In vivo quantification of cell–polymer interactions

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An *in vivo* rat model was developed to determine cell–polymer interactions under physiological conditions. Microporous tubular grafts, made of polytetrafluoroethylene, a polyetherurethane, a polyesterurethane and also a modified polyetherurethane were implanted intraperitoneally in rats. The grafts were filled with cultured rat smooth muscle cells prior to implantation.

At t = 0, 2 and 48 h, the grafts were evaluated macroscopically and also prepared for light microscopy and for cell count of their contents. At t = 0 no cellular attachment was observed on the lumenal side of the capsules. At t = 2 h a monolayer of smooth muscle cells could be observed on all materials except PTFE, on which only small patches of cells were observed. At t = 48 h a multilayer of cells was seen on all materials except PTFE. Cell counts at 48 h demonstrated no multiplication in the PTFE graft but a 1.4, 2.3 and 2.0-fold multiplication in the polyetherurethane, polyesterurethane and the modified polyurethane grafts respectively. These *in vivo* results show a clear linear relationship with our *in vitro* results in which it has been proved that cell spreading increased with increasing substratum surface free energy. This rat model allows the study of cell–polymer interactions *in vivo*, in a standardized way, under controlled physiological conditions.

Keywords: Polymers, cell–polymer interaction, microporous tubular graft, polyurethanes, polytetrafluoroethylene, surface free energy

Recently we developed an *in vitro* test model to study cell–polymer interactions[1-3]. The effect of substratum surface free energy on cell spreading and growth, and the influence of certain factors e.g. cellular and substratum surface charge[4-6], cell surface free energy and the effect of serum proteins on the adhesive process[7-11] were studied employing this model.

The aim of this paper was to determine the relevance of the observations with the *in vitro* model for *in vivo* situations. To do so, we designed an *'in vivo* cell culture' model which consisted of a microporous tubular graft, in which smooth muscle cells were seeded. After closure of the graft it was implanted intraperitoneally in rats and removed at 2 or 48 h for the evaluation of cellular adhesion and growth.

In such an *'in vivo* cell culture model' cell–polymer interactions can be evaluated in a well-controlled physiological environment. The results can directly be related to those obtained with the *in vitro* test as well as to *in situ* observations of implanted microporous vascular grafts[12,13].

MATERIALS AND METHODS

Tubular grafts. Four polymers (*Table 1*) were used for the preparation of microporous tubular prostheses (see *Figure 1*) with a length of 1 cm, 1.5 mm i.d. and a wall thickness of

Table 1 Polymer materials used as tubular grafts for implantation in the peritoneal cavity of wistar rats; pore sizes and surface free energies (γ_s)

Polymer	Abbreviation	Specification	Pore size (μm)	γ_s (erg.cm^{-2})
Polytetrafluoroethylene	PTFE	Goretex, USA	40	20
Polyetherurethane	PEU	Pellethane 75D, Upjohn, USA	30–100[a]	48
Polyesterurethane	PESU	5701 F1 Goodrich, USA	30–100[a]	53
Modified polyurethane	PU	Not available	30–100[a]	56

[a]Pore size gradient, ranging from 30 μm on the inside to 100 μm on the outside (see *Figure 1*).

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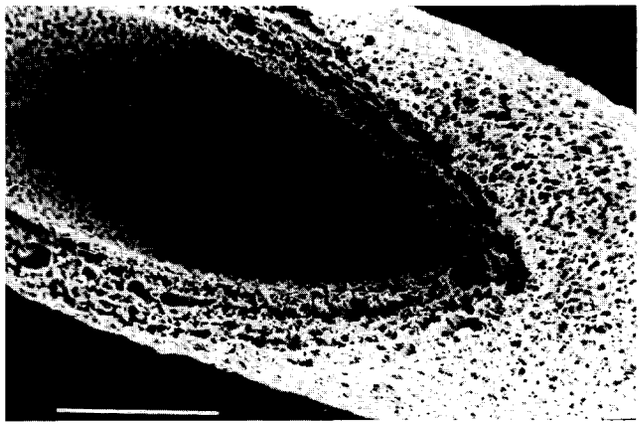

Figure 1 SEM of a tubular microporous graft showing large pores on the outside (100 μm) and small pores on the inside (30 μm). The bar denotes 1 mm.

