

Modulatory role of IL10 in endothelial cell damage and platelet adhesion

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Summary. This study explores the possibility of a regulatory role for cytokine IL-10 in platelet aggregation as an active vascular repair mechanism. Endothelial cells from human umbilical cord vein were cultured in the presence of different IL-10 concentrations (0-100 ng/ml). Platelet-rich plasma was then added to these cultures and allowed to act for 30 minutes. To rule out blood plasma involvement, washed platelets were also incubated with IL-10 (0-100 ng/ml). Changes in endothelial cell morphology were observed depending on the IL-10 concentration used; apoptotic cells appearing at the highest IL-10 concentration. Greatest platelet adhesion was noted at the highest IL-10 concentration. It was concluded that, in this *in vitro* model, low IL-10 levels do not affect cell viability or the pattern of platelet adhesion, but at high doses, this cytokine induces cell death and enhances platelet deposition.

Keywords: Apoptosis, Endothelial cells, IL-10, Platelets

Introduction

Neovascularisation or the growth of new vessels is frequently associated with diseases such as atheromatous plaques, tumours or proliferative diabetic retinopathy. In all these settings, the common factor is an anomalous proliferation of blood vessels, whose function is to supply the surrounding tissue. The vascular endothelium secretes large amounts of mediators that exert their action via several signalling pathways involved in regulating both vessel patency and blood clotting. Alterations in vascular endothelial function are induced by interactions among cells, cell and humoral factors and matrix components (Cotran and Mayadas-Norton, 1988; Simionescu and Simionescu, 1998). These signalling

molecules are starting to generate interest as prognostic factors for severe cardiovascular events.

The vascular endothelium has anticoagulating properties that prevent the onset and propagation of coagulation processes (Broze, 1992; Bourin and Lindahl, 1993; Esmon and Fukudome, 1995). On occasion, however, vascular injury sets off an opposite procoagulation, or inflammatory, response (Gimbrone et al., 1990; Marcus and Safier, 1993) and/or an immune reaction in which the endothelial cell receives a series of signals leading it to modify its response (Cavaillon, 1996; Buján et al., 1999; Parent and Eichacker, 1999).

Many of these modifying agents or conditions have been identified and include proinflammatory cytokines (LPS, IL-1, TNF- α), atherogenic substances (lipoprotein A and homocysteine), hypoxia, hyperthermia, viral infections and activated leukocytes or their products. These factors are able to alter the anticoagulating properties of endothelial cells and render them apoptotic or proadhesive (Pearson, 1993; Bombeli et al., 1999), thus contributing to thrombotic processes (Bombeli et al., 1997).

In a transient, limited way, cytokines are secreted during the immune response and exert their effects by binding to specific, high affinity receptors on the cell membrane of target cells. IL-10 is a pleiotropic cytokine involved in regulating lymphocyte and myeloid cell function. It is now known that this cytokine is produced by IL-4 and IFN γ -producing T-lymphocytes as well as by non-T cells (Assenmacher et al., 1994), and can exert immunostimulatory/suppressant effects on white blood cells, yet its effects on the endothelium remain unclear.

In the past few years, several pathologies associated with altered regulation of endothelial cell function have been described. Endothelial cells are the targets of activated inflammatory cells and are typically involved in the acute inflammatory processes that occur when damage is incurred by the vascular endothelium (Pober and Cotran, 1990; Davies and Hagen, 1994; Okamoto et al., 1996).

Given the close relationship between the endothelial cell and cytokines, this study was designed to evaluate

the direct effects of IL-10 on cultured endothelial cells, and to assess whether this cytokine is capable of modifying endothelial behaviour in situations of cell activation.

