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Monocyte adhesion and adhesion molecule expression on human endothelial cells on plasma-treated PET and PTFE *in vitro*

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The aim of this study was to evaluate *in vitro* the inflammatory potential of endothelialized surfaces of polyethylene terephthalate (PET) and polytetrafluorethylene (PTFE) after ammonia gas plasma modification. HUVECs grown on polystyrene and HUVECs stimulated with tumor necrosis factor (TNF-α) were used as controls. At day 1 and day 7, surfaces were evaluated for U937 cells and HUVECs using flow cytometry and immunohistochemistry. Plasma-treated PET (T-PET) and treated PTFE (T-PTFE) increased U937 cell adhesion compared to the negative control but this was not statistically significant. Maximal adhesion of U937 cells to HUVEC was observed on TNF-α stimulated endothelium with significant differences between day 1 and day 7. There was a small increase in U937 cell adhesion to plasma-treated PET compared to PTFE on both day 1 and day 7, but this was not statistically significant. Immunohistochemical staining demonstrated two patterns of distribution for monocyte adhesion on materials. On T-PET the cells were positioned in clusters attached to HUVECs and on T-PTFE the cells were randomly distributed on HUVECs and material. The effects of plasma-treated PET and PTFE on HUVEC adhesion and proliferation were also studied. On day 1 there were slight increases in the growth of HUVECs on both of T-PET and T-PTFE but this was not statistically significant. On day 7, cell number increased significantly on all of surfaces compared to the negative control. The results demonstrate that the plasma treatment of PET and PTFE with ammonia improves the adhesion and growth of endothelial cells and these surfaces do not exhibit a direct inflammatory effect in terms of monocyte adhesion. Plasma-treated PTFE enhances HUVECs growth and was less adhesive for monocytes as compared with treated PET. The monocyte adhesion to endothelial cells on surfaces can be used as a tool for the evaluation of material surface modification and further to study the mechanisms of cell to cell interactions in response to surfaces.

1. Introduction

The use of synthetic biomaterials as vascular grafts to replace diseased human blood vessels has increased tremendously over the past thirty years [1], polyethylene terephthalate (PET) and polytetrafluorethylene (PTFE) are two of the most commonly used. However, current small-diameter (< 6 mm) vascular grafts have a potential for thrombosis of the internal graft surface [2]. Surface modification is one possible methodology for improving the patency. Since normal vessels are lined by a monolayer of endothelial cells (EC), which are the ideal nonthrombogenic blood-contacting surface, it is believed that covering the surface of vascular prostheses with EC would combine the thromboresistance of endothelium with the availability of a prosthesis [1].

The endothelium is of crucial importance in the regulation of the homeostatic balance and plays a pivotal role in the inflammatory and immune response through the phenomena of leukocyte attachment and their subsequent transluminal migration to the site of inflammation. This recruitment of circulating cells is mediated by the expression and regulation on the surface of vascular endothelial cells of adhesion molecules and cytokines, the role of which is now partially elucidated [3, 4].

Although considerable knowledge is available on EC growth on materials, very few studies have concentrated on the EC response to materials by their expression of adhesion molecules [5, 6] and monocyte adhesion particularly on plasma-modified PET and PTFE. Previous work has shown that the expression of endothelial cell adhesion molecules was slightly increased on ammonia plasma-modified PET and PTFE [7]. In this study we have examined monocyte adhesion

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to endothelialized PET and PTFE surface with ammonia plasma modification.

2. Materials and methods

2.1. Materials and treatment

PTFE and PET were purchased from Goodfellows in 1 mm thick sheet form and punched into 12.5 mm diameter disks. Surfaces of PTFE were polished, then both materials were cleaned and plasma-treated using ammonia gas in an in-house built gas plasma rig [8]. After treatment the samples were stored in sterile de-ionized water and used within 5 days.

