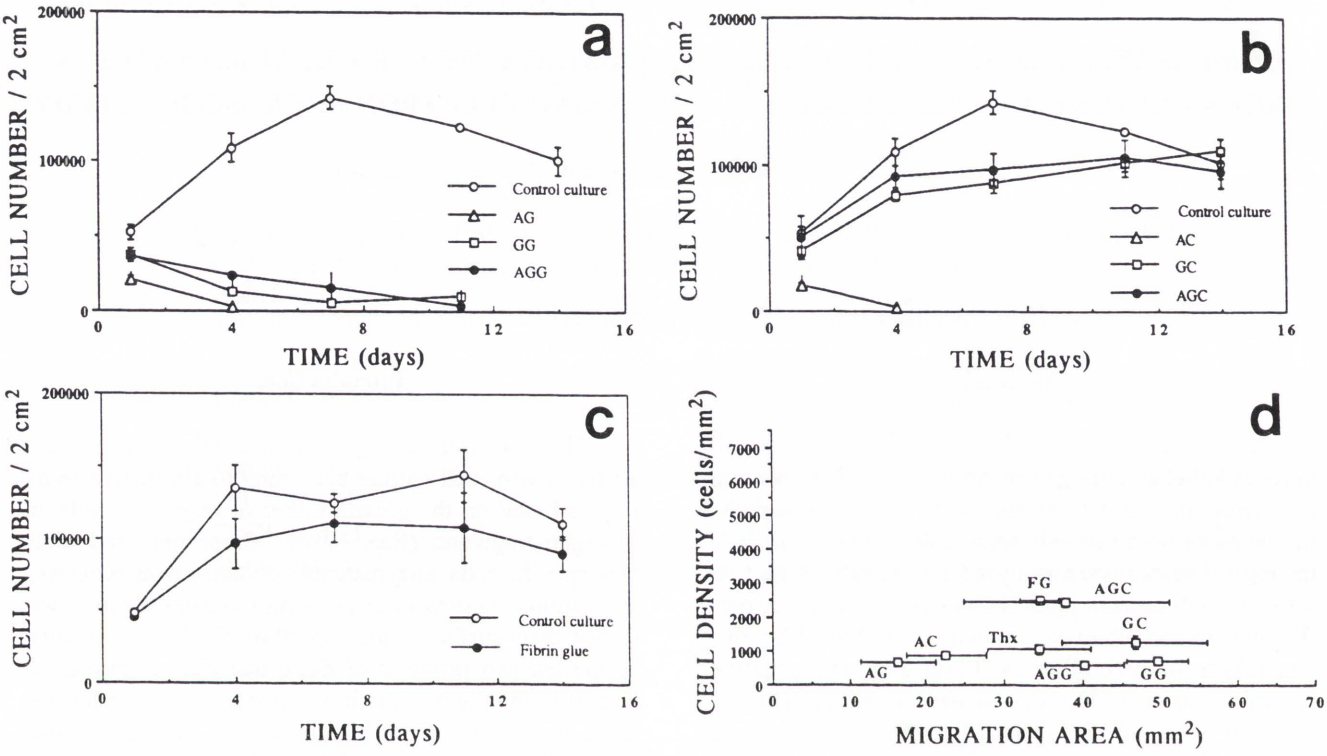

Figure 1. Endothelial cell growth on various substrates comparing dispersed cell and organ cultures techniques (mean values ± S.E.). **(a)** Cell growth on glutaraldehyde cross-linked membranes (AG, GG, AGG) in dispersed cell culture and on uncoated plastic surface (control culture). **(b)** Cell growth on carbodiimide cross-linked membranes in dispersed cell culture (AC, GC, AGC) and on uncoated plastic surface (control culture). **(c)** Cell growth on fibrin glue membrane in dispersed cell culture and on uncoated plastic surface (control culture). **(d)** Cell growth on each membranes in organ culture and on Thermanox® (Thx) as control culture.

As previously reported, we used the organ culture method to assess the cytocompatibility of protein membranes cross-linked by two chemical agents: glutaraldehyde or carbodiimide (Sigot-Luizard *et al.*, 1986, Duval *et al.*, 1990). These membranes were proposed for Dacron® vascular prostheses coating to improve their impermeability and to eliminate the preclotting step before implantation (Warocquier-Clérout *et al.*, 1987). Glutaraldehyde cross-linked membranes provide an example of material which cannot be analyzed with the dispersed cell culture technique because of the lethal effect of glutaraldehyde, whereas in organ culture, a cellular tissue succeeds in surrounding the explants. Determination of biological parameters of newly synthesized tissue, such as density and migration, analyzed by reference to a control material, gives a comparative assessment of the cytocompatibility. In order to compare the results provided by these two culture methods and for a better understanding of the differences, we propose to analyze the behavior of endothelial cells and to examine their morphology in different culture conditions using scanning electron microscopy (SEM).

Material and Methods

Tested materials

Fibrin glue with 100 UI/ml of human thrombin (Biocol® Biotransfusion S.A.) (**FG**).

Albumin membrane cross-linked by glutaraldehyde (**AG**).

Pigskin gelatin membrane cross-linked by glutaraldehyde (**GG**).

Albumin-pigskin gelatin membrane cross-linked by glutaraldehyde (**AGG**).

Albumin membrane cross-linked by carbodiimide (**AC**).

Pigskin gelatin membrane cross-linked by carbodiimide (**GC**).

Albumin-pigskin gelatin membrane cross-linked by carbodiimide (**AGC**).

Negative control (Thermanox® Lux corp.) (**Thx**).

Cross-linking of protein membranes

Fraction V bovine albumin (Sigma) and pigskin gelatin (Rousselot) were covalently cross-linked according to the following techniques using glutaraldehyde (GTA) or carbodiimide (CDI):

