The Leukocyte-Endothelial Cell Interactions are Modulated by Extracellular Matrix Proteins

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Key Words

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Abstract

Background: Endothelium is supported, in normal conditions, by a basement membrane composed, among others, by collagen IV and laminin. Changes in the basement membrane composition could induce changes in endothelial cell modifying their interactions with leukocytes. **Methods and Results:** Isolated polymorphonuclear cells (PMN) and peripheral blood mononuclear cells (PBMC) were added to cultured human umbilical endothelial cells (HuVEC) previously seeded on collagen IV, collagen I or gelatin. Adhesion of leukocytes to HUVEC and specific cytotoxicity were analysed. PMN adhesion and cytotoxicity were lower whereas those from PBMC were higher when HuVEC were seeded on collagen I, as compared with cells seeded on collagen IV. To analyse the mechanisms involved in these

phenomena, P-selectin, ICAM-1, VCAM-1 and MCP-1 expression were evaluated in HuVEC seeded on the different ECM components. P-selectin and mRNA expression of VCAM-1 were lower in cells seeded on collagen I. By contrast, MCP-1 expression was higher in collagen I. Collagen I-dependent effects were partially prevented when collagen I was treated with pepsin. ILK activity was lower in cells seeded on collagen I, whereas ERK 1/2 activity was enhanced. ILK overexpression reduced ERK 1/2 phosphorylation and this could promote the reduction in P-selectin and the increase in MCP-1. **Conclusion:** Collagen I decreased ILK activity and this would induce an increase in ERK 1/2 activity in HuVEC. As a consequence, the P-selectin content is diminished and, by contrast, the MCP-1 content is increased. The final effect is a lower recruitment of PMN and a higher adhesion of PBMC.

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Introduction

Endothelial cells are normally in contact with the basement membrane, a specialized layer of extracellular matrix (ECM), which is composed predominantly by laminin, collagen type IV (COL IV), entactin and heparan sulphate proteoglycans [1]. The loss of integrity of the basement membrane is a common feature from some physiological and pathological conditions such as wound repair [2], angiogenesis [3] and the remodelling of vessel walls characteristic of atherosclerosis [4], diabetes [5] or hypertension [6]. This fact promotes endothelial cells come into contact with another ECM proteins such as collagen type I (COL I), an interstitial collagen which is not typically found in basement membrane.

Multiple studies have supported that the dynamic interaction between cells and ECM controls many processes, including proliferation, migration and survival [7-9]. It is well documented that ECM components can modulate the phenotype of cells that are in contact with them across the interaction with specific cellular membrane receptors, named integrins [10]. Specifically, when are in contact with COL I, endothelial cells come into a migrating phenotype leading to new vessel formation [11]. On the other hand, the eNOS expression and activity are diminished by COL I in human umbilical cells (HuVEC) [12], whereas endothelin synthesis is enhanced [13].

Endothelium is the most active player in controlling leukocyte traffic [14]. Endothelial cells show a characteristic pattern of adhesiveness to leukocytes. In normal conditions, the endothelium is mainly an anti-adherent barrier preventing leukocyte migration from the blood vessels into the underlying tissues, and only in cases of inflammation or damage this barrier is physiologically removed. The activation of endothelium by cytokines [15] or vasoactive substances [16] promotes reorganization of the adhesion molecules leading to changes in leukocyte adhesion. The adhesion of leukocytes to endothelial cells induces the leukocyte activation and the consequent cytotoxic effects on endothelial monolayer [17].

Since ECM components can play a role in the modulation of endothelial function, the present experiments were designed to analyse whether the changes in the composition of ECM modulate the adhesiveness pattern of endothelial cells.

Materials and Methods

Cells

HuVEC were isolated using standard procedures [12, 18], plated onto gelatin-coated culture plastic dishes and grown at 37°C and 5% CO₂. M-199 medium (BioWhitaker, Verviers, Belgium), supplemented with antibiotics (penicillin and streptomycin), 20% foetal calf serum (FCS) and 25 mg/ml of endothelial cell growth factor, was used as culture medium. When cells reached confluence, they were harvested and replated at a 1:5 split rate. Experiments were performed with cells at passages 2 to 4.