300 to 400 μm. The grafts were prepared by a modified dipcoating technique described by Gogolewski *et al.*[17], except for PTFE grafts, which were available commercially.

Cells. An established cell line of rat smooth muscle cells was cultured in RPMI 1640 medium (Gibco) supplemented with 15% foetal calf serum (Gibco) and 100 u./ml penicillin-/streptomycin at 37°C and 5% CO_2 in humidified air. Every 2 d, cultures were subdivided by trypsinization [0.15% (w/w) trypsin (Difco) 1:250 in Ca/Mg free Hanks balanced salt solution].

Cell spreading. For cell spreading experiments 2 ml aliquots of trypsinized cells were plated, at a density of 5×10^4 cells-/ml, in 6-plate wells on the bottom of which the different polymer films were positioned. After 2 h incubation at 37°C, 2% glutaraldehyde in phosphate buffer was added for cell fixation. Each well was photographed to obtain 100 cells per substratum material on the photonegatives.

The surface area of the cells was used as a measure for cell spreading and was determined with an antiplotter using enlarged photonegatives. The spreading of cells on the different substrata was compared with the cell spreading on tissue culture polystyrene (100%) to obtain relative cell spreading values. Cell spreading was measured in the presence of 15% foetal calf serum in the medium.

Implantation. Trypsinized smooth muscle cells were centrifuged for 10 min at 1000 rpm. After decantation of the supernatant, 1 ml of RPMI medium was added and cells were resuspended. Cell count and viability test (trypan blue exclusion test) were performed subsequently. After another centrifugation step (10 min at 1000 rpm) the pellet was resuspended in rat serum at a concentration of 5×10^5 cells/ml. The cell suspension (0.1 ml) was put into the tubular graft which was then closed (mersilene 2-0, EH 6664 Ethicon).

The grafts were implanted at random in the peritoneal cavity of male wistar rats, weighing approx. 200 g, through a 2 cm midline incision under Fluothane anaesthesia. The experiments were performed in triplicate; each material was implanted 4-fold for each time period. Grafts filled with serum only functioned as a control to test cellular ingrowth from the outside.

Harvesting and evaluation. At $t = 0$, grafts were collected directly after cell seeding. At $t = 2$ h and 48 h prostheses were harvested from the abdominal cavity and prepared either for cell count or for light microscopy.

For: (i) cell count, the content of the grafts was trypsinized, the viability (trypan blue) was tested and cell count (Türk vital staining) was performed. A control experiment to test trypsinization efficiency was performed at $t = 0$, 2 and 48 h ($n = 4$). The trypsinized grafts were checked for cellular residues in and on the graft surface.

For each graft at t = 48 h the Growth Index (G.I.) of the smooth muscle cells was determined:

$$\text{G.I.} = {}^2\log \frac{\text{amount or number of viable cells at 48 h}}{\text{amount viable cells at implantation time}}$$

Cell counts are given as a mean of 12 determinations ± standard deviation (SD).

And (ii) light microscopy, the ends of the capsules were cut off and the capsules were fixed in a 0.1 M phosphate buffered (pH 7.4) solution of 2% glutaraldehyde. After dehydration in alcohol, the specimens were embedded in paraffin. Semi-thin sections (thickness 1 μm) were cut, using a glass knife, and subsequently stained with haematoxylin and eosin.

Surface free energy. Surface free energies (γ_s) were calculated according to the method of Busscher *et al.*[14], accounting for spreading pressure, at 37°C in humidified air. Measurements were performed on solid films of the corresponding polymers using contact angles of droplets of phosphate buffered saline (PBS), mixtures of PBS-/*n*-propanol and α-bromonaphthalene.

RESULTS

The grafts filled with serum only, did not show any cellular ingrowth into the lumen originating from the outside. Apparently the pore size gradient of the grafts prevented perigraft tissue ingrowth within this time interval.

The grafts could always be removed easily without major adhesions to surrounding tissues. Grafts evaluated at $t = 0$, showed no adhesion of smooth muscle cells to the lumenal surface of the graft (*Figure 2 a*). At $t = 2$ h a single or double layer of cells had adhered and spread on the lumenal surface of the polyurethane grafts (*Figure 2 b*). The PTFE grafts did not show spreading of cells although cells had adhered. At $t = 48$ h, all materials except PTFE showed multiplication of cells; the single layer had developed into a multilayer also showing infiltration into the pores of the lumenal wall (*Figure 2 c*). Cell counts of the trypsinized content of the grafts at $t = 48$ h showed an increase in cell numbers for all materials except for PTFE (see *Table 2*). After trypsinization no significant amounts of cellular remainings were found in or on the graft lattice.