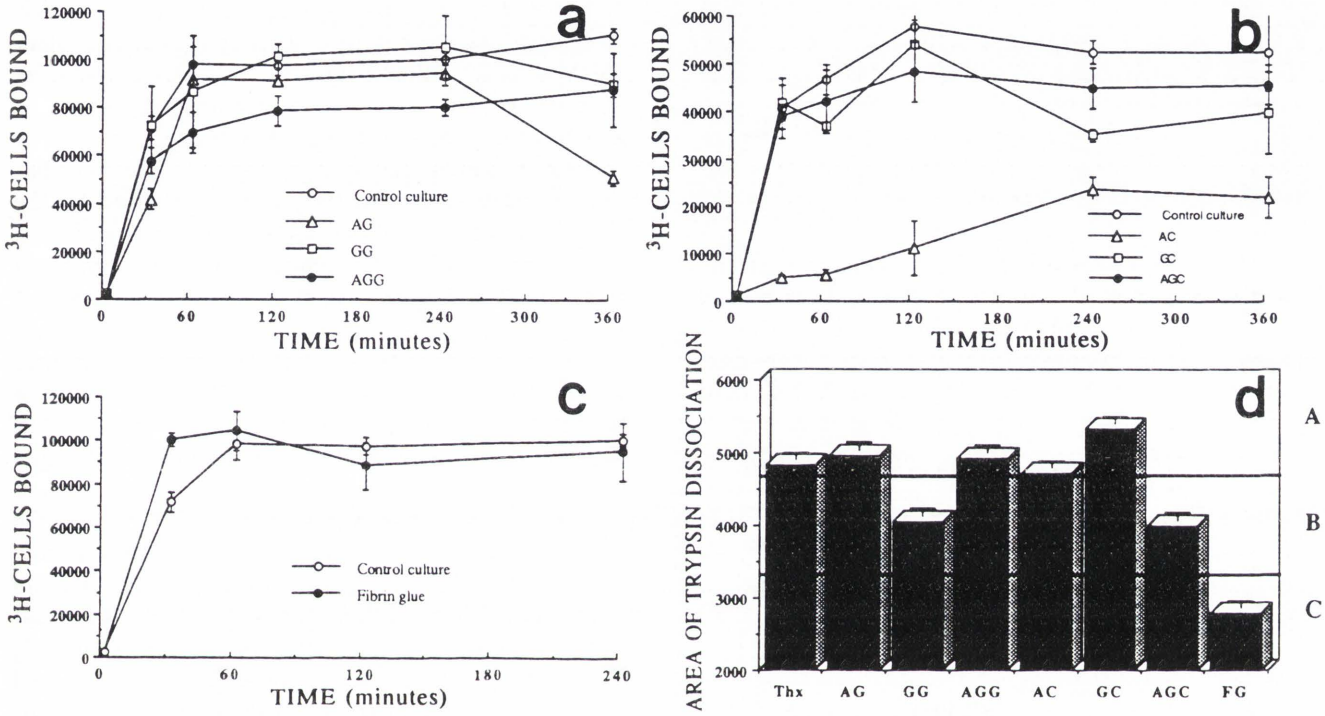

Figure 2. Endothelial cell attachment and adhesion on various substrates comparing dispersed cell and organ cultures techniques (mean values ± S.E.). (**a**) Cell attachment on glutaraldehyde cross-linked membranes (AG, GG, AGG) in dispersed cell culture and on uncoated plastic surface (control culture). (**b**) Cell attachment on carbodiimide cross-linked membranes in dispersed cell culture (AC, GC, AGC) and on uncoated plastic surface (control culture). (**c**) Cell attachment on fibrin glue membrane in dispersed cell culture and on uncoated plastic surface (control culture). (**d**) Cell adhesion on each membranes in organ culture and on Thermanox® (Thx) as control culture.

Cross-linking by GTA: Albumin (AG) and albumin + gelatin (AGG) membranes were made by adding 20% protein solutions in 0.02 M phosphate buffer pH 6.8 with 2.5% GTA. Gelatin membranes (GG) were made with 5% gelatin solution. Gelatin gel was first dried, then immersed into 1.25% GTA solution. In both cases, excess GTA was neutralized after cross-linking by adding 1% glycine solution.

Cross-linking by CDI: AC and AGC membranes were obtained by adding equal volumes of 20% albumin and 20% albumin + gelatin solutions respectively diluted in 0.02 M phosphate buffer pH 4.75 with 0.2 M 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide (Sigma) diluted in 0.02 M phosphate buffer pH 4.75. Gelatin membranes (GC) were post-reticulated by adding CDI solution after drying the gelatin gel.

Each solution was sterilized by filtration. The membranes were reticulated over a glass plate and allowed to air dry in a laminar flow hood. Final membranes measured 5 x 10 cm and were 200 μm thick (Duval *et al.*, 1986).

Fibrin glue membrane: This membrane is made by spreading, on a glass sheet, a mixture of concentrated hemostasis factors (fibrinogen) and human thrombin. This mixture was then allowed to clot.

Cell culture

Culture technique: Endothelial cells were obtained from aortas of 14 day chick embryos following the primary explant technique (Freshney, 1987). Aortas were cut into cylindrical fragments of 1 mm length, then split and layered into Nunc® plastic dishes so that the endothelium faced the plastic substratum. Explants were gently fed with medium 199 supplemented with 20% fetal calf serum (Boehringer-Mannheim) and incubated at 37 °C in 5% CO_2 humid atmosphere. After 6 days incubation, cells surrounding the explants were collected after a brief treatment by 0.125 % trypsin + 0.10 % ethylenediaminetetraacetic acid (EDTA). Explants were decanted in conical tube and discarded and cells were pooled by centrifugation at 100 g for 10 minutes. Harvesting of cells from 6 explants provided an average of 2 x 10^5 cells.

Cell growth rate assessment: Pooled endothelial cells were resuspended in medium 199 added with 20 % fetal calf serum, endothelial cell growth supplement

(ECGS 50 mg/ml, Sigma) and heparin (50 mg/ml, Sigma). They were seeded at 2 x 10^4 cells per cm^2 onto Nunclon® 4-well plates uncoated or coated respectively with glutaraldehyde or carbodiimide cross-linked proteins or with fibrin glue (Biocol®) as described previously. At 3 day intervals, cells were counted in a hemocytometer. Data are presented as the mean values of 4 counts ± standard error (SE).

Cell attachment assay: Six day old explant cultures which displayed a uniform cell layer, were labelled with 1 mCi/ml [6-^{3}H]thymidine (28 Ci/mmole, Dositek France). After 24 hours at 37 °C, the cells were trypsinized, washed with culture medium, centrifuged at 100 g for 5 minutes and resuspended in fresh culture medium. Labelled cells were seeded onto coated and uncoated plates at a density of 30,000 cells/cm^2. At time intervals up to 6 hours, medium was removed and the cell layer was rinsed twice with phosphate buffer salt (PBS) and then hydrolysed in 0.1 M NaOH. Extract was transferred to scintillation vial, neutralized with 0.1 M HCl and counted in aqueous scintillation fluid (ACS®II, Amersham) in spectrometer Beckman LS 8000. Cell radioactivity was plotted versus incubation time. Data points represent means of 4 measurements ± SE.

Organ culture

Culture technique: Aortic fragments from 14 day old chick embryo were layered, the endothelial side facing the substrate to be tested (Sigot-Luizard *et al.*, 1986), onto buffered agar medium containing:
M199 (Boehringer Mannheim) (37%) + fetal calf serum (Boehringer Mannheim) (10%) + Tricin (Merck) (2%) + L-Glutamin (Boehringer Mannheim) (2mM) + Bacto-Agar 1% in Gey saline solution (Difco) (50%).

After 7 days incubation at 37 °C, both surface structure and density of cell layers growing on the biomaterial from the initial explant, were compared with control samples results.

Cytocompatibility assessment: After neutral red staining, the surfaces of the cell layers were measured with stereo-microscope fitted with camera lucida and digitizing tablet connected to microcomputer. The cells were counted with a Multisizer® (Coultronics) and cellular density calculated after trypsin dissociation. Cell densities were plotted versus cell migration surfaces, each point being the average of 48 samples.

Adhesion test: The cells from layers were enzymatically detached with trypsin-EDTA (0.025%) to establish the curve of the percentages of released cells as a function of time (Duval *et al.*, 1988). By integration, we calculated the area included between curve and X-axis which determines the cell adhesion behavior. This area is inversely proportional to the cell adhesion on the biomaterial. A comparative results diagram with 3 different zones has been drawn: Zone A = area more than 4500 = weak cell adhesion; Zone B = area between 3000 and 4500 = medium cell adhesion; and Zone C = area less than 3000 = strong cell adhesion.