Human peripheral blood mononuclear cells (PBMC) and peripheral polymorphonuclear cells (PMN) were isolated from heparinized blood samples obtained from healthy volunteers. Blood was mixed with Ficoll-Paque (Pharmacia) and centrifuged to allow density gradient separation of the cells. PBMC were isolated from interface and PMN from the bottom fraction after dextran sedimentation and hypotonic lysis of red cells [19]. Upon isolation, cells were maintained in Mg⁺⁺ and Ca⁺⁺ free Hanks's balanced salt solutions (HBSS). The viability of cells obtained was assessed by trypan blue exclusion and was in every case 98.3 ± 1.2 %.

Collagen coating plates

Plates were coated with COL I, COL IV, pepsin treated COL I (COL I PEPS) or gelatin before seeding HuVEC. Acid soluble COL I and COL IV were purchased from Sigma (St. Louis, MO). Petri dishes were treated with a solution of 12.5 µg/ml collagen in bicarbonate buffer (Na₂CO₃ 15 mM, NaHCO₃ 35 mM, pH 9) for 16 hours at 4° C to allow the formation of a thin film of collagen as previously described [20]. Then, the collagen not attached to plastic was discarded and Petri dishes were washed with Hank's balanced solution to restore the pH 7.5. COL I was treated with 0.2 mg/ml pepsin for 18 hours at 4°C to remove telopeptides and added to plates as described above. 0.2 % gelatin was added to plates and allowed to dry at room temperature.

Adhesion assay

Adhesion assays were performed according to previously reported methods [18]. Briefly, PMN or PBMC were suspended in HBSS (20 x 10⁶ cells/ml) and radiolabelled by incubation with 30 µCi/ml Na⁵¹CrO₄ for 1 hour at 37°C. Then, the cells were washed twice with fresh HBSS (50 x g for 7 minutes, at 4°C) to remove unincorporated radioactivity and resuspended in HBSS supplemented with Ca⁺⁺, Mg⁺⁺, and 5% bovine serum albumin (BSA). HuVEC were plated on previously coated 24-well plates (1 x 10⁵ cells/well) 18 hours before to start the adhesion experiments.

Labelled PMN or PBMC were added to HuVEC at a ratio 5:1, and allowed to adhere for 30 minutes at 37°C. Afterward, supernatants were collected, cells were washed twice with HBSS and dissolved with 2N NaOH overnight at 37°C. Radioactivity from each well was measured in a gamma counter. Leukocyte

adhesion, expressed as percentage, was calculated as the ratio between cell lysate radioactivity and total counts (supernatant plus washing solution plus cell lysate radioactivity). In selected experiments, adhesion of PMN or PBMC to collagen coated plates, without endothelial cells, was measured as described above.

Cytotoxicity assay

The endothelial damage was assessed by ^{51}Cr release [21]. HuVEC were labelled with $\text{Na}^{51}\text{CrO}_4$ (5 $\mu\text{Ci}/\text{well}$) and cultured in 24-wells plates (10^5 cells/well), previously coated with the different ECM components, 18 hours before starting the experiments. Then, cells were washed twice with HBSS to remove unincorporated ^{51}Cr and were ready to use for experiments. PMN or PBMC isolated as described above were resuspended in HBSS supplemented with Ca^{++} , Mg^{++} , and 5% BSA and added at a 5:1 ratio (5×10^5 cells/well) on endothelial monolayers. The coculture was maintained for 4 hours and ^{51}Cr present in the supernatant was measured with a gamma counter. Total ^{51}Cr release was determined by incubating HuVEC with 1% Triton X-100 (Sigma). The specific cytotoxicity (CXs) was calculated as follow:

$$(1) \% \text{ CXs} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Analysis of mRNA expression by Northern blot