Materials and methods

Endothelial cell culture

Human EC were obtained from umbilical vein and cultured according to the methods described by Jaffe (1973) with some modifications (Buján et al., 1999). The vein was flushed several times with MEM under sterile conditions and "filled" with a 0.1% solution of type I collagenase (Worthington) in MEM (15 min at 37°C). Cells were cultured in medium 199 (Gibco BRL) supplemented with 20% bovine foetal serum (Gibco BRL), antibiotics (100,000 IU/ml penicillin/10,000 µg/ml streptomycin) (Gibco BRL), 10 mM HEPES (Gibco BRL), 2 mM L-glutamine (Gibco BRL), endothelial cell growth factor (20 µg/ml) (ECGF) (Sigma), and sodium heparin (90 µg/ml) (Roche) in a humidified 5% CO₂ atmosphere at 37°C. The endothelial cells were then seeded (5x10⁴ cells/cover slip) onto sterile glass coverslips (12 mm diameter) inside a multiwell plate with 1 ml of culture medium and grown for 72 h to confluence. At this stage, the EC were stimulated for 12, 24, 48 or 72 h by directly adding IL-10 (1-100 ng/ml) (a gift from Schering-Plough Research Institute, Kenilworth, New Jersey) to the culture medium. Untreated endothelial cells in culture served as the control group.

Platelet-rich plasma (PRP)

Blood was collected from volunteers into tubes containing 3.8% sodium citrate solution at a proportion of 1:10, and centrifuged in conical tubes at 200g for 10 min. The platelet-rich plasma (PRP) was then collected and the number of platelets counted in an automatic haemocytometer (Haematology Analyser System 9000; SERONO DIAGNOSTICS, Spain).

After culture with the different IL-10 concentrations, the EC were incubated with 100 µl of PRP (5x10⁴) for a further 30 min. Following incubation, the supernatant was used to estimate the number of non-adhered platelets.

To establish whether the IL-10 effects on platelet adhesion could be ascribed to the cytokine itself or to changes produced in the endothelial cell after stimulating with the cytokine, in a second set of experiments, platelets were preincubated with the cytokine alone before adding to EC cultures.

In situ DNA fragmentation

The *in vitro* identification of apoptotic cells was performed by modification of the TUNEL method (Negoescu et al., 1996). This method is based on the *in situ*

detection of fragmented nucleosomal DNA characteristic of apoptosis, by the specific binding of deoxynucleotides to the exposed 3'-OH-ends of the fragmented chromatin. Samples were subjected to microwave irradiation (SANYO EM-704T) for 5 min (350W) in 0.01M citrate buffer (pH 6). Fragmented DNA was then determined using a commercial kit (Calbiochem, CN Biosciences Inc., USA).

Scanning electron microscopy (SEM)

Cultures were fixed in 3% glutaraldehyde (2 h at 4°C) and then placed in Millonig buffer (pH 7.3). Following their dehydration in a graduated acetone series, reaching critical point in a Polaron E-3000 with CO₂, they were metallised in palladium gold and examined under a Zeiss 950 DSM scanning electron microscope. Images were recorded using an ILDFORD FP4 instrument (ILDFORD Limited Mobberley Cheshire, UK).

Statistical analysis

Proliferation and death data were expressed as means ± standard deviation. The Mann-Whitney U-test was used to compare data corresponding to the different treatments. The level of significance was set at p < 0.05.

Results

Morphological effects of IL-10 on EC

When the EC were cultured in the presence of IL-10 concentrations in the range of 1-25 ng/ml, no morphological alterations were observed with respect to controls.

Endothelial cells grew to form a stable monolayer, typical of this cell type. Some cells were firmly attached to the substrate. These cells showed nuclei and nucleoli. Several intercytoplasmic bridges could be observed between neighbouring cells.

As the cytokine dose was increased to 50 ng/ml or 100 ng/ml, we were able to note changes in endothelial cell morphology and behaviour, most changes appearing after treatment with the highest IL-10 concentration. Alterations included a lower cell density and absence of a confluent monolayer, with cells showing signs of damage (Fig. 1). Cells adopted a rounded shape, became detached from the substrate and sometimes showed nuclear extrusion. It was also possible to find signs typical of different stages of apoptosis such as nuclei with fragmented, peripheral chromatin and bubbling of the cytoplasm.