Extracellular matrix preparation: After incubation of 7 days at 37 °C, the substrates covered by the cell layers were rinsed into the PBS and into a 20 mM glycine buffer pH 9.6 containing phenyl methyl sulfonyl fluoride (PMSF) 1 mM (Sigma). They were immersed in 1% Nonidet P 40 (Sigma) in glycine buffer (pH 9.6, PMSF 1mM) during 10-15 minutes at room temperature, to burst the cell membranes. Then the substrates were washed with 10 mM Tris-HCl buffer pH 7.5, with PBS and fixed in 3% glutaraldehyde in Rembaum buffer pH 7.4.

Scanning electron microscopy: Cell layers and the extracellular matrix on different biomaterials with both cell culture and organ culture techniques were fixed in 3% glutaraldehyde in Rembaum buffer (pH 7.4), dehydrated in graded alcohols, critical-point dried from CO_2 (Polaron Instrument Inc.), sputter-coated with gold (Polaron Instrument Inc.) and examined using a JEOL (model JSM 840) scanning electron microscope.

Results

Figure 1 summarizes the endothelial cell growth on glutaraldehyde and carbodiimide cross-linked protein membranes in organ culture or in dispersed cells culture. The proliferation of isolated cells was drastically reduced on glutaraldehyde membranes compared to uncoated surface (Fig. 1a) and the number of surviving cells decreases more rapidly on albumin membrane (AG). After 10 days incubation, nearly all cells died. Except for albumin membrane (AC), carbodiimide membranes allowed slower cell growth rate compared to control but cultures were able to reach confluence in 2 weeks (Fig. 1b). Cultures performed on fibrin glue (FG) and uncoated surface (Fig. 1c) show comparable growth behavior.

In organ culture, cell layers grown from explants incubated on glutaraldehyde membranes exhibited slightly lower cell density than that from control explants on plastic surface (Thx) (Fig. 1d). Migration areas increased on gelatin and albumin + gelatin membranes cross-linked by glutaraldehyde (GG, AGG). With the exception of albumin membrane (AC), carbodiimide membranes enhanced both cell migration and density referring to control sample Thermanox® (Thx). Fibrin glue Biocol® (FG) clearly stimulated cell growth (Fig. 1d).

We attempted to explain the effect of the substrate on cell behavior by analyzing attachment and adhesion

strengths. In dispersed cell culture, kinetics of attachment of prelabelled cells give a comparative assessment of attachment strengths during early phase of culture.

Figure 2a shows that cells adhered at similar rates to glutaraldehyde membranes and control surface during the first hours post seeding. Cells started to detach from gelatin and albumin membranes cross-linked by glutaraldehyde (GG, AG) after 4 hours. Kinetics curves were quite similar on both gelatin, albumin + gelatin membranes cross-linked by carbodiimide (GC, AGC), and plastic surfaces, whereas both the rate of attachment and the number of cells attached were significantly reduced on albumin membranes cross-linked by carbodiimide (AC) (Fig. 2b). Otherwise, kinetics of cell attachment appeared slightly enhanced on fibrin glue during the first hour following cell seeding (Fig. 2c); then, become similar to the control.

In organ culture, adhesion strength of cells to substrate were evaluated after 7 days culture by measuring the kinetics of cells released during trypsin treatment. The diagram shown in Fig. 2d demonstrates that cell adhesion to glutaraldehyde and carbodiimide membranes was similar to Thermanox® (Thx) except for gelatin membrane (GG) and albumin + gelatin membrane (AGC). Fibrin glue displayed stronger adhesion than other substrates tested.

Scanning electron microscopy of cell monolayers grown on the different substrates has been performed in an attempt to further analyze the growth alterations. The micrographs presented in Fig. 3 illustrate the most striking differences observed in the growth of cells cultured on glutaraldehyde or carbodiimide cross-linked membranes. The morphology of dispersed cell cultures changed depending on membranes (Figs. 3a-f) and it can be seen that even after 11 days of incubation, cells were still scattered on glutaraldehyde cross-linked membranes (Figs. 3e-f) and could not spread as well as on carbodiimide cross-linked gelatin or albumin + gelatin membranes (Figs. 3c-d). Cell monolayers from organ culture kept a conformation quite similar to that shown on Thermanox® (Thx) (Figs. 3g-l). We observed a continuous tissue of well spread cells which tended to a more elongated shape on carbodiimide cross-linked membranes (Figs. 3i-j). Dispersed cells seeded on both albumin membranes displayed an altered morphology which explained the inhibition of growth (Figs. 3e-f) unlike organ culture which appears less sensitive and allowed for the formation of a quasi unaltered cell monolayer (Figs. 3k-l).

Dispersed cells have a similar morphology on both fibrin glue Biocol® (FG) and Thermanox® (Thx) in agreement with the growth studies (Figs. 3a-b). As seen in Figs. 3h-j (FG, GC, AGC), closely joined cells form a cellular tissue in organ culture which can be compared to the endothelium *in situ* of embryonic chicken artery (Fig. 4a).

Morphological analysis of extracellular matrix observed after solubilization of cell culture samples in a non-ionic detergent, provides a complementary technique to compare the response of cells to substrate. For both types of cell culture, no secretion of matrix product was detected on uncoated plastic surface as well as in dispersed cell cultures on both glutaraldehyde cross-linked membranes. A loose network of fibrous material can only be seen on carbodiimide cross-linked gelatin and albumin + gelatin membranes (GC, AGC) (Figs. 5b-c). In contrast, all the membranes seem to enhance secretion of a uniform fibrous network from organ culture even on albumin membranes (Figs. 5e-f).

It is interesting to note that large amount of extracellular matrix were secreted by cell cultures on fibrin glue Biocol® (FG) (Fig. 5a) while the cells from organ cultures produced only a thin matrix network on this substrate (Fig. 5d). Fig. 5f reveals the similarity of the fibrous network with the filamentous structure of subendothelium *in situ* (Fig. 4b).

Discussion

Analysis of these results will be more conclusive by determining what is comparable and what is different in cell behavior cultured according to cell and organ culture methods on different materials.

Similar results are the following:

Compared to control cultures, stronger cell attachment were observed with fibrin glue Biocol® (FG) as measured in the early phase of the culture, and stronger cell adhesion were also observed as measured after 7 days of culture. Moreover, cell growth was enhanced in organ culture whereas it could not exceed the confluent cell density in cell culture because of contact inhibition. Whether this inhibition process is responsible for the high secretion of extracellular matrix on fibrin glue Biocol® (FG) needs to be further investigated.

Cell attachment and cell adhesion to control surfaces and to carbodiimide cross-linked membranes except albumin, were comparable. Both culture methods exhibited lower growth on all the glutaraldehyde cross-linked membranes than on the other substrates. Whatever the reticulating agent used, albumin always gave the lowest growth values.

Divergent results are the following:

Migration of cells grown in organ culture was higher on glutaraldehyde cross-linked gelatin and albumin + gelatin membranes (GG, AGG) compared to Thermanox® (Thx) whereas single cells in cell culture could not adhere for longer than a few hours and then progressively detached from substrate.