HuVEC were seeded on plates previously coated with the different collagens at a density of 10^5 cells/cm 2 and allowed to adhere for 24 hours at 37°C. Then, cells were disrupted in solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5 % sarcosyl, 0.1 M 2- mercaptoethanol). Total RNA was isolated by repeated phenol-chlorophorm extractions and isopropanol precipitations as described by Chomczyshy and Sacchi [22]. Total RNA (20 mg per line) was denatured by heating in formamide/formaldehyde at 100°C for three minutes and electrophoresed through 1% agarose gel with 0.66 M formaldehyde, transferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK) by capillary blotting, and fixed by UV-crosslinking. All hybridisations were carried out in a rotating drum in a temperature-controlled oven. The membranes were prehybridised at 65°C for 2 hours in 10% dextran sulphate, 1% SDS, 1M NaCl and denatured salmon sperm DNA. cDNA inserts were radiolabelled with 5 μCi (^{32}P) deoxycytosine 5'triphosphate (Ci/mmol, Amersham) using a radiolabelling system (Ready to go, Pharmacia, Uppsala, Sweden). The probes used were ICAM-1, VCAM-1 and monocyte chemoattractant protein, MCP-1 [23-25]. Blots were hybridised overnight in the same buffer used for prehybridisation, with 10^6 cpm/ml probe at 65°C. The membranes were washed twice for 5 minutes in 2x SSC and 1% SDS at 65°C. Auto-radiographs were performed with intensifying screens (X-OMAT, Kodak) at -80°C for 5-6 hours. The densitometric analysis of the films exposed was performed with an Apple scanner and appropriate software (NIH Image from the National Institutes of Health).

Protein extraction and western blot analysis

Total protein extracts were prepared by cell lysis with 0.7 ml RIPA buffer composed of 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.2), 1 mM PMSF, 0.2 mM sodium orthovanadate, and 1 $\mu\text{g}/\text{ml}$ aprotinin, leupeptin, and pepstatin. The resulting solution was spun at 12,000 rpm for 30 min at 4°C. To perform immunoblots, total cell extracts (20 $\mu\text{g}/\text{lane}$) were size-fractionated by SDS-PAGE on 7.5% polyacrylamide gels and transferred in 20% methanol to polivinylidene difluoride membranes. The presence of P-selectin was detected by using a mouse anti-P-selectin antibody and the peroxidase conjugated goat anti-mouse-Ig as secondary antibody. Activation and expression of different kinases were analyzed with specific antibodies, particularly rabbit anti-human phospho-GSK-3 β Ser 9 (P-GSK-3 β) and rabbit anti-human GSK-3 β (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-human phospho-ERK 42/44 (Thr 202/Tyr 204) (P-ERK) and anti-human ERK 42/44 (Cell Signaling Technology), rabbit anti human ILK (Upstate Biotechnology, Lake Placid, NY, USA), and rabbit anti-rat-MCP-1 (Chemicon, Temecula, CA, USA). The results were developed with the Supersignal system and autoradiography. The films were then scanned and analyzed using appropriate software (NIH Image 1.55 from the National institutes of Health, Bethesda, MD, USA). The blots were stripped and reblotted against mouse anti- β -tubulin antibody (Sigma) to guarantee that equal amounts of protein were loaded in any case.

Analysis of P-selectin expression in non fixed cells by Flow Cytometry

The expression of P-selectin on the surface of HuVEC was assayed by flow cytometry. Cells were seeded on plates previously coated with the different extracellular matrix proteins and, 24 hours later, they were harvested with trypsin-EDTA and centrifuged 1200 x g, 5 minutes at 4°C. Pellets were washed in PBS containing 1%BSA, 0.01% NaN_3 and 1% FCS (PBS-staining) twice and incubated with 20 $\mu\text{g}/\text{ml}$ mouse anti-P-selectin antibody in PBS staining for 20 minutes at 4°C in darkness. Then, cells were centrifuged 1200 x g for 5 minutes, rinsed with PBS and incubated with a FITC conjugated anti-mouse-Ig as secondary antibody, for 20 minutes, 4°C, in darkness. Finally, cells were washed and resuspended in PBS-staining and analysed in a flow cytometer with a 488 nm argon laser (FASCcalibur, Becton Dickinson, NJ, USA).