2.2. Cell culture

HUVECs were harvested from freshly obtained human umbilical cord using the collagenase isolation technique described by Jaffe *et al*. [9]. The cell growth medium consisted of 50% 199 medium and 50% RPMI-1640 (v/v,) supplemented with 20% fetal calf serum (FCS, v/v), penicillin–streptomycin (100 U/mL and 100 μg/mL, respectively), amphotericin (0.25 mg/mL), and 4 mML-glutamine (Gibco). The cultures were grown at 37 °C in an atmosphere of 5% CO_2. At confluence, cells were passaged with 0.05% trypsin and 0.02% ethylene diaminetetra-acid (EDTA, Sigma). For experiments, HUVECs from the first passage were pooled from several umbilical cords (to avoid batch to batch variability of cells, but tested as independent passage). The phenotype of the endothelial cell (EC) was confirmed using monoclonal antibodies for PECAM-1 (Pharmingen, USA) and von Willebrand factor (Serotec Ltd, UK) and visualized and quantified using immunohistochemistry and flow cytometry (FACS). Monoclonal antibody anti α-smooth muscle actin (α-SMA, Sigma) was used to determine the contamination of smooth muscle cells. Promonocytic U937 cells [10] were obtained at unknown passage from European Collection of Cell Cultures (ECACC, Wiltshire, UK) and maintained in suspension culture in RPMI 1640 with 10% FCS and 2 mML-glutamine. They were passaged every 3 days after achieving a density of 4×10^5 cells/ml.

2.3. HUVECs contact with PET and PTFE and monocyte adhesion

Confluent endothelial cells were detached with trypsin-EDTA for less than 3 min, washed in cell culture medium and resuspended in 1 ml of fresh medium with a cell density at 1×10^5. Suspensions were seeded on materials placed in 24 well-plate for 1 day and 7 day periods. To compare plasma-treated PET and PTFE, all the assays were conducted with a negative control and positive control using polystyrene coverslips (Starstedt Ltd, UK). The negative control was cells cultured in cell culture medium alone while in the positive control, cells were stimulated with tumor necrosis factor (TNF-α) at a concentration of 10 ng/ml.

Adhesion studies were performed with the promonocytic cell line U937, which has been established as a useful model for monocytes in adhesion studies [11]. To test monocyte adhesion to gas-plasma-treated PET and PTFE after growth with HUVECs, U937 cells were centrifuged and resuspended at density of 10 cells/ml, and then 100 μl of cells were added to the different surfaces on day 1 and day 7 and allowed to adhere at 37 °C for 4 h in a 5% CO_2/95% air incubator. At the end of the incubation period, the medium from each well was aspirated and the materials coverslips were transferred to fresh 24 well-plates. The monolayer was gently washed three times with cold PBS. Preliminary experiments indicated that three washes of the monolayer decreased negative control adhesion by 50–100% (compared with a single wash), while effecting a minimal decrease ($< 2\%$) in TNF-α stimulated adhesion. The HUVECs proliferation and monocyte adhesion were analyzed by FACS and immunohistochemistry.

2.4. Immunofluorescence flow cytometry

In order to analyze HUVECs, U937 cells and the effects of plasma-treated PET and PTFE on the monocyte adhesion after endothelialization, FACS was used. The cells on surfaces were collected and resuspended with 1% bovine serum albumin (BSA, Sigma, UK) in PBS. Single-cell suspensions (20 μL) were incubated for 15 min at 4 °C with 1 μL of one of the following mouse anti-human monoclonal antibodies: leucocyte function associated-1 (LFA-1, CD 11a), PECAM-1 (CD31), Von Willebrand factors and α-smooth muscle actin (α-SMA). All were conjugated with one of the following: fluorescein isothiocyanate (FITC), R-phycoerythrin (RPE), or Cy-chrome (CyC). An isotype control (IgG1-κ) was used as the negative control. All antibodies were obtained from Pharmingen Europe. Subsequently, cells were diluted with 200 μL filtered saline sheath fluid (FACS-Flow, Becton Dickinson, Oxford, UK) and 30 000 events were collected using a FACSort flow cytometer running the Lysis II program (Becton Dickinson, San Jose, California). The percentage of positive cells was determined by comparison with the isotype control. U937 cell and HUVEC numbers were quantified by the cell expression of CD 11a and CD31.