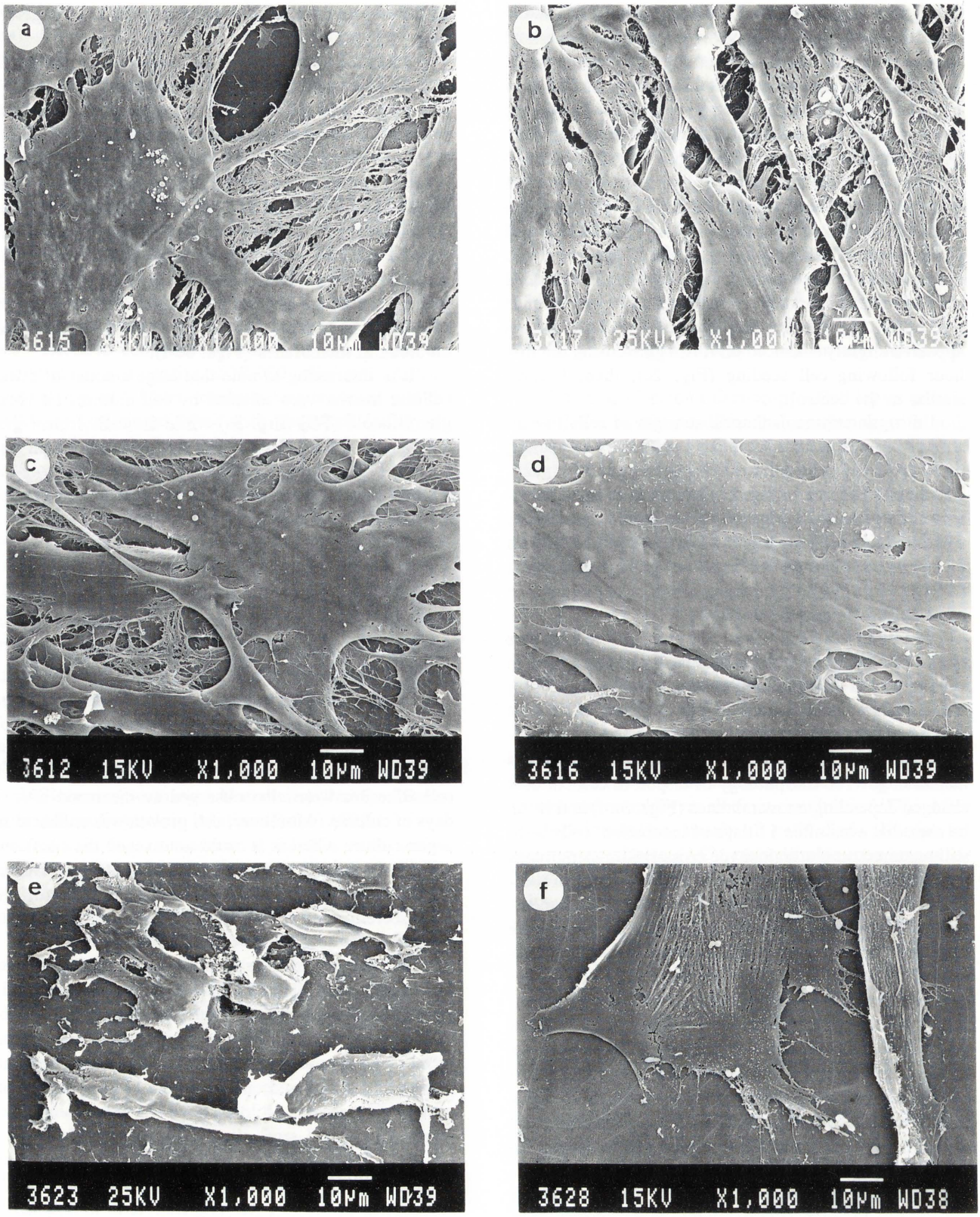

Figure 3 (a-f). Scanning electron micrographs of endothelial cells in cell culture on different substrates. Bar = 10 µm. **(a)** Culture-treated plastic dish surface; **(b)** FG; **(c)** GC; **(d)** AGC; **(e)** AG; **(f)** AGG.

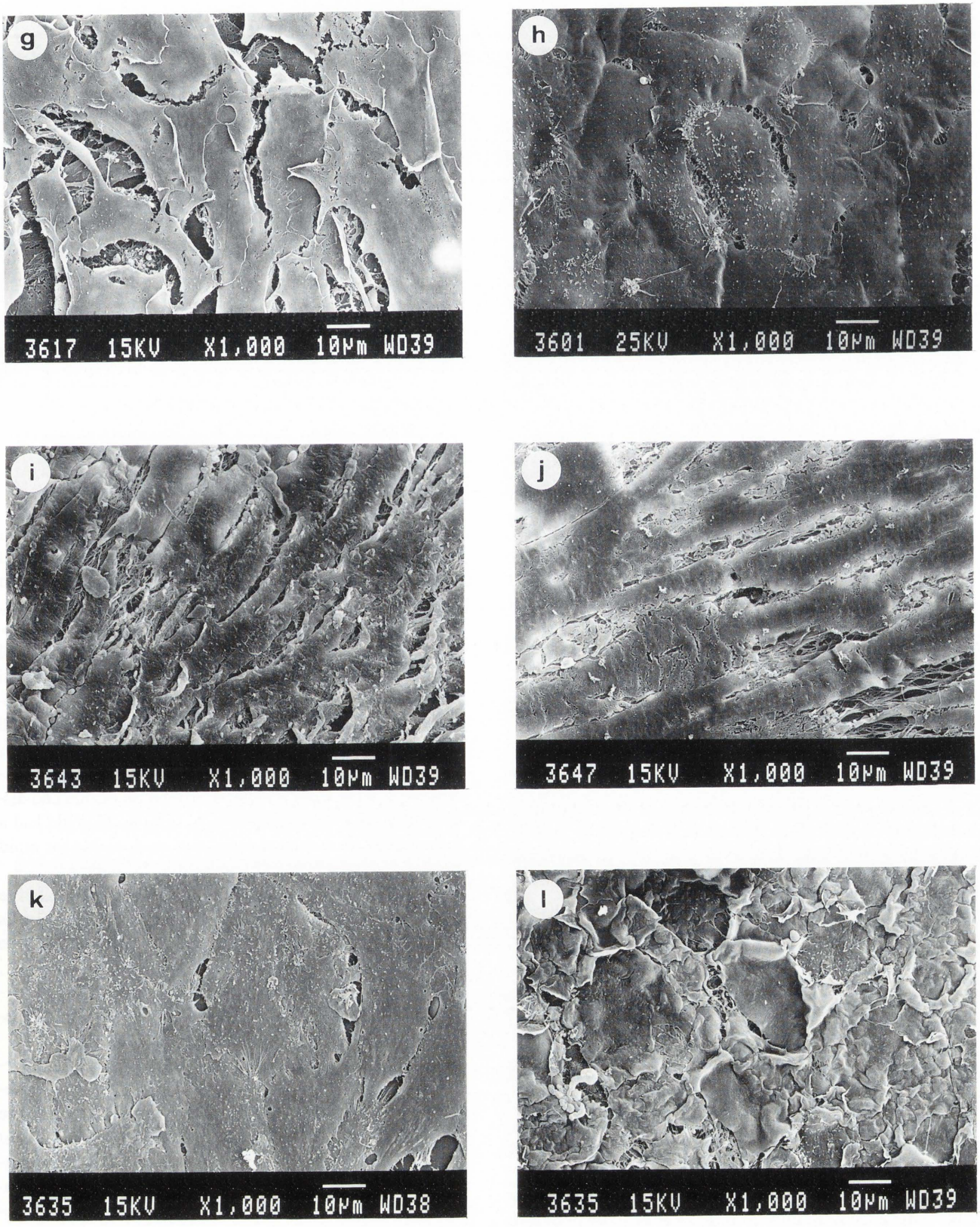

Figure 3 (g-l). Scanning electron micrographs of endothelial cells in organ culture on different substrates. Bar = 10 µm. (**g**) Thermanox®; (**h**) FG; (**i**) GC; (**j**) AGC; (**k**) AG; (**l**) AGG.