Transfection of the active mutant of ILK

In selected experiments cells seeded on COL I or COL IV were transfected with a plasmid containing an active mutant of ILK (WT-ILK, kindly provided by S.Dedhar from British Columbia University, Vancouver, Canada) or with the empty vector (pcDNA 3) with Lipofectamine (Invitrogen, San Diego, CA) according with de manufacturer's recommendation. Subconfluent (60%) HCM were incubated 8 h with 1 μg cDNA plasmid and 5 μl Lipofectamin reagent. Proteins were extracted 16 hours later.

Fig. 1. Adhesion of polymorphonuclear cells to unstimulated HuVEC seeded on different culture substrates and cytotoxic effect. Panel A: Polymorphonuclear cells were labelled with ^{51}Cr and added (5×10^5 cells/well) to HuVEC seeded (10^5 cells/well) on different ECM components. Results are the mean $\pm$ SEM from 8 different experiments. * $P < 0.05$ COL I vs COL IV. Panel B: HuVEC were labelled with ^{51}Cr and seeded on wells previously coated with different ECM components. Eighteen hours later polymorphonuclear cells were added and incubated for 4 hours at 37°C . ^{51}Cr release was measured in the supernatants. Results are the mean $\pm$ SEM from 8 different experiments. * $P < 0.05$ COL I vs COL IV.

	Gelatin	COL I	COL IV	P-COL I
PMN	39.7±10.05	36.8±7.57	40.8±13.9	38.8±5.0
PBMC	37.0±6.39	46.3±5.6	48.9±7.5	47.9±1.46

Table 1. PMN and PBMC adhesion to ECM components without endothelial cells. Results shown are mean $\pm$ SEM of percentage of adhesion from 6 different experiments.

Fig. 2. Adhesion of polymorphonuclear cells to HuVEC stimulated with $\text{TNF-}\alpha$. Polymorphonuclear cells were labelled with ^{51}Cr and added to HuVEC seeded in different ECM components, previously treated with $10 \mu\text{g/ml}$ $\text{TNF-}\alpha$ (hatched bars) for 4 hours. Results are the mean $\pm$ SEM from 4 different experiments. * $P < 0.05$ COL I vs COL IV. # $P < 0.05$ non-stimulated vs stimulated cells in the same culture substrate.

Statistical analysis

Results shown are the mean $\pm$ SEM of a variable number of experiments (see Legends to the Figures). Northern blot densitometry data were corrected for variations in gel loading by accounting for the relative intensities of the 18S band. Western blot densitometry data were corrected for variation in gel loading by accounting for the relative intensities of the β -tubulin, total ERK 1/2 or total GSK-3 β . As $n < 10$, non-parametric statistics were used for comparisons (Friedman's and Wilcoxon's test). A $p < 0.05$ was considered statistically significant.

Results

Adhesion of PMN to HuVEC was lower when cells were seeded on COL.

HuVEC were grown on different matrix components, and then PMN isolated from healthy volunteers were added to assess the adhesion rate to endothelial monolayers for 30 minutes. Results show a different pattern of adhesiveness of these leukocytes as a function of the culture substrate. PMN adhesion to endothelial monolayers was lower when endothelial cells were seeded on COL I as compared with cells seeded on COL IV (Figure 1 A). To evaluate the specific cytotoxicity promoted by PMN on endothelial cells seeded on different culture substrates, the ^{51}Cr release was quantified in the supernatants of HuVEC exposed for 4 hours to PMN. Results show again a different pattern of toxicity depending on the matrix component tested. PMN

Fig. 3. VCAM-1 and ICAM-1 mRNA expression in non-stimulated HuVEC seeded on different ECM components. HuVEC were seeded on different ECM components and incubated for 24 hours at 37°C. Then, total mRNA was extracted and VCAM-1 (panel A) or ICAM-1 (panel B) expression was evaluated by Northern blot. Each panel shows the densitometric analysis of 6 different experiments (mean ± SEM) and a representative experiment. * $P < 0.05$ COL I vs COL IV.

have a minor cytotoxic effect when HuVEC were seeded on COL I (Figure 1, panel B).