Cell proliferation and death

Using an antibody against the cell proliferation factor (PCNA), we were able to observe a large number of cells with nuclear labelling in control cultures. The

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TUNEL method was then used to evaluate cell damage induced by the culture conditions, and a low proportion of damaged cells was confirmed.

In the presence of IL-10, changes in the pattern of endothelial cell proliferation and death were observed (Table 1).

After 24 h of culture, adding IL-10 (10 ng/ml) to the medium gave rise to a reduction in the proportion of cells in proliferative phase and an increase in the TUNEL positive cell fraction. This difference persisted at 72 h of culture.

Increasing the IL-10 concentration to 100 ng/ml affected both these variables. The proliferative population diminished from 12 h onwards, and became stable at levels below that shown by the control group at each of the follow-up times. In contrast, the proportion of damaged cells increased with time of exposure to the cytokine.

A dose-dependent induction of nuclear damage, assessed as apoptosis, could be observed in the EC. This induction became statistically significant at 24 h using the 100 ng/mL IL-10 concentration and, at 48 h of

incubation when concentrations of 10 ng/ml were applied.

Table 1.a. Proportion of proliferative cells (PCNA) per thousand. **b.** percentage TUNEL positive cells (* $p < 0.05$).

a	PCNA (%)			
	12 h	24 h	48 h	72 h
Control	112.87±6.21	114.10±5.38	69.69±3.25	66.32±4.53
IL-10 (10 ng/ml)	93.74±7.58	63.93±4.04*	57.41±5.98*	42.45±3.15*
IL-10 (100 ng/ml)	86.50±5.24*	73.20±6.36*	42.58±3.81*	38.58±4.77*

b	TUNEL (%)			
	12 h	24 h	48 h	72 h
Control	2.03±0.19	2.2 ± 0.21	3.28±0.35	7.63±0.28
IL-10 (10 ng/ml)	3.1±0.62	5.74±3.75*	9.79±2.54*	11.52±1.13*
IL-10 (100 ng/ml)	3.81±0.47*	10.31±3.21*	14.17±4.39*	17.08±4.42*

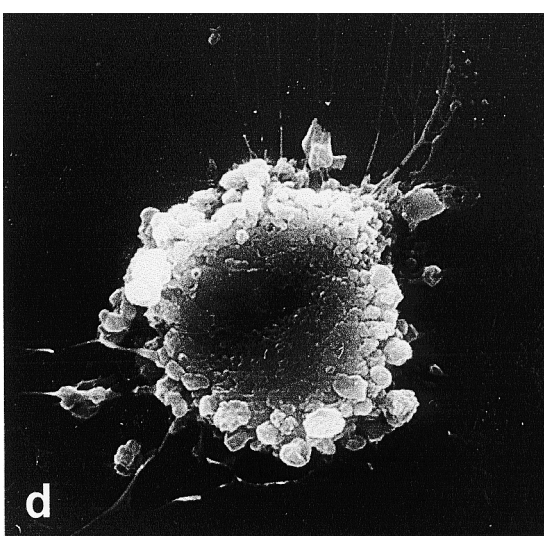
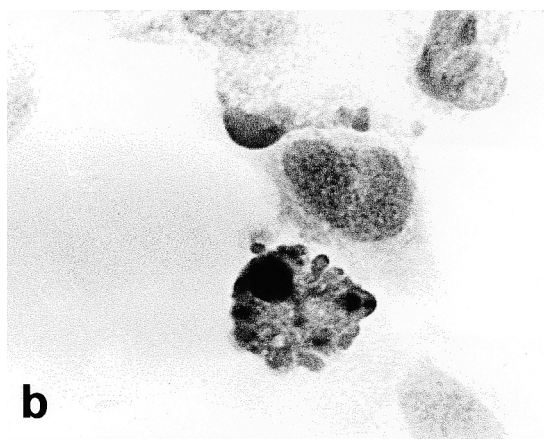
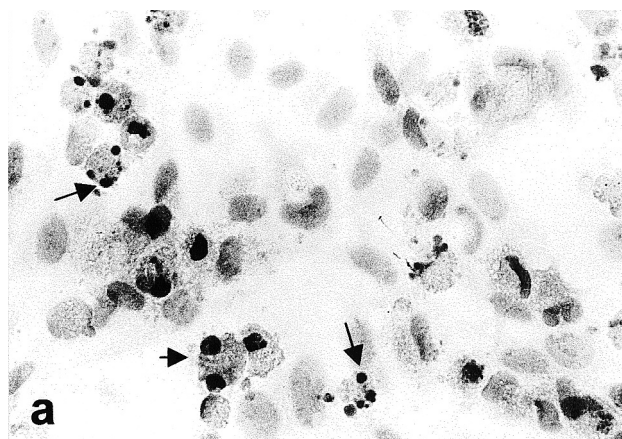