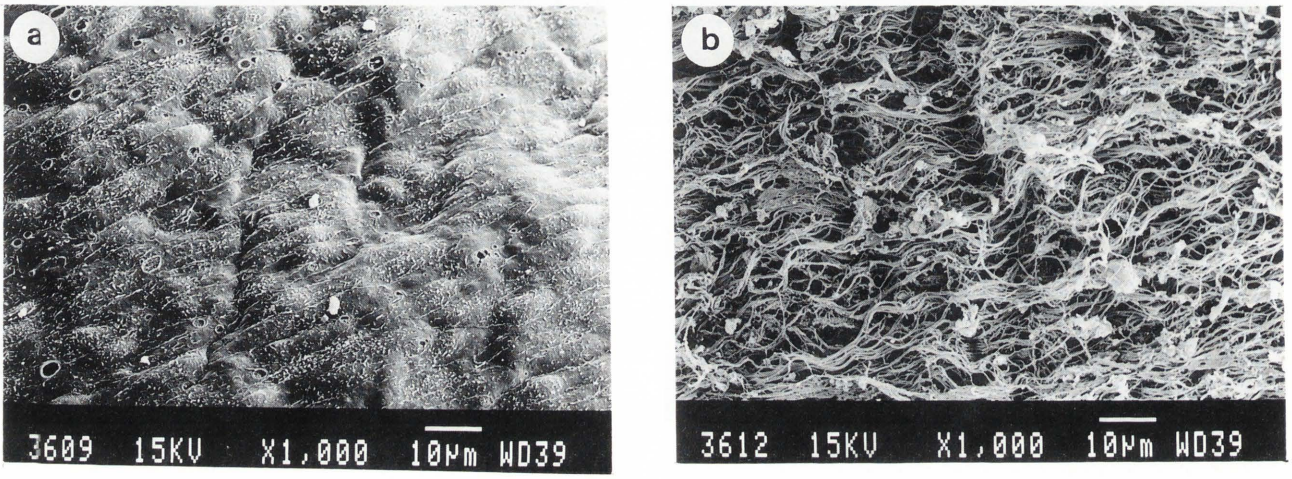

Figure 4a. Cell morphology of an endothelium *in situ* of chick embryo artery. **Figure 4b**. Sub-endothelium layer shape after removing cells. Bar = 10 μm.

All the tested membranes allowed extracellular matrix to be secreted from explants in a comparable amount although Thermanox® (Thx) prevented any secretion; dispersed cells culture did not synthesize cellular matrix except when they were grown on carbodiimide cross-linked gelatin or albumin + gelatin membranes (GC, AGC) which presented a small amount of fibrous material.

Considering the results provided by organ culture, it can be concluded that the most cytocompatible membranes are those which show growth parameters higher than Thermanox®. This criterium allows the selection of fibrin glue Biocol® (FG), gelatin and albumin + gelatin cross-linked by carbodiimide (GC, AGC). In dispersed cell culture, the best membranes are those which provide kinetic curves similar to control. Again the same substrates respond to these criteria: fibrin glue Biocol® (FG), gelatin and albumin + gelatin cross-linked by carbodiimide (GC, AGC). It is noteworthy that these three substrates enabled the secretion of extracellular matrix. Absence of matrix on culture treated plastic surfaces might result from the ionizing surface treatment to improve cell culture.

As a result of this study, cross-linked albumin can be considered as a highly toxic material. However, *in vivo* experiments of implantation of prostheses coated with carbodiimide or glutaraldehyde cross-linked albumin provided attenuated results which depended on the reticulating agent used (Ben Slimane *et al.*, 1988). Carbodiimide cross-linked albumin membranes enhanced the migration of endothelial cells after implantation in dog thoracic aorta, whereas glutaraldehyde cross-linked membranes induced an inflammatory reaction. Similar observations have been made after implantation in rat abdominal cavity (Ben Slimane *et al.*, 1986). Organ culture experiments confirmed these results. In contrast, dispersed cell culture was too sensitive to the physicochemical properties of material and toxicity of chemical products such as glutaraldehyde and consequently it did not allow such a discrimination. The other glutaraldehyde cross-linked membranes (GG, AGG) reduced cell growth compared to Thermanox® (Thx). Furthermore, cell density was lower in organ culture but cells continued growing throughout the incubation period and displayed an unaltered morphology. Glutaraldehyde had a lethal effect on single cells and was responsible for rapid cell release which prevents cytotoxicity assessment.

Previous work reported a similar comparative behavior of human umbilical vein endothelial cells (HUVEC) grown on glutaraldehyde and carbodiimide cross-linked membranes (Warocquier-Clérout *et al.*, 1990). Cell proliferation occurred at lower rate on carbodiimide membranes but was inhibited by glutaraldehyde.

Chemical products which have to be tested in pharmacology, and which might have an effect on biomaterials, can now be routinely analyzed by conjugating the possibilities of both these culture techniques (Adolphe *et al.*, 1991). They are complementary applications because organ culture may approach *in vivo* cell environment by maintaining cellular interactions and morphology, while dispersed cell culture may provide a more specific response of a type specific cell to a given material (Warocquier-Clérout *et al.*, 1991).