These results were obtained in non-stimulated HuVEC. To evaluate whether the effect was comparable after stimulation, a proinflammatory cytokine, Tumor Necrosis Factor- α (TNF- α), was added to the cells four hours before starting the adhesion experiments. TNF- α increased significantly the adhesion of PMN to HuVEC in all culture substrate analysed, and the differences between COL I and COL IV remained (Figure 2).

To evaluate the specificity of the results obtained, three kinds of control experiments were performed. First, the intrinsic cytotoxicity of the ECM components was

Fig. 4. Cellular content and surface expression of P-selectin in unstimulated HuVEC seeded on different ECM components. Panel A: HuVEC were seeded on different ECM components, proteins were extracted 24 hours later, and immunoblot for P-selectin was performed. Bar graphs represent the densitometric analysis of 4 different experiments (mean ± SEM). A representative western blot is shown. Panel B: HuVEC were seeded on different ECM components and 24 hours later harvested, incubated with anti-P-selectin antibody and secondary FITC conjugated-IgG, and analysed by flow cytometry. Numbers represent the percentage of fluorescent cells and the fluorescence intensity in each case and are the mean ± SEM of 5 different experiments. A representative experiment is shown. * $P < 0.05$ COL I vs COL IV.

Fig. 5. Effect of a blocking anti-P-selectin antibody on the polymorphonuclear cells adhesion. Non-stimulated (black bars, panel A) or TNF- α stimulated (hatched bars, panel B) HuVEC seeded on different ECM proteins were treated with a blocking anti-P-selectin (grey bar in both panels) antibody for one hour before adding the labelled PMN. Results are the mean $\pm$ SEM from 4 different experiments. * $P < 0.05$ COL I vs COL IV. # $P < 0.05$ blocking anti-P-selectin treated cells vs untreated cells.

Fig. 6. Intracellular mechanism involved in the regulation of P-selectin expression in COL I seeded cells. Panel A: HuVEC were seeded on COL or COL IV substrate culture, proteins were extracted 24 hours after and immunoblots for different kinases with specific antibodies were performed. Representative blots are shown. Panel B: HuVEC were seeded on COL I and transfected with a plasmid containing an active mutant of ILK (WT-ILK), 24 hours later proteins were extracted and immunoblot for P-selectin was performed. Bar graphs represent the densitometric analysis of 4 different experiments (mean $\pm$ SEM). A representative western blot is shown. # $p < 0.05$ vs COL I. Panel C: HuVEC were seeded on COL I or COL IV and treated with 50 μ M PD98059 (PD) for 24 hours. Proteins were extracted and immunoblot was performed with anti- P-selectin antibody. Bar graphs represent the densitometric analysis of 3 different experiments (mean $\pm$ SEM). A representative western blot is shown. * $p < 0.05$ COL I vs COL IV; # $p < 0.05$ vs COL I. Panel D: HuVec were seeded on COL I or COL IV and tranfected with a plasmid containing an active mutant of ILK, 24 hours later proteins were extracted and immunoblot for P- ERK 1/2, ERK 1/2 and ILK were performed. Representative blots are shown.

evaluated. HuVEC seeded on different ECM components, without leukocyte addition, showed a similar spontaneous release of ^{51}Cr (Gelatine: 28.2 ± 2.3 %; COL IV: 29.6 ± 2.5 ; COL I 30.1 ± 3.6 ; COL I PEPS: 31.5 ± 1.3). Second, the possible contribution of the substrate culture to the adhesion results was tested. For that purpose, in selected experiments, PMN or PBMC were added to wells previously coated with the matrix components without endothelial cells and allowed adhere for 30 min. No differences were found in the adhesion percentage of PMN or PBMC to the matrix used (Table 1). Third, the specificity of results was supported by disrupting the fibrillar structure of COL I with pepsin. In these conditions, the differences observed in the COL I cultured cells disappear (COL I PEPS in all figures).