2.5. Immunohistochemistry

An avidin biotin complex based immunostaining protocol was performed to visualize and confirm the quantified expression from the FACS analysis. After HUVECs were cultured on plasma-treated PET and PTFE with controls for a period of 1 and 7 days and then cocultured with U937 cells for 4 h, they were fixed with 95% methanol (Sigma) at room temperature for 10 min and dried in air. The cultures were stained and fixed on the same day. The cells were pre-treated with 1% normal horse serum in PBS (Sigma) for 20 min before incubation with one of the following primary mouse anti human monoclonal antibodies CD31, CD11a, α-SMA (Serotec Ltd, UK), diluted 1 : 100 or 1 : 1000. The secondary antibody used was a Biotinylated Rabbit Anti-mouse Immunoglobulin (DAKO A/S, Denmark, dilution 1 : 200).

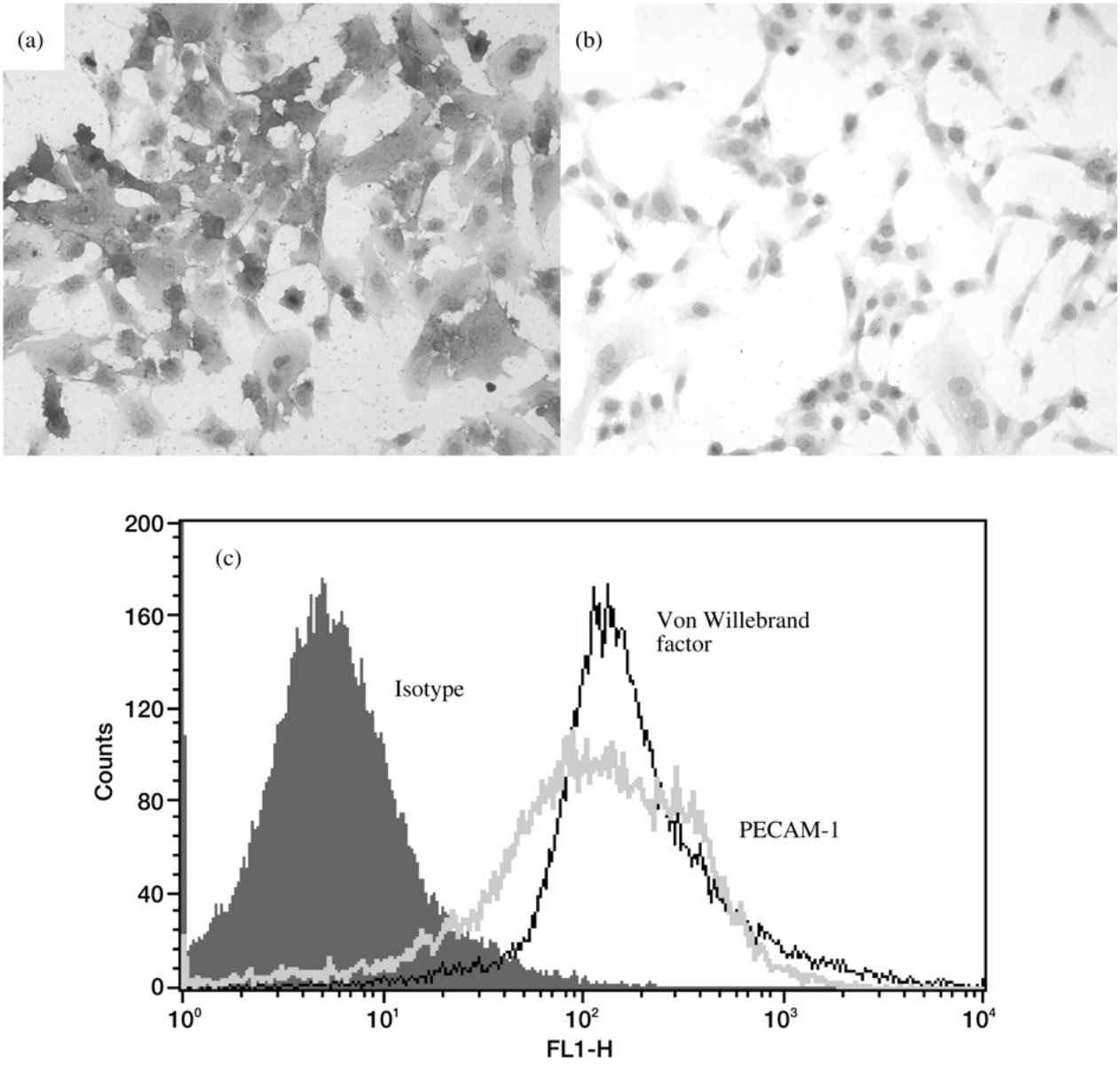

Figure 1 HUVECs were characterized by immunohistochemical staining for PECAM-1 (a) but not for α-SMA (b) FACS analysis (c) showed over 98% purity with two specific markers, PECAM-1 (open gray curve) and Von Willebrand factors (open black curve). Solid curve represents the negative control. Data from one of five similar experiments is presented.

2.6. Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences, Inc, Chicago) by ANOVA and Waller-Duncan's multiple comparison tests were applied to detect differences between groups. Four or five repeats per material per time period were used for the statistics. In all statistical evaluations $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Characterization of cells

HUVEC and U937 cells were characterized by the expression of specific adhesion molecules using immunohistochemistry and FACS analysis before they were used in contact with materials. Fig. 1a shows the morphological features of HUVEC cultures by immunostaining. Almost all of the cells stained positive for the specific endothelial cell marker PECAM-1 (CD 31) and Fig. 1b negative for α-SMA (a marker for the identification of smooth muscle cells which are the most common contaminant of HUVEC isolations and cultures). The purity of these cultures was also determined by FACS analysis. One typical profile of HUVEC cultures is presented in Fig. 1c. Over 98% of the cells were stained positive for PECAM-1 and Von Willebrand factor. Once the cell purity was confirmed, they were then used for the experiments.

U937 cells were also characterized by the expression of adhesion molecules using immunohistochemistry and FACS analysis. Fig. 2a shows the morphological features of U937 cells nearly 100% stained positive for CD11a on a cytospin preparation slide and FACS analysis in Fig. 2b.

3.2. Monocyte adhesion

The adhesion of monocytes to endothelium on plasma-treated PET and PTFE was performed after a series of preliminary experiments, such as the culture of U937 cells on different surfaces alone or coculture cells on