A plot of the G.I. of the cells at 48 h *versus* the graft surface free energy can be projected on a plot of *in vitro* cell

Table 2 Surface free energy (γ_s) in erg.cm^{-2}, mean cell count per capsule ± standard deviation (× 10^4) and Growth Index (G.I.) of four tubular prosthetic materials at t = 48 h after implantation

Polymer	γ_s	Mean cell count (± SD)	Growth Index
PTFE	20	5.0 ± 1.5	0
PEU	48	13.8 ± 2.5	1.4
PESU	53	22.8 ± 2.6	2.3
PU	56	20.0 ± 4.1	2.0

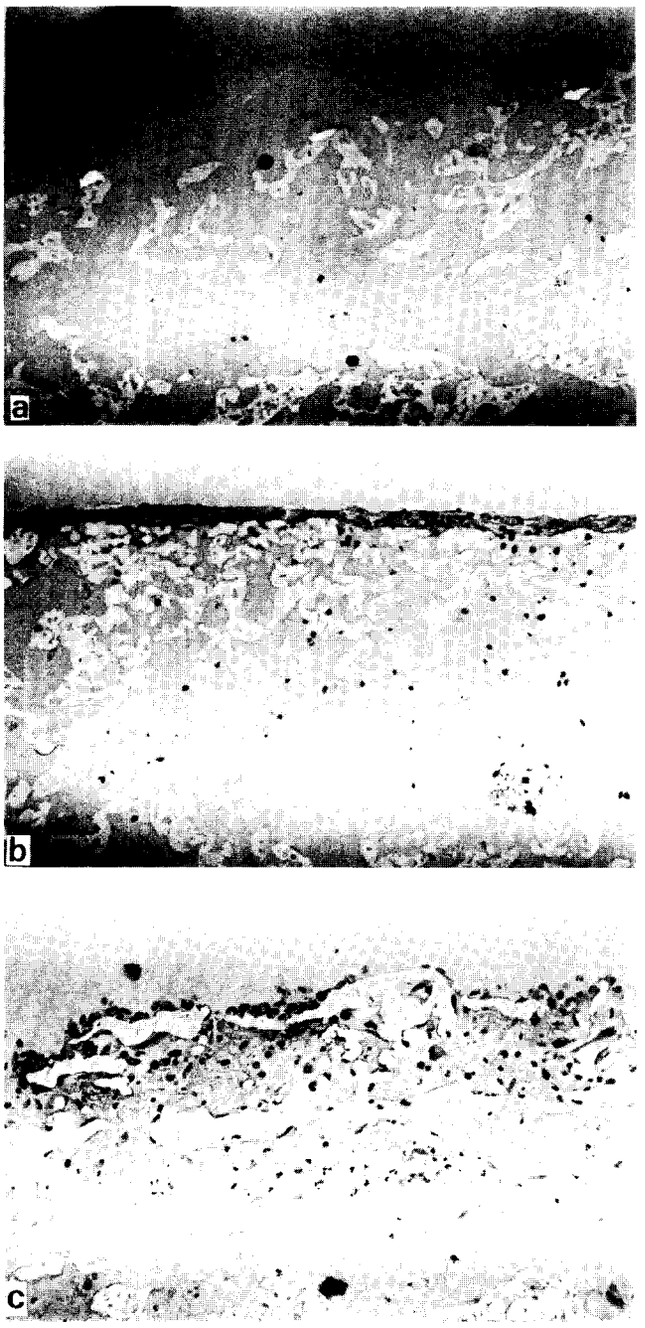

Figure 2 Haematoxylin and eosin staining of a PU graft at t = 0 (a), 2 h (b) and 48 h (c) after intraperitoneal implantation. The bar denotes 100 μm.

spreading *versus* surface free energy[3] and suggests a similar sigmoid relationship (*Figure 3*).

DISCUSSION

The results of this study demonstrate that our newly designed *in vivo* rat model provides a simple, physiological, quantitative and reliable test system. The results obtained with this *in vivo* system correlate well with results obtained with our *in vitro* model[3].