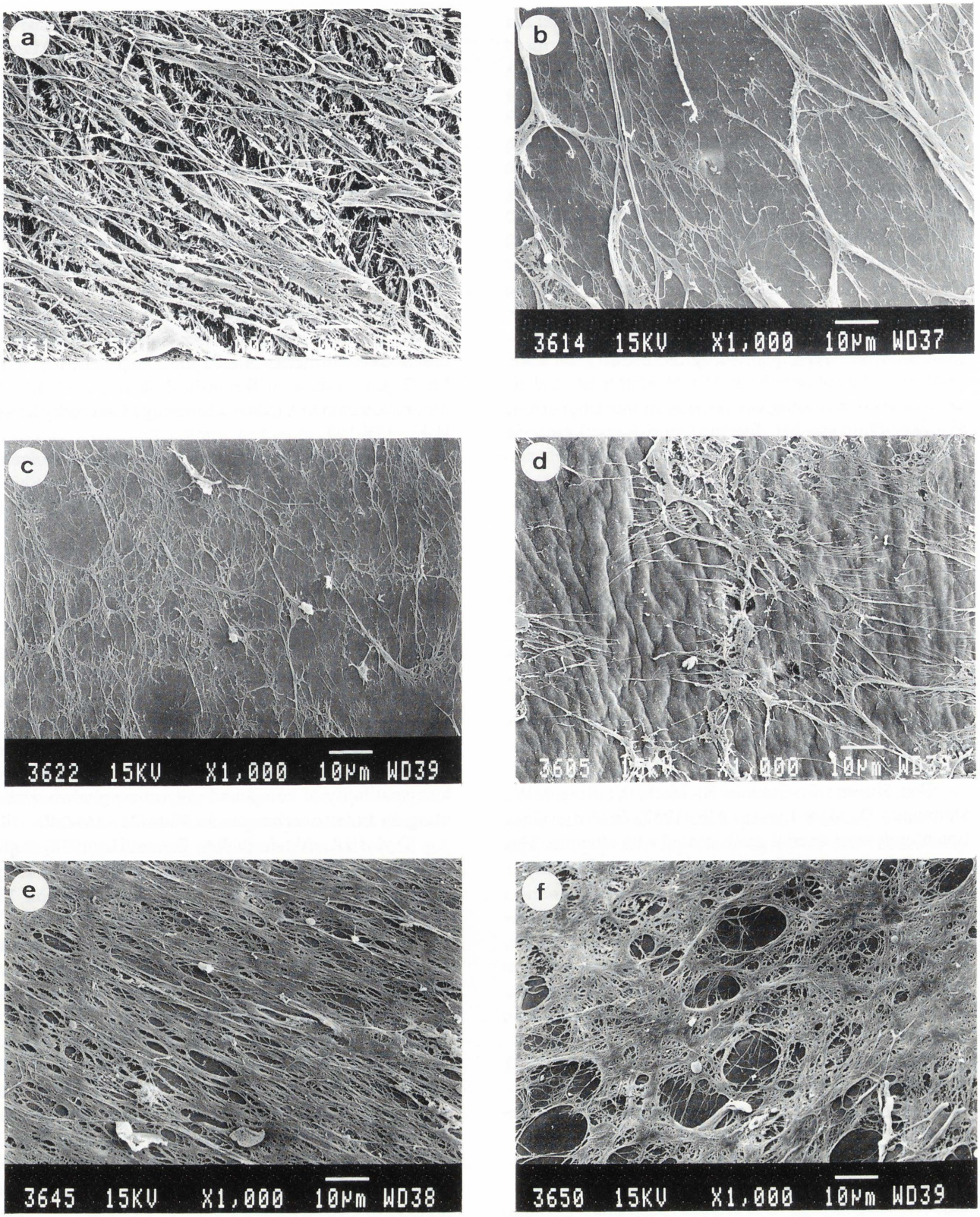

Figure 5. Scanning electron micrographs of extracellular matrix from dispersed cell (**a-c**) and organ (**d-f**) cultures on different substrates. Bar = 10 μm.
Cell culture: (a) FG; (b) GC; (c) AGC. **Organ culture**: (d) FG; (e) GC; (f) AGC.

Conclusion

Organ and dispersed cell cultures are complementary techniques useful to investigate the cytocompatibility of vascular prosthesis materials. Scanning electron microscopy has provided documentation of the cell morphology and behavior to elicit material which might be able to replace *in vivo* vascular endothelium. Therefore, fibrin glue Biocol®, gelatin, and albumin + gelatin cross-linked by carbodiimide, are proposed for coating vascular prostheses.

Acknowledgement

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References

Adolphe M, Thenet S, Vincent F, Demignot S (1991) Monolayer, tridimensional and immortalized cell line cultures as models in pharmacology and toxicology. Toxic. In Vitro **5**, 367-371.

Ben Slimane S, Guidoin R, Roy PE, Friede J, Hébert J, Domurado D, Sigot-Luizard MF (1986) Degradability of cross-linked albumin as an arterial polyester prosthesis coating in *in vitro* and *in vivo* rat studies. Biomaterials **7**, 268-272.

Ben Slimane S, Guidoin R, Merhi Y, King MW, Domurado D, Sigot-Luizard MF (1988) *In vivo* evaluation of polyester arterial grafts coated with albumin: The role and importance of cross-linking agents. Eur. Surg. Res. **20**, 66-74.

Duval JL, Sigot-Luizard MF, Sigot M (1986) Studies of endothelial cells adhesion to glutaraldehyde or carbodiimide cross-linked proteins. In: Biological and Biomechanical Performance of Biomaterials. Christel P, Meunier A, Lee AJC (eds.). Elsevier, Amsterdam. Advances in Biomaterials **6**, 269-274.

Duval JL, Letort M, Sigot-Luizard MF (1988) Comparative assessment of cell/substratum static adhesion using an *in vitro* organ culture method and computerized analysis system. Biomaterials **9**, 155-161.

Duval JL, Letort M, Sigot-Luizard MF (1990) Fundamental study of cell migration and adhesion toward different biomaterials with organotypic culture method. Observation of two cell components implicated in these events: actin and integrin. In: Clinical Implant Materials Heimke G, Soltész U, Lee AJC (eds.). Elsevier, Amsterdam. Advances in Biomaterials **9**, 93-98.

Freshney RI (1987) Disaggregation of the tissue and primary culture. In: Culture of Animal Cells. A Manual of Basic Technique. A R Liss, New York, Chap. 9, 113-114.

Hegyeli AF (1973) Use of organ cultures to evaluate biodegradation of polymer implant materials. J. Biomed. Mater. Res. **7**, 205-214.

McAuslan BR, Johnson G (1987) Cell responses to biomaterials I: adhesion and growth of vascular endothelial cells on poly(hydroxyethyl methacrylate) following surface modification by hydrolytic etching. J. Biomed. Mater. Res. **21**, 921-935.

Rae T (1976) The use of foetal rat knee joints in organ culture as a means of measuring the tolerance of tissues towards materials used for orthopaedic implants. In: Organ Culture in Biomedical Research. Balls M, Monnickendam MA (eds.). Cambridge University Press, U.K., 179-185.

Rae T (1986) Tissue culture techniques in biocompatibility testing. In: Techniques of Biocompatibility Testing. Williams DF (ed.), CRC Press. Chap. 3, p. 81-93.

Ratner BD, Horbett T, Hoffman AS (1975) Cell adhesion to polymeric materials: implications with respect to biocompatibility. J. Biomed. Mater. Res. **9**, 407-422.

Sigot-Luizard MF, Duval JL, Bourdillon C (1980) Etude de l'influence du pHi de membranes protéiques sur l'affinité cellulaire en culture *in vitro* (Study of the effect of pI of protein membranes on cellular affinity in *in vitro* culture). C.R. Acad. Sc. Paris **290**, 423-426.

Sigot-Luizard MF, Lanfranchi M, Duval JL, Ben Slimane S, Sigot M, Guidoin R, King M (1986) The cytocompatibility of compound polyester-protein surfaces using an *in vitro* technique. In Vitro **22**, 234-240.

Taylor JA, Abodeely RA, Fuson RL (1973) Rapid screening of biomedical polymers by two methods of tissue culture. Trans. Am. Soc. Artif. Intern. Organs **19**, 175-178.

Warocquier-Clérout R, Sigot-Luizard MF, Legendre JM (1987) F.p.l.c. analysis of the leakage products of proteins cross-linked on Dacron® vascular prostheses. Biomaterials **8**, 118-123.

Warocquier-Clérout R, Lee YS, Penhoat J, Sigot-Luizard MF (1990) Comparative behaviour of L-929 fibroblastic and human endothelial cells onto cross-linked protein substrates. Cytotechnology **3**, 259-269.

Warocquier-Clérout R, Penhoat J, Sigot-Luizard MF (1991) IL-1-induced procoagulant and fibrinolytic activities of human endothelium grown on carbodiimide cross-linked proteins. Cytotechnology **7**, 151-158.

