Interleukin-6 expression by cultured human endothelial cells in contact with carbon coated polyethylene terephthalate

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In order to evaluate if carbon coated polyethylene terephthalate (C-PET) could favor inflammatory reactions, the expression of interleukin-6 (IL-6) by cultured human umbilical vein endothelial cells was tested *in vitro*. The cultures were put in contact with C-PET for 1, 24, 48 and 72 h. The same cells cultured on tissue culture-treated polystyrene without biomaterials were tested as the negative control; the same cells incubated with LPS were the positive control. The level of IL-6 in the conditioned medium was tested by enzyme immunoassay; the mRNA expression was evaluated by RT-PCR with specific primers. The cultures incubated with C-PET produced non significantly different amount of IL-6 compared to the negative control and did not induce the expression of IL-6 specific mRNA. LPS induced a significantly higher release of IL-6 in the medium and the expression of mRNA after 24, 48 and 72 h. We conclude that C-PET does not stimulate the synthesis of IL-6 and therefore does not favor inflammatory reaction through the release of this cytokine.

Introduction

Different cell types, such as polymorphonuclear cells, monocytes, macrophages, lymphocytes, fibroblasts, platelets and endothelial cells, are involved in the response to the foreign materials implanted into the human body. These cells release chemical mediators, such as IL-6, that induce a flogistic reaction.

IL-6 stimulates mesenchimal cell recruitment and proliferation, controls inflammation, interferes in immune reactions, induces bone resorption [1]. It is released by many cell types, such as lymphocytes, macrophages, bone marrow stromal cells, fibroblasts and endothelial cells. IL-6 production by endothelial cells is stimulated by LPS, interleukin-1, Tumor Necrosis Factor, histamine [2], endothelin [3], activated protein C [4] and thrombin [5]. Also some artificial materials could induce IL-6 release. In previous experiments, an increased production, even if unsignificant, by endothelial cells in contact with uncoated polyethylene terephthalate was demonstrated [6].

The requirements of vascular grafts for a good incorporation in the tissues are blood compatibility and the ability to favor the growth of endothelial cells. In order to improve blood compatibility, the material surface is coated with substances inert to platelets and coagulation factors, such as carbon. Carbon coating is obtained by a physical vapor deposition technique at low temperature (40–200 °C), during which groups of carbon atoms are transferred from a pyrolytic carbon target to texturized polyester fibers. The deposition technique at low temperature permits the carbon coating of polyester fibers, without altering physical and handling characteristics. The carbon film is about 0.5 μm thick and presents structural properties similar to those of pyrolytic carbon. It has tetrahedral and graphitic bondings, as in the turbostratic structure typical of pyrolytic carbon [7], and has a large tendency to adsorb proteins irreversibly, in spite of its chemical inertness [8]. The film of adsorbed proteins, which do not interact with platelets nor partecipate in blood coagulation, favor blood compatibility [9, 10, 11].

In order to give impermeability to the graft and avoid preclotting, carbon coated polyethylene terephthalate is impregnated with collagen from bovin tendon. The collagen form a thin and continuous superficial film on the graft.

The aim of this research is to evaluate if carbon coated polyethylene terephthalate impregnated with collagen induce the expression of IL-6 from endothelial cells.

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Materials and methods

Materials

Polyethylene terephthalate texturized as double velour, impregnated with collagen and coated with carbon (Carbograft) (C-PET) on both internal and external face, was tested (Sorin Biomedica, Saluggia, Italy). About 100% of the PET surface was coated by carbon. The material was sterilized with ethylene oxide and stored at room temperature.

Medium and buffer

The culture medium consisted of complete medium, an equal mixture of Medium RPMI 1640 (Imperial) and Medium 199 (Gibco), supplemented with 25 mM Hepes buffer (Imperial), 2 mM L-glutamine (Biological Industries), 100 UI/ml Penicillin (Pool Industries), 100 μg/ml streptomycin (Pool Industries) and 20% Fetal Calf Serum (FCS) (Boehringer Mannheim) [12]. The same batch of FCS was used for the primary cultures and for the experiments.