COL I induces a decrease in the VCAM-1 mRNA expression and P-Selectin content

To investigate the mechanisms involved in these phenomena, changes in the expression of adhesion molecules promoted by COL I were evaluated in HuVECs. The mRNA expression of VCAM-1 (Figure 3 A) was down-regulated in COL I seeded cells, whereas ICAM-1 mRNA expression (Figure 3 B) was not modified depending on the culture substrate. COL I induced a decreased cellular content of P-selectin (Figure 4 A), as well as of its surface expression (Figure 4 B). In order to assess the role of P-selectin down-regulation in the decreased PMN adhesion to HuVEC seeded on COL I, resting (Figure 5 A) or TNF- α stimulated cells (Figure 5 B) were treated with a blocking anti-P-selectin antibody for one hour before adding the PMN. Results show that the adhesion of PMN to cells was partially inhibited by the blocking antibody in all ECM components tested. In the presence of the antibody, no differences were detected between both collagen types.

The decreased P-selectin expression in COL I was dependent on ILK and ERK 1/2 activities

To analyse the intracellular mechanisms involved in the regulation of P-selectin content by COL I, the expression of several kinases was evaluated in HuVEC seeded on COL I or COL IV. Specifically, integrin linked kinase (ILK), and extracellular signal regulated kinase (ERK 1/2) and glycogen synthase kinase 3 β (GSK-3 β) expression was analysed. COL I promoted a down-regulation of ILK content (Figure 6 A) and its activity, since Phospho-GSK-3 β (P-GSK-3 β) was also diminished in COL I. By contrast, expression of Phospho-ERK 1/2

Fig. 7. Adhesion of peripheral blood mononuclear cells to non-stimulated HuVEC seeded on different culture substrates and cytotoxic effect. Panel A: Peripheral blood mononuclear cells were labelled with ^{51}Cr and added (5×10^5 cells/well) to HuVEC seeded (10^5 cells/well) on different ECM components. Results are the mean $\pm$ SEM from 8 different experiments. * $P < 0.05$ COL I vs COL IV. Panel B: HuVEC were labelled with ^{51}Cr and seeded on wells previously coated with different ECM components. Eighteen hours later peripheral blood mononuclear cells were added and incubated for 4 hours at 37°C . ^{51}Cr release was measured in the supernatants. Results are the mean $\pm$ SEM from 8 different experiments. * $P < 0.05$ COL I vs COL IV.

(P-ERK 1/2) was enhanced in COL I respect to COL IV (Figure 6 A).

The role of ILK in the effect of COL I on the P-selectin content was elucidated by transfecting cells seeded on COL I with a plasmid containing an ILK active mutant (wild type ILK, WT-ILK) [26]. Expression of P-selectin was increased in this condition. (Figure 6 B).

In the same way, the role of ERK 1/2 on P-selectin decrease was analysed by, treating HuVEC seeded on COL I with the specific inhibitor PD 98059 [27]. This inhibitor induced an increase in P-selectin content (Figure 6 C), indicating that ERK 1/2 is repressing the P-selectin expression in COL I.

Fig. 8. MCP-1 expression in non-stimulated HuVEC seeded on different culture substrates and mechanism involved in its regulation. Panel A: HuVEC were seeded on different substrate culture and 24 hours later total RNA and proteins were extracted to analyse the mRNA expression by northern blot (left panel) and the protein content by western blot (right panel). Bar graphs represent the densitometric analysis of 4 different experiments (mean $\pm$ SEM). A representative experiment is shown in each case. * $p < 0.05$ COL I vs COL IV. Panel B: HuVEC were seeded on different culture substrate and treated with 50 μ M PD98059 (PD) for 24 hours (right panel) or transfected with a plasmid containing the active form of ILK (WT-ILK, left panel). Twenty-four hours later protein were extracted and western blots for MCP-1 were performed. Bar graphs represent the densitometric analysis of 3 different experiments (mean $\pm$ SEM). A representative western blot is shown in each case. * $p < 0.05$ COL I vs COL IV; # $p < 0.05$ vs COL I.