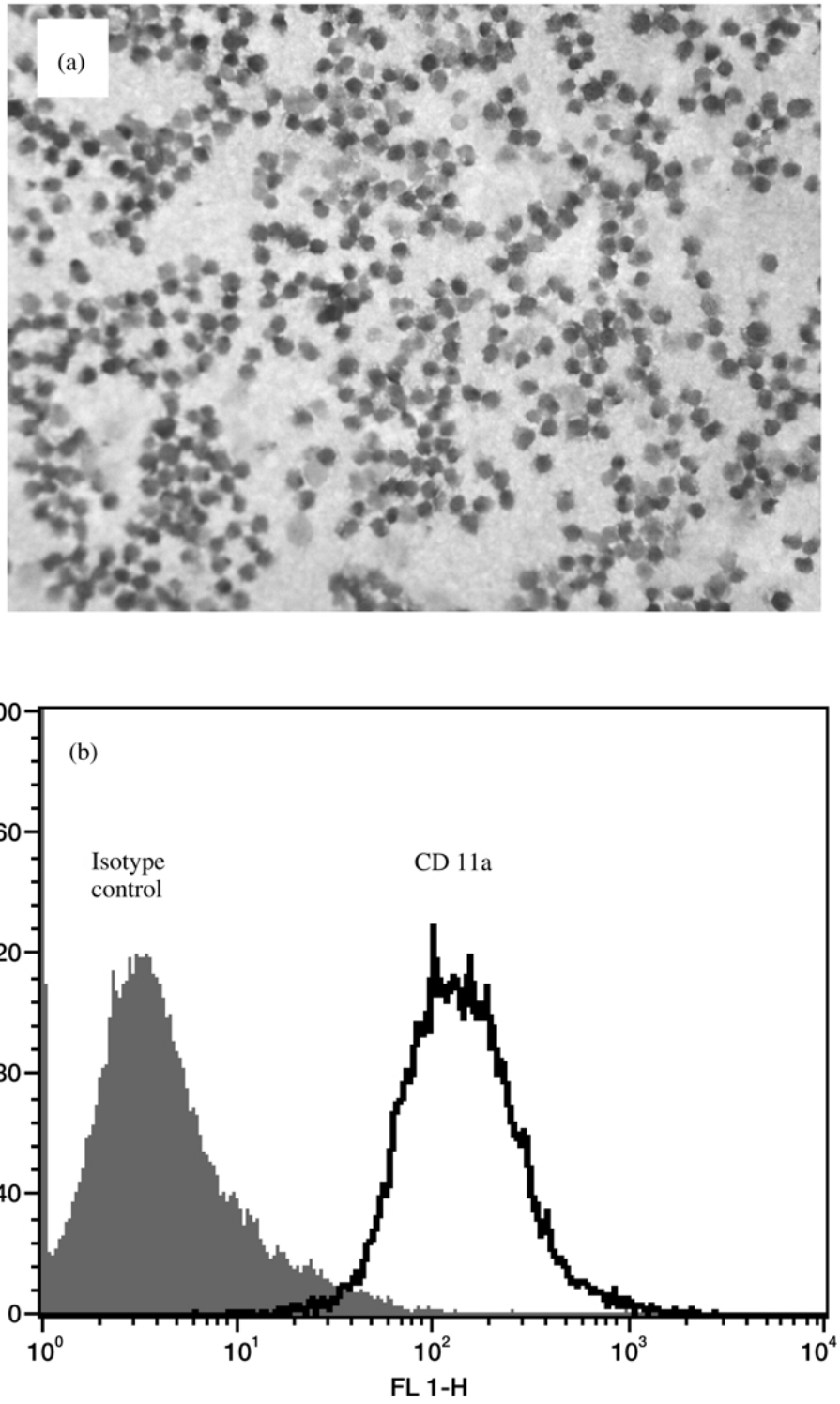

Figure 2 U937 were characterized by immunohistochemical staining for CD 11a (a) and FACS analysis showed nearly 100% express CD11a (b). Solid curve represents the negative control and open curve represents CD11a positive staining. Data from one of five similar experiments is presented.

different surfaces including untreated PET and PTFE. U937 cells normally grow in suspension and they did not spontaneously adhere on treated or untreated surfaces, but when HUVECs were able to grow on these materials, they express adhesion molecules which caused the adhesion of U937 cells. HUVECs did not grow on untreated PET and PTFE and so there was no adhesion of U937 (result not shown). The results illustrated in Fig. 3 show a typical profile of FACS analysis for U937 cells adhesion to different surfaces. The peaks on the right hand side represent U937 cell numbers and are expressed as a percentage of total cell numbers for CD11a: control 23.69%, TNF-α 72.77%, treated PET 33.78% and treated PTFE 26.40%. Although treated PET and PTFE had shown a slight increase in U937 cell numbers, TNF-α caused maximal U937 cell adhesion. The data analysis from 4–5 experiments was presented in Fig. 4. U937 cell adhesion and HUVEC proliferation were presented individually in Figs 4a and b. On day one, 8524 U937cells/cm^2 were adhered to the control and 58114 U937cells/cm^2 on TNF-α stimulated HUVECs which gives a 5.5-fold increase (Fig. 4a) which was a statistically significant difference. U937 adhesion to plasma-treated PET and PTFE was slightly higher compared to the control (increase 1.12 and 0.65 fold), but not a statistically significant difference. Day 7