Fig. 1. EC incubated with IL-10 (100 ng/ml) for 24 h. **a.** Note the large number of apoptotic bodies (arrows). Light microscopy. x 400. **b.** Detailed view of the previous micrograph showing nuclear fragments typical of apoptosis. Light microscopy. x 630. **c.** Retracted EC. Scanning electron microscopy. x 2000. **d.** EC forming apoptotic bodies (bubbles) characteristic of cell death. Scanning electron microscopy. x 3,000

Reversibility of the IL-10 effect

These data suggest that IL-10 induces damage to the EC which is dependent on the time of incubation. To assess whether this effect was reversible, we incubated

Table 2. Assessing cell proliferation using the PCNA and TUNEL methods. Values correspond to 24 h of culture with the cytokine and a further 24 h in its absence (*p<0.05).

	PCNA(%)	TUNEL(%)
Control	69.69±3.25	3.28±0.35
IL-10 (10 ng/ml)	118.54±9.87*	6.4±1.05*
IL-10 (100 ng/ml)	114.26±10.53*	10.25±2.14*

EC for 24 h with two concentrations of IL-10 (10 and 100 ng/ml) and then removed the cytokine from the medium leaving the culture to incubate a further 24 h in M-199 medium alone.

Removing the cytokine from the medium induced the recovery of cell proliferation rates to values similar to controls while cell damage, measured as the apoptotic cell fraction, was not significantly different to that recorded after 24 h of culture (Table 2). These cell death rates were, nevertheless, lower than those observed after 48 or 72 h of continuous culture with IL-10.