To delimitate the relation between ILK and ERK 1/2 activities, the P-ERK 1/2 and total ERK 1/2 expression were assayed in HuVEC seeded on COL I or COL IV transfected or not with WT-ILK. Results showed that P-ERK was diminished in presence of active ILK whereas no changes were found in total ERK content (Figure 6 D). As a control of the efficiency of transfection the blots were reblotted with anti-ILK antibody (Figure 6 D). Expression of ILK was enhanced when cells were transfected with WT-ILK.

Adhesion of PBMC to HuVEC was higher when cells were seeded on COL I

PBMC were isolated from healthy volunteers and added to a monolayer of HuVEC seeded on COL I or COL IV. After 30 minutes of incubation rate of adhesion was evaluated. In contrast, with the PMN results, the adhesion of PBMC to endothelial cells was higher when the cells were seeded on COL I (Figure 7 A). Moreover, PBMCs were significantly more cytotoxic when HuVEC were seeded on COL I (Figure 7 B).

COL I induces the up-regulation of MCP-1 expression in HuVEC

To investigate the possible agent that is mediating in this effect. The MCP-1 expression was analysed in HuVEC seeded on different culture substrate. The mRNA expression of MCP-1 and the protein content were increased by COL I (Figure 8 A). The intracellular kinases involved in this effect were evaluated by using different experimental approaches. HuVECs seeded on COL I and transfected with WT-ILK show an increase in MCP-1 content (Figure 8 B, left panel). By contrast, the expression of MCP-1 was inhibited in presence of PD 98059 (Figure 8 C, right panel).

Discussion

Many studies have reported that ECM plays a role in the modulation of the behaviour of resident cells. Changes in the composition of ECM can promote changes on different aspects of cell phenotype. In normal conditions, endothelial cells are not in contact with collagen I. However, in some pathological conditions, collagen I could be present at the subendothelial space [29]. The purpose of this study was to evaluate whether changes in ECM composition at the vessel walls could promote changes in the interactions between leukocyte and endothelial cells.

Experiments were performed on confluent monolayers of HuVEC seeded on different ECM components. Cultured cells synthesize their own extracellular matrix. For this reason, experiments were performed 24 hours after cell seeding to minimize interferences of newly synthesized ECM components. The results obtained show that COL I modifies the interactions of endothelial cells with leukocytes, when compared with COL IV and with the standard culture substrate for endothelial cells, gelatin. Specifically, the adhesion of PMN to HuVEC seeded on COL I decreased whereas that of PBMC increased.

PMN are the first leukocytes to migrate specifically across the endothelium adjacent to the inflammation site, hours before monocytes or lymphocytes [30]. The initial event in the inflammation process is the appearance, on the endothelium adjacent to the damaged tissue, of new adhesion molecules that recruit the leukocytes from the circulation. The first molecule implied is P-selectin which is released from Weibel-Palade bodies after endothelial stimulation [31]. After the rolling phase, other adhesion molecules are required to promote the firm adhesion of

leukocyte to HuVEC, mainly ICAM-1 and VCAM-1 [32]. In our experimental conditions, the cellular content and the surface expression of P-selectin, as well as VCAM-1 mRNA expression, were diminished in non-stimulated endothelial cells seeded on COL I with respect to COL IV, whereas no changes were found in the ICAM-1 mRNA expression. These changes in the expression of adhesion molecules are important because demonstrate the role of the environment on the basal expression of these molecules in the endothelium, in absence of any inflammatory or anti-inflammatory event. The adhesion of PMN to HuVEC was partially inhibited when P-selectin was blocked with a specific antibody, and the differences between collagens disappeared in these conditions. Therefore, the lower P-selectin expression in the surface of HuVEC, and also probably the diminished expression for VCAM-1, could be responsible for the lower PMN adhesion rate to non-stimulated endothelial cells cultured on COL I.