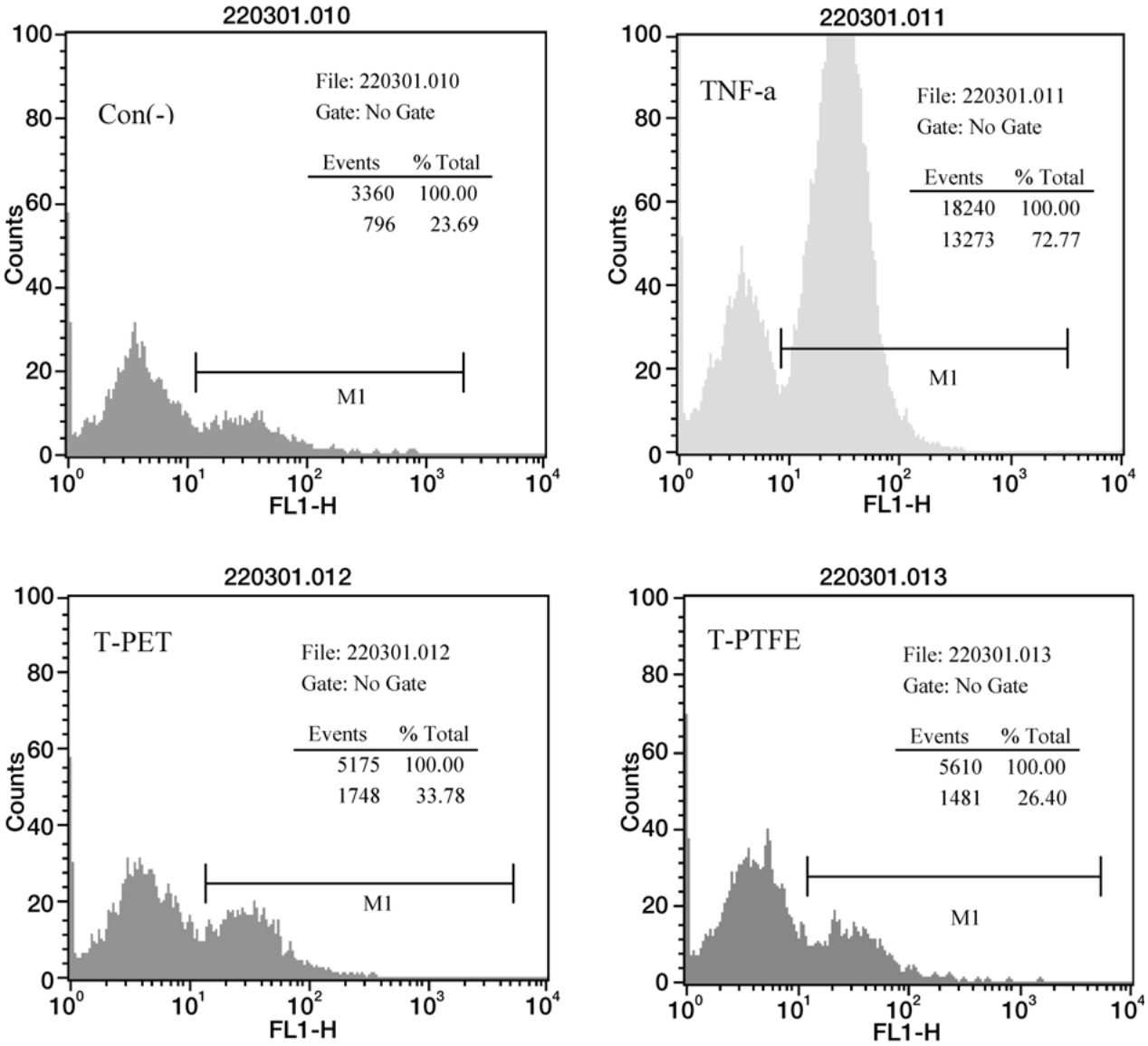

Figure 3 A typical profile FACS analysis of U937 adhesion on different surfaces for 1 day. Cells were monodispersed by trypsin-EDTA and incubated with monoclonal antibodies as described in Section 2.4. The peaks on the right hand side represented U937 cell numbers and are expressed as a percentage of total cell numbers for CD 11a. (a: CON (−): negative control, b: TNF-α: positive control, c: T-PET: treated PET, and d: T-PTFE: treated PTFE). Figures from one of five similar experiments is presented.