Discussion with Reviewers

W.Y. Koo: The authors' technique for harvesting cells involves putting down the aortic explants with the endothelium facing the plastic substratum. In this position, are the endothelial cells injured? Is serum supplemented during the 6 days of incubation? Do smooth muscle cells grow out from the explant onto the dish in addition to the endothelial cells? Some of the scanning photomicrographs (Figs. 3a,b,c,g,h,l) show overlapping of cells which is not a characteristic feature of endothelial cells in general. It would be helpful if the authors could demonstrate that a pure culture of endothelial cells was obtained, for example, by staining the cells for Factor VIII-related antigen or by staining for acetylated-LDL uptake. Antibodies to α-smooth muscle actin would identify any smooth muscle cells.

Authors: The cell culture technique for harvesting endothelial cells was performed in accordance to current primary explant technique (Freshney, 1987). The organ culture routinely performed, provides a majority of endothelial cells as identified by staining the cells for Factor VIII-related antigen (Sigot-Luizard *et al.*, 1986). However, it is possible that some smooth muscle cells from the tunica media grew out of the vessel since organ culture maintains the integrity of the tissues and their mutual interactions. Similarly, cell suspension prepared by the primary explant technique could also contain comparable mixed cell population since neither enzymatic technique for harvesting cells nor culture medium enabled the discrimination of cell phenotype. The aim of this work was to compare the results provided by two culture techniques in order to assess the cytocompatibility of various substrates and purity of endothelial cell culture is not an absolute requirement since it would not reproduce *in vivo* environment.

W.Y. Koo: A number of cross-linked biomatrices were tested for cytotoxicity. It would be helpful to include a control study where the same matrices were used but non-cross-linked by any agents.

Authors: It is not possible to include a control study with the non-cross-linked matrices because albumin and gelatin are soluble proteins at 37 °C. They need cross-linking agent to become insoluble and to constitute solid membranes.

W.Y. Koo: The authors are comparing the cytotoxicity of two cross-linking agents: glutaraldehyde and carbodiimide. In their experiments, are the authors sure that the proteins tested were cross-linked to the same extent by both agents? This is most important because if one agent caused more cross-linking than the other, then the authors are not testing for cytotoxicity of the substrates but rather, they would be testing for the ability of cells to survive on substrates cross-linked to different degrees.

Authors: The biochemical processes of cross-linking of proteins by glutaraldehyde or carbodiimide are quite different: the glutaraldehyde links the amine radicals of the proteins to form a tridimensional network. In contrast, carbodiimide acts as a catalyst to activate carboxyl groups of proteins which bind amine radicals, then secondary products of carbodiimide are released from cross-linked protein network. The difference in the cytocompatibility of the substrates seem to be due to the presence of the cross-linking agents in the membranes rather than to the degree of reticulation. It could be argued that carbodiimide concentration of 0.2 M provided optimal conditions for high degree of reticulation meanwhile glutaraldehyde concentration was calculated to strengthen protein reticulation and to minimize the amount of remaining free-aldehyde groups. The toxicity of glutaraldehyde is now well known and unpublished data from our laboratory demonstrated that careful rinsing of the carbodiimide cross-linked membranes could reduce the leakage of carbodiimide to a non-toxic level.

W.Y. Koo: Could the extracellular matrix elaborated by organ cultures originate from vascular smooth muscle cells rather than endothelial cells? This may also explain why endothelial cells from organ cultures attached and grew better than dispersed cells cultures.

Authors: This is an interesting question for discussion. Whatever the percentage of vascular smooth muscle cells present in our organ cultures, it could be thought that they secrete growth factors or matrix products which modulate endothelial cell growth unless a process of reverse cell interaction takes place. Primary culture of explant by preserving the integrity of cell tissues help cell metabolism to resume after seeding whereas enzymatic treatment used in dispersed cell culture caused the solubilization of the extracellular matrix and injuries of cell membrane which modify the cell behavior. These technical differences may explain why endothelial cells from organ cultures attached and grew better than dispersed cell cultures.

J.M. Schakenraad: Why is it a restriction of the cell culture model that it is sensitive for physico-chemical material properties. Does that not also provide information on the biocompatibility of a material?

Authors: In the early phase of cell-material contact, physico-chemical properties of the material defined by Grinnell [Grinnell F (1978) Cellular adhesiveness and extracellular substrata. Int. Rev. Cytol. **53**, 65-144] as pH, isoelectric point pI, van der Waal's forces, ionic forces, wettability etc. may interact to inhibit single cell attachment causing a subsequent rapid cell death. Organ

culture can modify these characters by secreting extracellular matrix products which allowed cell migration to be delayed and then rebuilt the *in vivo* environment. This is well illustrated by albumin membranes: dispersed cells are not able to adhere and they die rapidly; explants from organ culture appeared surrounded with a limited cell layer of living cells. So, cell culture indicates an early cytotoxic effect, possibly not due to the physico-chemical properties of the membrane which can be overcome in the organ culture conditions.

J.M. Schakenraad: If your aim is stated as: comparing the cell culture and the organ culture models, why do you then choose all proteinaceous materials, of which you give no physico-chemical data. Since you also conclude that these physico-chemical material properties influence your cell culture test, I think it is necessary to have an idea about the material properties.
Authors: These proteinaceous membranes have been chosen because of their possible application for coating vascular polyester prostheses. Among their physico-chemical properties, we studied the influence of the pI on their cytocompatibility (Sigot-Luizard *et al.*, 1980). This parameter revealed some electric charge oppositions among these materials and it seemed to have no influence on their cytocompatibility in organ culture. Stability of the membranes have been controlled in *in vitro* physiological conditions but as stated in answer to W.Y. Koo, we do not know the degree of cross-linking. However, an attempt to evaluate the degree of cross-linking by measuring the shrinkage temperature of membranes showed a minimum of shrinkage for AG membrane at water-bath temperatures as high as 88 °C.

J.M. Schakenraad: What do the authors think is the reason for the different behaviour of the same cells in a different test model towards these materials.
Authors: We think that the main reason for different behavior of the same cells in a different test model towards these materials is an eventual secretion of extracellular matrix products by the whole cell tissue in organ culture and the maintenance of the organ structure which preserves the original environment of the cells.

J.M. Schakenraad: Apparently, in France Thermanox® is a negative control material. However it is not allowed as such in USP (United States Pharmacopoea), BSI (Britsh Standard Institute) or ISO (International Standard Organization). Why then use Thermanox® as "the golden standard"?
Authors: With regard to ISO international norms, the AFNOR (Association Francaise de NORmalisation) norms have the same value as BSI or USP national norms. All the national norms have been referenced in ISO norms, consequently Thermanox® can be considered as a negative control as defined in the AFNOR norm.

G. Pasquinelli: Do you know whether GC and AGC can remain sufficiently stable in the early phase of graft implantation thus avoiding any significant blood loss from the graft? On the other hand, can the substrate be readsorbed within 7-14 days as conventional G and A coatings do?
Authors: The stability of GC and AGC has been tested *in vitro* (Warocquier-Clérout *et al.*, 1987) but the *in vivo* study has yet to be done.

G. Pasquinelli: Apart from the agents which are used to cross-link the substrate, albumin *per se* always provided unsatisfactory results (however, this contrast with the corresponding SEM investigation). Would you comment on this?
Authors: As we answered to the first remark of Dr. J.M. Schakenraad, the surface properties of albumin membranes inhibit the adhesion of single cells and they are responsible for the abnormal shape of the few attached cells (Fig. 3e). In contrast, the organ culture appears less sensitive to the surface properties of the material and allows the formation of a limited cell layer. It is suggested that an early secretion of extracellular matrix neutralizes the surface properties of albumin to allow subsequent cell migration. We intend to study the kinetic of this secretion in organ culture to understand the mechanisms of cell migration in these conditions.