The buffer used to wash endothelial cells consisted of 0.14 M NaCl (Carlo Erba), 0.004 M KCl (Riedel-de-Haen), 0.001 M glucose (Carlo Erba), 0.001 M K monobasic phosphate (Carlo Erba), 0.01 M Na bibasic phosphate (Carlo Erba) [13]. It was sterilized by filtration.

Endothelial cell cultures

The experiments were performed on human endothelial cells (HUVEC), isolated from the umbilical vein with enzymatic treatment with collagenase according to the method of Jaffe *et al*. [13]. The cultures were incubated at 37 °C in 5% CO_2 and 95% air.

Contact between endothelial cells and material

Nine experiments were performed using cultures from different isolates. For each experiment endothelial cells from a single umbilical vein were used.

The cells, at the 2nd passage, were detached with a solution of 0.05% trypsin–0.02% EDTA (Sebam). The proteolytic action of the enzyme was blocked with the addition of medium (v/v) supplemented with 20% FCS, then the suspension was centrifuged and the pellet was resuspended in fresh complete medium.

The cell suspension was seeded on 1.5 cm diameter tissue culture-treated polystyrene wells at the concentration of $1.0–1.5 \times 10^5$/well. From each culture 12 wells were prepared. The cells were incubated at 37 °C in 5% CO_2 and 95% air.

After 24 h, the medium was thrown. In 4 wells, named as negative control, 1.5 ml of complete medium were added. In 4 wells, named as LPS, 1.5 ml of complete medium with endotoxin (LPS) at the final concentration of 10 μg/ml (from *E. coli* 055:B5, Sigma) were added. In 4 wells, named as C-PET, 2.5 cm^2 pieces of C-PET and 1.5 ml of complete medium were added.

The cultures were incubated at 37 °C, in 5% CO_2 and 95% air. A series (respectively 1 negative control, 1 culture with C-PET and 1 culture with LPS) was incubated for 1 h, a series for 24 h, a series for 48 h and a series for 72 h.

After incubation, the conditioned media were collected, centrifuged at 2500 r.p.m. for 10 min and stored at −70 °C until IL-6 assay. RNA was isolated and reverse transcripted into cDNA, which was amplified with specific primers for IL-6.

Interleukin-6 assay

IL-6 was determined on the conditioned culture medium by enzyme immunoassay using a commercial kit (Cytoscreen-Human IL-6, BioSource International, Inc., Camarillo, California, U.S.A.). The minimum detectable dose was 2.0 pg/ml. Standards were used at the following concentrations of IL-6: 500, 250, 125, 62.5, 31.2, 15.6, 0 pg/ml. The standard curve was linear till 500 pg/ml. The assay was performed according to the indications of the producer, on a single analytical run. The wells were pre-coated with an antibody specific for human IL-6. The addition of standards and unknown samples was immediately followed by the addition of a biotinylated second antibody. The IL-6 of the sample or standard bound simultaneously to the immobilized antibody on one site and to the solution phase biotinylated antibody on the second site. The reaction was demonstrated by the addition of streptavidin-peroxidase and of the substrate solution. The optical density was read at 450 nm (Spectra III microplate spectrophotometer, SLT Labinstruments, Austria). The optical density of the ''blank'' was subtracted from standards and samples, then the calibration curve was calculated, according to the function of linear regression (software Soft 2000 version 5.x, connected to the spectrophotometer).

RNA isolation

Total cellular RNA was isolated from endothelial cell cultures using the RNeasy Mini Kit (Quiagen GmbH, Hilden, Germany). This method combined the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. The cells were lysed directly in the culture wells and homogenized in the presence of highly denaturing guanidinium isothiocyanate-containing buffer, to which 2β-mercaptoethanol was previously added in the proportion 1 part of 2β-mercaptoethanol and 100 parts of buffer. 70% ethanol was added to provide binding conditions, then the samples were applied to a spin column with a silica-gel-based membrane, which adsorbed RNA. Contaminants were removed with three wash spins with buffer, then RNA was eluted in diethylpyrocarbonate (DEPC) treated water.