showed very similar results and patterns on plasma-treated PET (T-PET) and PTFE (T-PTFE). U937 cell adhesion was slightly increased compared to the negative control (0.86 and 0.56-fold increase Fig. 4a). Maximal adhesion of monocytes to HUVEC was observed on TNF-α stimulated endothelium (1.22-fold increase). The differences were not statistically significant on T-PET and T-PTFE, while TNF-α induced a significant increase in U937 cell adhesion in contrast to the negative control which was statistically significant among groups ($p < 0.05$). Interestingly, on both day 1 and day 7, U937 cell number adhesion to T-PET was slightly increased in contrast to T-PTFE, but not statistically significant. Two patterns of distribution of monocyte adhesion on the materials were viewed. On T-PET the cells were positioned in clusters on cells and on T-PTFE the cells were randomly and evenly distributed.

3.3. HUVEC adhesion and proliferation

The effects of plasma treatment of PET and PTFE on HUVEC adhesion and proliferation were analyzed by FACS. On day 1 (Fig. 4b), there was a 0.25-fold increase in cell numbers adhered on ammonia plasma-treated PET compared to the control, and a 0.5-fold increase for treated PTFE. There were no statistical differences between the control and T-PET, T-PTFE, but significant differences can be seen between TNF-α stimulated cells compared to the others and gave a 1.16-fold increase of cell number compared with control. On day 7, the cells did proliferate significantly on all surfaces in contrast to the 1-day time point. The positive control TNF-α showed significant maximal increases: 136%, in comparison to the negative control. The growth of HUVECs on T-PET and T-PTFE was also enhanced to 71% and 81% in contrast to the control and was a statistically significant difference.

4. Discussion

The complexity of the host implant interface involves ECs but also host growth factors, cytokines and circulating cells which are able to react with ECs to generate an inflammatory response. This phenomenon is