Platelet adhesion

Endothelial cells cultured for 24 h in M-199 and

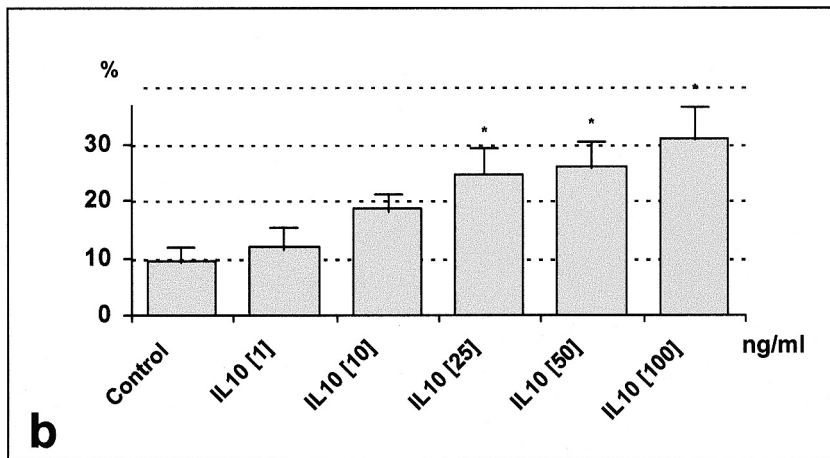
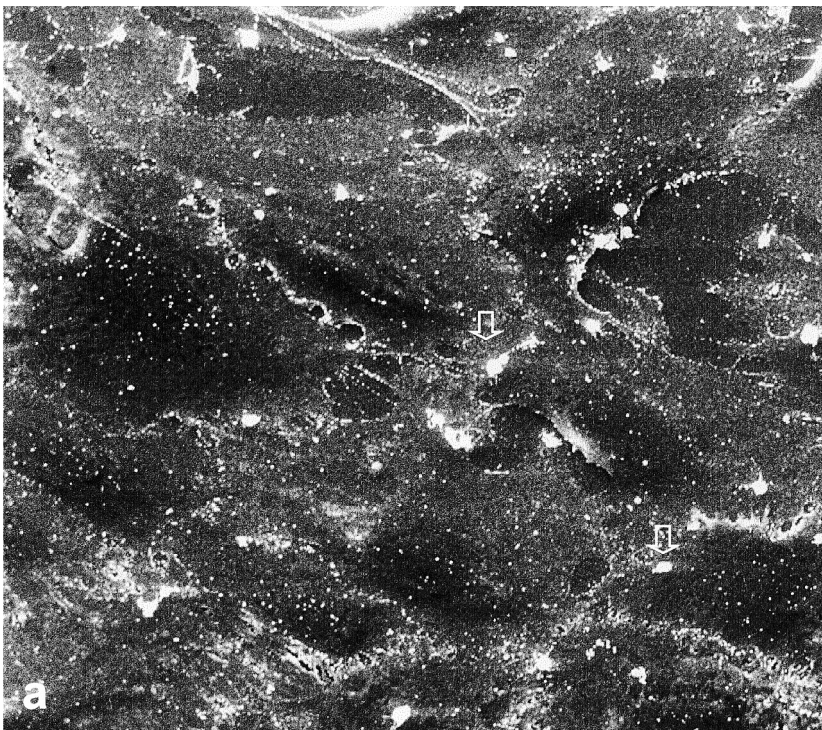


Fig. 2. a. Platelets adhering to the endothelial monolayer (arrows). Scanning electron microscopy x 1,000. b. The proportion of platelets adhering to the endothelial monolayer increases with the IL-10 concentration added to the culture medium (*p<0.05).

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placed in contact with washed platelets showed no procoagulation activity. Platelet deposits could be observed on the cells yet these did not become activated during the 30 min of exposure, confirming the good *in vitro* antiadhesive capacity of umbilical vein EC (Fig. 2a).

When platelet-rich plasma was added to EC previously incubated with different concentrations of IL-10 for 24 h, no significant differences were shown among treatment groups. However, if we consider the diminished viability attributable to the presence of the cytokine, an increase in platelet adhesion depending on the IL-10 concentration may be inferred (Fig. 2b).

The addition of increasing IL-10 levels leads to a selective adhesion pattern on the EC. An IL-10 concentration of 1 ng/ml did not modify endothelial adhesion capacity with respect to controls, yet at increased doses (10 and 25 ng/ml), this variable was affected. Platelet adhesion started with their positioning on the lateral edges of the cell (Fig. 3a) and along the cytoplasmic projections as a "string of beads" (Fig. 3b).

High cytokine (50 and 100 ng/ml) levels induced maximum platelet adhesion. The number of cells with their entire cytoplasm covered with platelets gradually increased (Fig. 3c), especially cells with a different morphology to control cells. These cells had

Table 3. Number of platelets adhering to the EC. Data expressed per thousand.