G. Pasquinelli: Figs 3e-f show details which can be eventually related to bacterial contamination. Did the authors perform any assays to rule out bacterial contamination? With regard to the adverse effect of glutaraldehyde, do you think that the presence of residual free aldehydic radicals may be, at least, in part, responsible for the cell failure to adhere and grow on the corresponding substrates?
Authors: Cells have been observed in SEM on day 11 and cultures were further incubated at 37 °C up to day 14 without appearance of bacterial contamination. We think that small points observed in SEM are artifacts.

The residual free aldehydic radicals are surely responsible for the cell failure to adhere and grow on the membranes cross-linked by glutaraldehyde. This toxicity is well-known and led us to look for another cross-linking agent such as carbodiimide. However it is possible to limit these residual radicals by rinsing the membranes in glycine solution after cross-linking reaction.

G. Pasquinelli: By looking over Figs. 3g-l, FG and AG seem to provide the best cell linings. The latter finding sharply contrasts with results provided by the functional assays. Would you comment on this subject?

Authors: Here is the typical difference between cell and organ culture. The albumin cross-linked by glutaraldehyde allows a weak cell migration but the morphologic aspect of the tissue is comparable to that on control culture Thermanox® or fibrin glue. Nevertheless, cell growth is drastically reduced, as also reported by van Wachem *et al.* [van Wachem PB, Vreriks CM, Beugeling T, Feijen J, Bantjes A, Detmers JP, van Aken WG (1987) The influence of protein adsorption on interactions of cultured human endothelial cells with polymers. J. Biomed. Mat. Res., **21**, 701-718], and the phenomenon by which single cells were released from albumin membranes after an early adhesion process is still unexplained.

G. Pasquinelli: Further, unlike FG, it appears that GC and AGC could facilitate the achievement of cell polarity. This finding is further substantiate by the amount of matrix deposited as well as by the pattern of matrix arrangement on such substrates as illustrated in Figs 5. Do you have any explanation for this?
Authors: This observation is difficult to explain but further demonstrates the cytocompatibility of these materials. The carbodiimide has been eliminated from these membranes by extensive washing and the cells are directly in contact with polymerized gelatin deprived of toxic residues. Thus gelatin membrane behaves as collagen coating which has been widely described to promote monolayer expansion and to influence cell polarity.

L.M. Waples: Your way of assessing attachment and/or adhesion in cell and organ culture is different between the two culture types. For cell culture, you looked at extracts from culture using PBS and 0.1 M NaOH for hydrolysis. How can you compare results from this method to the trypsin-EDTA treatment used for cell adhesion assessment in organ culture? Techniques have to be similar or ideally, identical in order to compare directly. Furthermore, attachment of cells to a substrate is a different parameter than adhesion strength of the cells to a substrate. Please clarify these items.
Authors: Analysis of endothelial cell behavior on biomaterials led us to observe phenomenon occurring during the very early phase of cell-material contact. In cell culture, we had the possibility to measure the kinetics of cell attachment by a using classical technique of solubilization of pre-labelled cells which provided accurate quantitative results. Both measures of the percentage of bound cells and of the rate of cell attachment vary depending on surface properties of substrates. In organ culture, early interaction of biomaterials could not be observed before cells had escaped from explants. This is the reason why we only gave a measure of adhesion strengths in a late growth phase. Fibrin glue appeared to enhance the rate of cell attachment and to increase adhesion strength in organ culture compared to the plastic control surface. However, attachment and adhesion processes were not affected in a similar way on other substrates. Carbodiimide cross-linked albumin decreased the rate of cell attachment and the amount of bound cells but did not affect the adhesion. Thus, this suggests that attachment and adhesion involved different molecular mechanisms and the two culture types provide complementary aspects of cell-substrate interactions.

L.M. Waples: Please comment on the statistics performed on the cell growth or cell attachment data presented in Figures 1 and 2.
Authors: The results of cell growth and cell attachment were subjected to Student's "t" test to determine the statistical significance. With a level of significance set up at $p < 0.05$, it appeared that cell growth on all the glutaraldehyde membranes and on both albumin membranes differed from control. On carbodiimide membranes (AGC, GC) significant decrease was observed on day 7 compared to control, then some cells began to detach from confluent control culture and on day 11 all the confluent cultures had similar densities. Cell growth on fibrin glue did not statistically differ from control.

As to kinetics of cell attachment, we found a significant increase on fibrin glue after 30 minutes incubation, then results did not differ. The rate of cell attachment was similar on all the membranes when compared to control except after 1 hour post seeding on glutaraldehyde cross-linked albumin + gelatin and on carbodiimide cross-linked albumin.

L.M. Waples: Please discuss why cell growth and cell attachment differed on the different membranes.
Authors: Experiments herein reported did not provide biochemical arguments to explain the differences in cell growth and cell attachment on the membranes. However, SEM observation of the extracellular matrix and of the morphological changes of cells indicated the involvement of the secretion of extracellular proteins and probably a rearrangement of the cytoskeleton which have to be further investigated by other methods.

L.M. Waples: It is not entirely clear from this study that you have measured cross-linked albumin to be toxic! Your measured parameters are cell growth and cell attachment. There could be decreased cell proliferation as opposed to cell death.
Authors: Cross-linked albumin membranes displayed a surface toxicity which hindered cells from spreading or proliferating so that the attached cells rounded and progressively detached from these membranes.

L.M. Waples: While it is good to look at cell attachment, adhesion and growth on a given substrate, other parameters must be evaluated before a substrate is labelled cytotoxic:

a) You mentioned in the introduction about using cell culture to test leached substances from a biomaterial. It would be worthwhile to perform this test with your substrate. This way, if cell death is occurring you might be able to determine what factor, the material or its leached substances, is cytotoxic. Note: there is an ASTM test method for obtaining extracts from implant materials.

b) It would be very useful to perform a cytotoxicity assay. Many ways exist to do this; load the cells with a label and look for the labels release or measure an enzyme released upon lysis such as LDH.

This information, a & b, coupled with your current work would allow better assessment of the substrates and better comparison between organ and cell culture techniques. Otherwise it is difficult to say with your current data that organ and cell culture yield comparable results. Furthermore, insufficient data exist to label a given substrate cytotoxic or non-cytotoxic.

Authors: We agree with you that the biocompatibility of a material has to be tested first by determining the cytotoxicity of leached substances by using the standardized assays that you mentioned. Our cross-linked membranes had undergone classical cytotoxicity tests and a chromatographic analysis of the leached products (Warocquier-Clérout *et al.*, 1987). A detailed protocol of elution of the membranes has been set up to lower the amount of leached secondary degradation products of carbodiimide to a level which does not affect cell proliferation (to be published).

The main purpose of this work was to analyze some biological parameters (attachment, adhesion, growth, migration) which were accessible respectively in cell culture or in organ culture or both in order to assess the cytocompatibility of a biomaterial. Different aspects of endothelial cell behavior were thus studied by using these techniques and classification of the substrates, which responded to the best criteria of biocompatibility as defined in each method, gave the same results.