Reverse transcription reaction

Immediately after isolation total RNA was reverse transcripted into cDNA with Molony murine leukaemia virus (MMLV) reverse transcriptase (Advantage RT-for-PCR Kit, Clontech Laboratories Inc., Palo Alto, CA, U.S.A). The entire population of mRNA molecules was converted into cDNA by priming with an oligo $(dT)_{18}$ primer. cDNA was stored at −70 °C until amplification.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in a total volume of 50 µl containing 0.4 µM of each primer (Amplimer Set, Clontech Laboratories Inc.), 0.8 mM of dNTPs, 0.8 mM Tris-HCl (pH 7.5), 1.0 mM KCl, KlenTaq-1 DNA polymerase with TaqStart Antibody (1.1 µg/µl) (Advantage cDNA PCR Kit, Clontech Laboratories Inc.), 39 µl H2O and 1 µl of cDNA sample. The primers were the following: 5′ primer: 5′ ATGAACTCCTTCTCCACAAGCGC3′; 3′ primer: 5′ GAAGAGCCCTCAGGCTGGACTG3′.

In each PCR run a specific positive control template was added (Amplimer positive control, Clontech Inc.). Amplification conditions were 30 cycles of 94 °C for 25 s, 60 °C for 45 s and 72 °C for 2 min, and an additional extension cycle at 72 °C for 7 min (DNA Thermal Cycler GeneAmp 9600, Perkin Elmer-Cetus, Norwalk, CT).

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA levels were assayed on the negative samples as an internal control to verify the efficiency of cDNA synthesis.

Electrophoresis

10 µl of each sample of PCR reaction were tranferred into a tube containing 1 µl of 6X loading buffer (0.25% bromophenol blue and 40% (w/v) sucrose in water). The samples were analyzed, along with the positive controls, by electrophoresis on 2.0% agarose gel (Sigma) containing 0.5 µg/ml etidium bromide (Sigma). The positive control had 628 base pairs. The gels were examined by UV light and then photographed.

Statistics

The results of the immunoassay, expressed as pg/ml, were multiplied by the ml of the supernatant in each culture and then divided by the number of cells in the inoculum, in order to obtain the mean amount of IL-6 released by each cell (pg/cell).

The statistical evaluation was performed using the StatView 4.5 software for Macintosh (Abacus Concepts, Inc.). The results were expressed as arithmetic mean $\pm$ standard error. The effect of the material on IL-6 release was estimated by the Mann-Whitney U test. A p value < 0.05 was considered statistically significant.

Results

IL-6 concentration in the conditioned medium

IL-6 release was constantly low in the negative controls and in the cultures in contact with C-PET. Significant differences between the controls and the cultures in contact with C-PET were not demonstrable. After 1 h incubation with LPS, IL-6 release did not differ from the negative control, but after 24, 48 and 72 h was significantly higher (Table I).

mRNA specific for IL-6

Only a qualitative evaluation of mRNA was performed. PCR results demonstrated that endothelial cells expressed mRNAs encoded by the gene for IL-6 when stimulated. The amplified products were confirmed to be those of the gene transcript by the detection of a 628 bp band.

The cultures seldom expressed IL-6 specific mRNA after 1 h. In negative controls and in the cultures in contact with C-PET, IL-6 specific mRNA was not demonstrable after 24, 48 and 72 h. Most cultures incubated with LPS expressed mRNA after 24, 48 and 72 h (Fig. 1a and 1b).

Discussion and conclusion

Cytokine assay is important for the evaluation of the effects of medical devices on the immune system, as suggested by the standard ISO 10993, Biological evaluation of medical devices–Part 4: Selection of tests for interactions with blood [14]. Our previous experience demonstrated that chromium released from orthopedic implants increased slighty the IL-6 production in mononuclear cells of healthy donors [15], while a significant increase was observed in mononuclear cells of patients with Cr-Co prosthesis [16]. Moreover in patients with aseptic loosening of hip prosthesis the serum levels of IL-6 were higher than in healthy donors [17].