	18 h treatment	18 h treatment + 6 h M-199	24 h treatment	24 h treatment + 24 h M-199
M-199	78.4±8.39	75.4±4.19	90.3±8.4	75.4±4.19
IL-10 [10 ng/ml]	75.4±4.19	68.7±5.00	81.4±7.65	63.5±6.52
IL-10 [100 ng/ml]	60.6±8.40	69.5±4.20	72.4±8.39	63.5±6.52

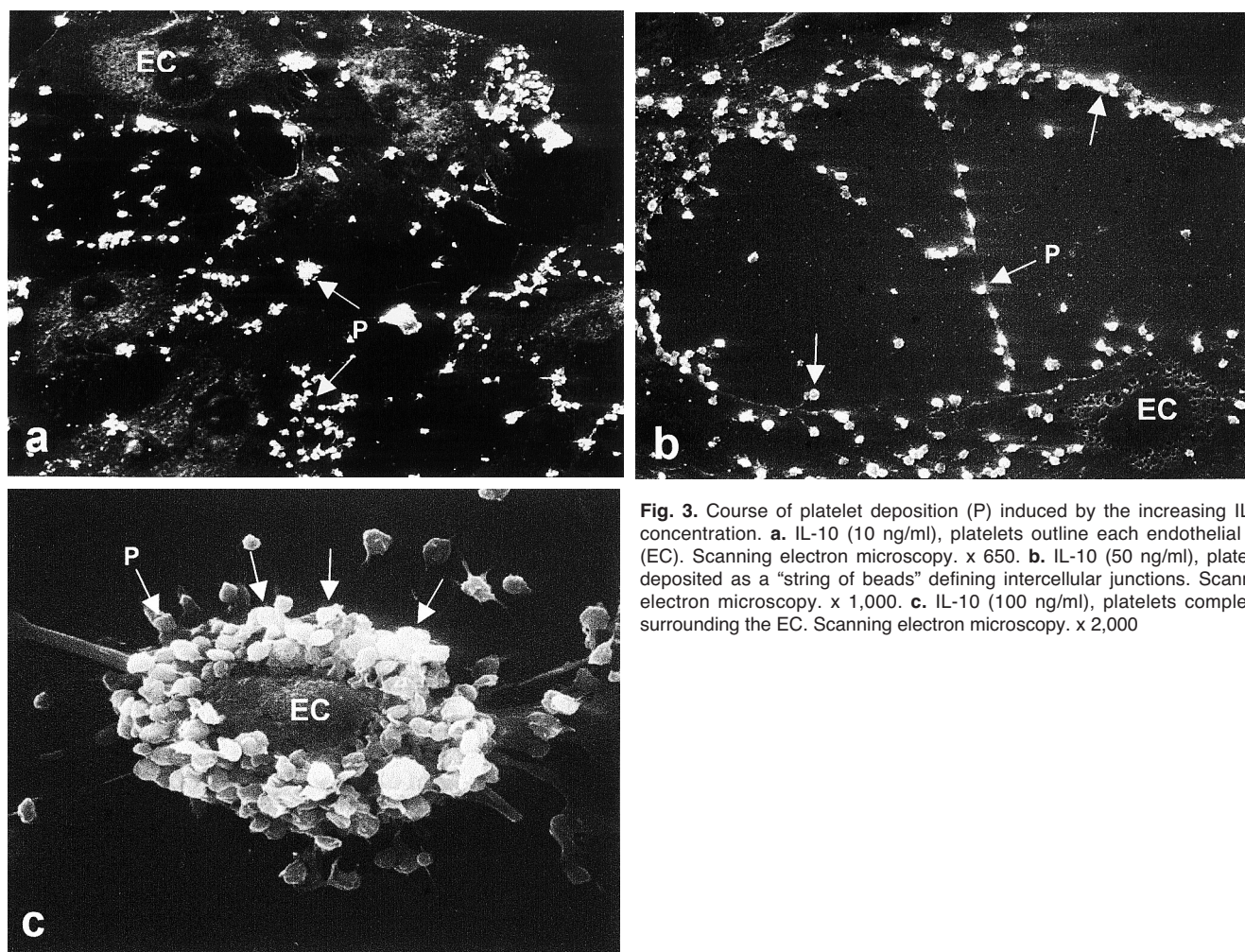


Fig. 3. Course of platelet deposition (P) induced by the increasing IL-10 concentration. **a.** IL-10 (10 ng/ml), platelets outline each endothelial cell (EC). Scanning electron microscopy. x 650. **b.** IL-10 (50 ng/ml), platelets deposited as a "string of beads" defining intercellular junctions. Scanning electron microscopy. x 1,000. **c.** IL-10 (100 ng/ml), platelets completely surrounding the EC. Scanning electron microscopy. x 2,000

morphological features characteristic of cell death; they became retracted and came away from the glass, and their cytoplasmic bubbles sometimes showed cells that expelled their nucleus.

To establish whether IL-10 acts on platelets or on the endothelium itself, we performed a series of experiments in which pre-washed platelets were incubated with different IL-10 concentrations (0, 10 and 100 ng/ml) and then added to the EC.

The results revealed that the pattern of platelet adhesion to the unstimulated EC was similar to that observed when unstimulated platelets were added to EC from the control group. The platelets maintained their rounded shape and barely showed the pseudopodic protrusions characteristic of their activated state.

Reversibility of the IL-10 effect

Since IL-10 was incapable of exerting any modulatory adhesion effect on the platelet itself yet could modulate the antiadhesive function of the vascular endothelium, we decided to explore the reversibility of the process. To this end, the EC cultures were stimulated with IL-10 over a sufficiently long period to induce a cell response. After 18 or 24 h, the cytokine was removed from the medium and incubation was continued for a further 6 or 24 h respectively. Table 3 shows the results obtained.

No significant differences in the number of platelets adhering to the cultured cells were observed among groups.