In vitro cell spreading is related to substratum surface free energy in a sigmoid way (*Figure 3*); polymers with high γ_s show good cell spreading whereas polymers with low γ_s show poor cell spreading. The transition from poor to good cell spreading occurs on surfaces having a γ_s of about 45 erg.cm^{-2}. To evaluate whether cell spreading *in vitro* correlates with cell growth in the *in vivo* model, presented in this paper, we plotted the relative cell spreading *versus* the Growth Index (*Figure 4*). A linear relationship (R = 0.9) can be noted. Substratum surface free energy seems to be a major determinant for cell-polymer interactions, not only under standardized *in vitro* cell culture conditions but also in a physiological environment[15]. Even more important, this new *in vivo* rat model provides data which correlate also with *in situ* experiments performed by van der Lei *et al.*[12,13]. These authors implanted two similar types of microporous tubular grafts of different surface free energy in a disected part of the rat abdominal aorta. PTFE vascular grafts of low surface free energy (γ_s = 20 erg.cm^{-2})[3] showed poor attachment and poor overgrowth of tissue in and over the graft. A high surface free energy substratum (polyurethane-/PLLA (poly-L-lactic acid): γ_s = 56 erg.cm^{-2})[3] showed complete overgrowth with smooth muscle cells and endothelial cells and also ingrowth of perivascular tissue in the graft wall.

In conclusion, the results obtained in this *in vivo* rat model correlate closely both with results from *in vitro* and *in situ* models as well as with predictions made on the basis of surface free energy determinations.

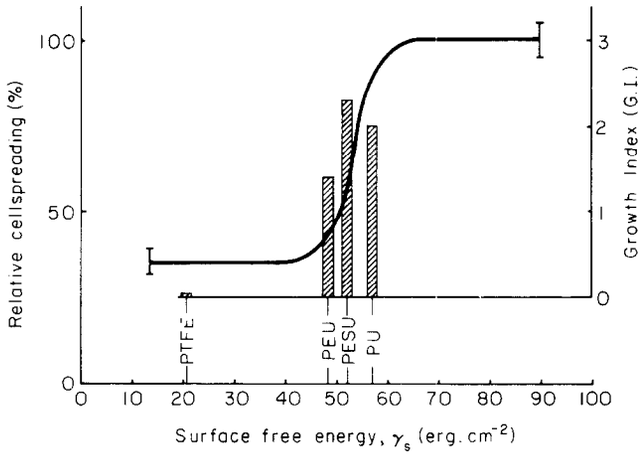

Figure 3 The relationship between relative cell spreading in vitro (in the presence of serum proteins[3]), the in vivo Growth Index determined in this study and substratum surface free energy.

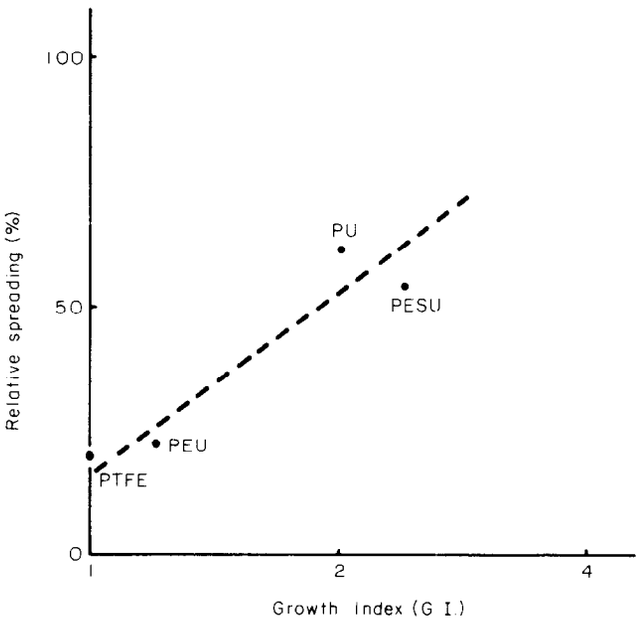

Figure 4 Correlation diagram of the relative spreading of smooth muscle cells in vitro versus Growth Index of smooth muscle cells in the in vivo cell culture model. (R = 0.9).

This *in vivo* rat model enables us to screen graft material characteristics, to test the effect of protein coatings on biomaterials and to test cell seeding techniques in an inexpensive convenient way with a fast experimental outcome.

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