The chemical composition of the device influences the IL-6 production. Increased levels were demonstrated in cultures of monocytes/macrophages and WBC incubated with Dacron [18], while ePTFE induced a significantly lower release [19].

We evaluated if carbon coating could induce IL-6 production by endothelial cells. The expression of

TABLE I Arithmetic mean, standard error and median (brackets) of IL-6 in the conditioned medium after contact with C-PET

	Negative control (pg/cell) $\times 10^{-3}$	LPS (pg/cell) $\times 10^{-3}$	C-PET (pg/cell) $\times 10^{-3}$
1 h incubation	0.038 $\pm$ 0.022 (0.002)	0.024 $\pm$ 0.014 (0.002)	0.048 $\pm$ 0.029 (0.002)
24 h incubation	0.051 $\pm$ 0.035 (0.024)	3.120 $\pm$ 1.188 * (5.0)	0.042 $\pm$ 0.025 (0.002)
48 h incubation	0.072 $\pm$ 0.040 (0.072)	3.871 $\pm$ 1.187 * (5.0)	0.073 $\pm$ 0.041 (0.020)
72 h incubation	0.117 $\pm$ 0.011 (0.118)	3.909 $\pm$ 1.149 * (5.0)	0.091 $\pm$ 0.024 (0.104)

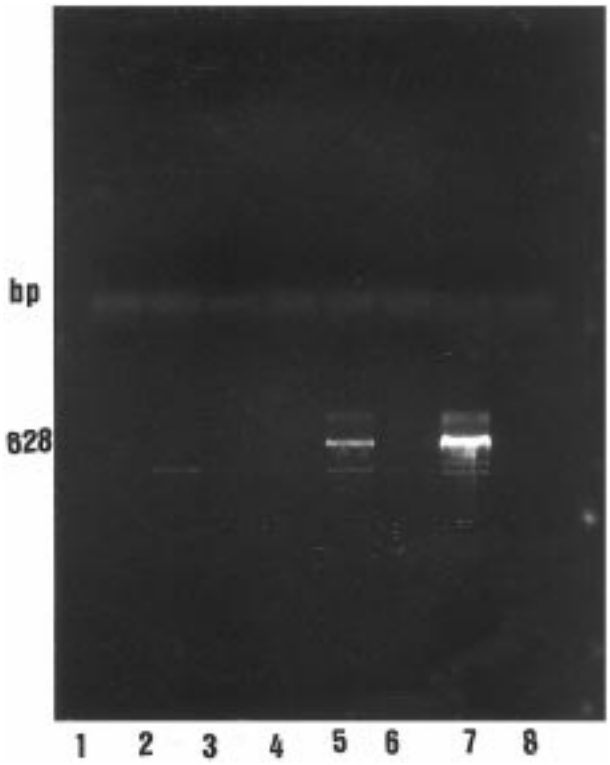

Figure 1(a) Expression of mRNA specific for IL-6 after 1 and 24 h incubation. Lane 1: negative control after 1 h; lane 2: culture incubated with LPS for 1 h; lane 3: culture in contact with C-PET for 1 h; lane 4: negative control after 24 h; lane 5: culture incubated with LPS for 24 h; lane 6: culture in contact with C-PET for 24 h; lane 7: positive control template of PCR (628 bp); lane 8: negative control of PCR (distilled H_2O).