Discussion

Once the vascular endothelium has been damaged, its capacity for controlling the flow of cell constituents and solutes is severely compromised. In this setting, mechanisms that favour tissue repair are required, such as enhancing platelet adhesion to damaged cells, thus protecting the affected zone. If this were the case, EC cultures treated with cell damage-inducing cytokines *in vitro* should increase their capacity to adhere to platelets. It was recently demonstrated in *in vitro* (Bombeli et al., 1998) and *in vivo* (Li et al., 1996) studies, that under certain conditions, EC develop properties that make them adhesive towards platelets. Once activated, platelets rapidly adhere and become associated to form large aggregates that coat the endothelial surface. This platelet adhesion is mediated by adhesion molecules that implicate the GPIIb/IIIa protein (Bombeli et al., 1998) and several endothelial receptors. Moreover, the same situation in which the endothelium becomes proadhesive can occur when it is the endothelium that is activated, for example, by proinflammatory cytokines (Frenette et al., 1998) or if it becomes apoptotic (Bombeli et al., 1999). Thus, it is not only platelets that undergo modulation, the endothelium itself can trigger the adhesive response.

Interaction of IL-10 with the endothelium is scarce

and has been barely investigated. It appears to be a weak stimulus for the expression of chemokines and IL-6 in some mouse cell lines, and amplifies the actions of IL-1 and TNF- α on the EC (Sironi et al., 1993). IL-10 was first described as an immunomodulatory cytokine, its antiinflammatory capacity being among its most well known properties when acting on human umbilical vein EC in the presence of LPS (De Beaux et al., 1995). IL-10 inhibits the expression of adhesion and histocompatibility molecules (Vora et al., 1994; Chatelain et al., 1998). It also inhibits the production of IL-6 in macrophages, while it stimulates the synthesis of this cytokine in the EC (Sironi et al., 1994).