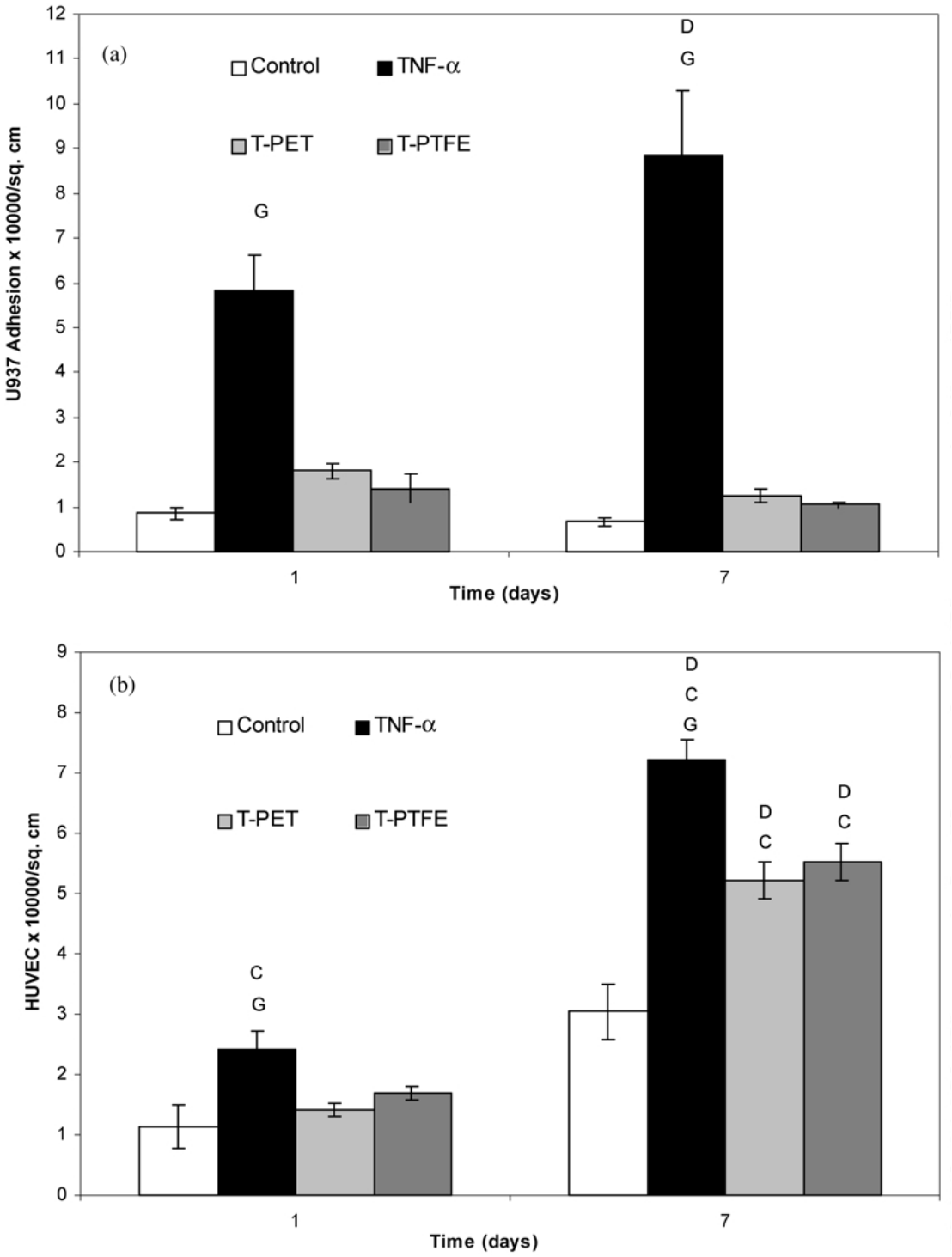

Figure 4 Adherent U937 cells (a) and HUVECs (b) per a square centimeter on surfaces for 1 day and 7 days were analysed by FACS. The details were described in Materials and Methods. The letters over the bar represent statically significant differences, G between groups, D between day 1 and day 7, and C difference to control. Data presented as the mean ± SD of four to five different experiments with three samples per experiments.

directly linked to a marked leukocyte recruitment which depends upon adhesive molecule expression by the endothelium. All these inter-dependent mechanisms could be modulated by the environmental conditions created by ECs. Indeed, the physical/chemical characteristics of biomaterials created by contact with the host cells can produce a spectrum of reactions among which is the expression of adhesion molecules. To predict such a phenomenon, *in vitro* studies of endothelial cell behavior in contact with biomaterials were reported as far as vascular graft endothelialization to improve patency is concerned [5, 6, 12, 13]. Most investigations focus on the studies of cell attachment after modification of surfaces [14, 15]. We have recently investigated expression of adhesion molecules on EC growth on plasma modification PET and PTFE [7]. Previous findings indicated that plasma-treated surfaces did not stimulate ECs compared to the controls shown by the fact that there was no significant increase in expression on ECs of ICAM-1, VCAM-1, E-selectin and P-selectin on treated surfaces; but a greater difference was seen between TNF-α stimulated cells and the control. This result was further confirmed by monocyte adhesion to the surfaces in this study. These results demonstrate that the expression of

pro-inflammatory adhesion molecules should come from ECs in contact with material surfaces, as this would result in the recruitment of inflammatory cells, such as granulocytes and monocytes which could damage the endothelial monolayer by the subsequent release of cytokines and oxygen radicals [16].

Previous papers [7, 15] have shown that the adhesion of human endothelial cells on PET and PTFE polymers is always low or even impossible, when their surfaces have a hydrophobic nature. This inability to support cell growth enhances the possibilities to observe the changes of cell growth induced by surface modification [7, 17]. The results from the HUVEC adhesion and proliferation demonstrated that ammonia plasma treatment could be used to modify PET and PTFE surfaces to support HUVEC growth. Surface treatment resulted in a significant increase in cell adhesion to treated PET and treated PTFE respectively compared to the control.

5. Conclusion

Gas plasma treatment of PET and PTFE produced a material modification which provided good support for EC adhesion and proliferation with a slight increase in monocyte adhesion compared to the control. Plasma treated PTFE has been demonstrated to be a better surface for HUVECs growth and less adhesive for monocytes as compared with treated PET. The monocyte adhesion to endothelial cells on surfaces can be used as a tool for the evaluation of material surface modification and further to the study of the mechanisms of cell to cell interactions in response to surfaces.

Acknowledgments

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