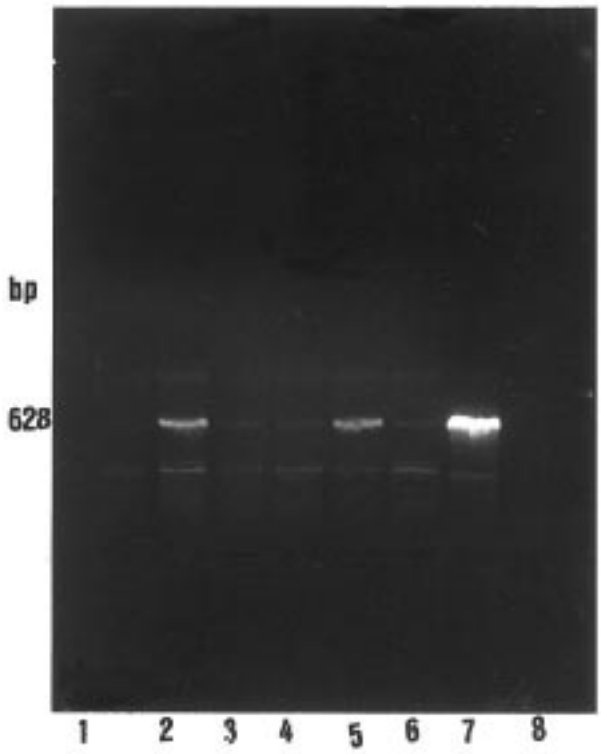

Figure 1(b) Expression of mRNA specific for IL-6 after 48 and 72 h incubation. Lane 1: negative control after 48 h; lane 2: culture incubated with LPS for 48 h; lane 3: culture in contact with C-PET for 48 h; lane 4: negative control after 72 h; lane 5: culture incubated with LPS for 72 h; lane 6: culture in contact with C-PET for 72 h; lane 7: positive control template of PCR (628 bp); lane 8: negative control of PCR (distilled H_2O).

specific mRNA was compared to the IL-6 levels in the conditioned medium.

Endothelial cells were tested because they are involved in the tissue response to artificial implants. In vascular grafts endothelium plays an important role for the incorporation in the human body. Surface treatments are performed on materials in order to improve compatibility, such as the coating with isotropic carbon. By LDH assay in the conditioned medium, it was demonstrated that carbon is less harmful to endothelial cells than uncoated Dacron and Teflon [20].

Our results showed a large variability in the release of IL-6, presumably linked to the fact that for the experiments we did not use established lines but different primary cultures. The mRNA expression after contact with C-PET was compared both with the same cultures in contact with tissue culture-treated polystyrene only [i.e. negative control] and with the same cultures stimulated with LPS.

Some negative control cultures expressed IL-6 specific mRNA after 1 h, but the protein level in the conditioned medium was low. This early expression was transient and probably caused by the handling of the culture. After 24, 48 and 72 h negative controls did not express mRNA and IL-6 concentration in the medium was low and close to the detection limit of the method.

The expression of mRNA after incubation with LPS is early after 1 h, and persists for the other incubation times, causing IL-6 levels in the conditioned medium higher than the negative controls. The expression of IL-6 specific mRNA and the higher levels of the protein in the cultures incubated with LPS are according to other authors [21, 22].

The contact with C-PET modifies neither mRNA expression nor IL-6 release compared to the negative control. This behavior could be a positive event because does not favor inflammatory reaction near the graft. The low IL-6 release could explain the scarce inflammatory reaction demonstrated in rats implanted with carbon coated vascular implants [23].

The absence of a significant IL-6 increase after contact with C-PET could avoid some side effects of uncoated PET. In fact uncoated PET induces thrombosis as well as an inflammatory response demonstrated by the adhesion of neutrophils [24] and the release of IL-6 and TNF [18]. In order to reduce thrombosis and inflammation, the surface of PET is variously modified. The treatment with a fluoropolymer inhibits inflammation and thrombus generation [25]. By our results, also the coating with pyrolytic carbon could inhibit inflammation, even if the study of other cells and chemical mediators is necessary. Pyrolytic carbon could reduce also the immune and proliferative reactions that IL-6 determines.

In conclusion, the obtained results suggest that pyrolytic carbon coating does not increase IL-6 expression and therefore does not favor inflammation through the release of this cytokine.

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