Today, the participation of IL-10 in proinflammatory phenomena *in vivo* is widely accepted. Experiments in mice transgenic for IL-10 have suggested that this cytokine can activate the vascular endothelium, provoking leukocyte adhesion and their exit from the blood vessel (Fiehn et al., 1997), these results overlapping the actions of cytokines such as IL-1 and TNF- α (Cavaillon, 1996). The latter stimulate proinflammatory activity on the endothelial cell surface, increasing the expression of adhesion molecules on white blood cells.

To evaluate the degree of toxicity induced by IL-10 in the present model, once stimulated, the EC were incubated for a further 24 h in the absence of this cytokine. The process was found to be reversible, indicated by the significant reduction in the rate of apoptosis as well as the late recovery of the proliferation rates of cultures previously stimulated with IL-10. Thus, we could argue that if IL-10 can induce morphological and functional changes characteristic of apoptosis, its protective effect against cytotoxic agents is difficult to understand, although a paradoxical effect in the absence of other more potent stimuli cannot be excluded.

There is, however, no reference in the literature to IL-10 modulation in the normal EC, in terms of effective dose or its effects on the cell's functional properties. Beneficial roles attributed to this cytokine during cell damage are, on the one hand, an ability to modulate the course of the cell cycle in the presence of other cytokines such as TGF- β (Heimark et al., 1986) and, on the other, an ability to modulate the expression of proinflammatory cytokines such as IL-1, IL-6, IL-8 and TNF- α (Poole et al., 1995). These well known IL-10 effects, need not translate to the EC, since most refer to its action on the immune cells themselves.

The findings described above, prompted our question of what might happen in pathological settings in which endothelial behaviour is altered (angiogenesis, neoplasia or vasculitis). If the state induced by cytokines were irreversible, we would be left with an altered endothelium without the capacity to respond to injury. Our demonstration of the ability of the EC to revert to the normal state once the cytokine has been removed from the medium sheds some light on the question posed.

There have also been descriptions of apoptosis in

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cells of the vascular endothelium mediated by LPS activation (Eissner et al., 1995) or in thrombotic processes highly related to platelet deposition (Dang et al., 1999). Our findings are consistent with reports by some authors (Dang et al., 1999) in that we noted abundant platelet deposition, especially in cells that were damaged or showed signs of nuclear fragmentation in the form of cell death or apoptosis. The IL-10 effect of inducing the programmed cell death of EC may also be related to the release of molecules such as antibodies into the extravascular space or to the transport, via the blood flow, of remnants of these damaged cells to a secondary lymph gland such as the spleen. It is at this site that antigens produced by the cell remnants will be used to develop a distant immune response mediated by antibodies. Though the physiological meaning of IL-10 inducing apoptosis is unknown, a possible interpretation is that it is capable of "unsticking" damaged cells from the vessel wall. These would be coated with platelets and subsequently withdrawn from the circulation in places, such as the spleen, where there are platelet receptors (Fujita et al., 1998; Chang et al., 1999). This could be a useful mechanism for removing immunostimulatory cells from the site of inflammation and transferring them to a place where they would be phagocytosed by "professional" antigen-presenting cells. Thus, apoptotic cell remains would serve as an antigenic stimulus to set off immune responses far from the site of inflammation.

In basal conditions and in the absence of IL-10, endothelial cells do not allow platelet adhesion, yet as the cytokine concentration increases, there seems to be a selective, orderly stimulus for platelet adhesion receptors. The concentration of this cytokine needed to activate EC is within the range 10 to 100 ng/ml; the incubation time needed for optimal cell stimulation appearing to be an important factor (Sironi et al., 1993). We noted positive correlation between IL-10 concentration and the somatic site of platelet deposition. This could be explained by activation or modification of platelet receptors such that platelet adhesion to the endothelial membrane can occur in a sequential manner. Our data indicate that at high doses, IL-10 could be an essential factor for the control of endothelial cell proliferation in several pathological processes. This cytokine might therefore be a potential therapeutic candidate for use in diseases involving endothelial proliferation (e.g., diabetic retinopathy) or in tumour processes, as an inhibitor of angiogenesis.

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