The PMN adhesion was also evaluated in cells previously stimulated with TNF- α . TNF- α has been recognized as a central mediator of inflammation [33] able to increase the PMN adhesion by increasing the expression of diverse adhesion molecules in HuVEC [34]. TNF- α treatment produced a marked increase in the PMN adhesion to HuVEC. The increment in adhesion was about a 150 % in all culture substrate tested indicating that the response to a proinflammatory stimulus was not substrate-dependent. Blocking P-selectin antibody also decreased the PMN adhesion to stimulated HuVEC in all culture substrate tested.

When PMN were added to wells previously coated with the matrix components without endothelial cells and allow adhere for 30 minutes, no differences were found in the adhesion percentage, indicating that the differences found in PMN adhesion to HuVEC were not dependent on the direct adhesion of PMN to matrix. In order to evaluate whether the observed effects on adhesion and cytotoxicity were specific for COL I in the fibrillar form, a pepsin treatment was performed. In our experimental conditions, when COL I was treated with pepsin, statistical differences between COL I and COL IV regarding PMN and PBMC adhesion and cytotoxicity disappeared. Additionally, this treatment restored the changes in the cellular content and surface expression of P-selectin, VCAM-1 and MCP-1 mRNA expression detected in HuVEC seeded on this interstitial collagen. This experimental approach is normally used in this type of studies [35, 36]. Pepsin removes the aminotelopeptide sequences of collagen type I, disrupts the intermolecular

crosslinking between telopeptides and the triple helix structure. The resulting fibrils of pepsin-treated COL I are thicker than normal [37].

Probably as a consequence of the reduced adhesion to endothelial cells, PMN-promoted diminished cytotoxicity on HuVEC seeded on COL I with respect to those seeded on COL IV or gelatine. This effect was not dependent on a possible intrinsic toxic effect of the ECM components, since the ^{51}Cr spontaneous release from HuVEC was similar in all substrate culture used. The physiopathological relevance of these findings could be very important, because a lower recruitment of neutrophils in response to traumatic injury or bacterial infection in the damaged vessel walls would determine a minor immunological primary response and an impaired bacteriological protection.

In contrast with PMN results, the adhesion rate of PBMC and their cytotoxicity were higher on HuVEC seeded on COL I. This supports the specificity of the COL I-induced phenotypic changes in HuVEC since different leukocytes interact in different way with endothelial cells. To investigate the mechanism involved in this effect, we evaluated the expression of a chemokine which is released by endothelial cells specifically to increase the recruitment of monocytes, the monocyte chemoattractant protein, MCP-1 [38]. Effectively, cells seeded on COL I produced higher amounts of MCP-1 than those cells seeded on COL IV. The increase in MCP-1 could be enough to increase the adhesion of PBMC to cells [39]. These findings could be very relevant in the context of the some pathological conditions, such as atherosclerosis, which involves vascular remodelling with appearance of COL I at the subendothelial space [29], since COL I could be responsible, at least in part, of the increase in monocytes adhesion and migration through the endothelial monolayer contributing to the atherosclerotic process.

The differences found in the adhesion of PMN and PBMC depending of the substrate culture were a consequence of a different expression pattern of the several intracellular signalling pathways. ILK is a key component of the integrin signalling complex that functions interacting with other kinases to transmit cues from the extracellular matrix to intracellular environmental [40]. We have demonstrated that ILK expression and activity were down-regulated in HUVEC seeded on COL I, whereas phospho-ERK 1/2 expression was increased. The modulation of ILK by COL I have been previously studied by our group and others in different cellular types

[12, 40, 41]. The role of these kinase pathways on P-selectin and MCP-1 expression were evaluated and results show that ILK is up-regulating P-selectin expression and down-regulating the expression of MCP-1 and these effects are mediated by the ERK 1/2 kinase pathway. Specifically, ILK seems to be repressing ERK 1/2 activation, since the transfection of cells with the ILK active form promotes a fall in the ERK 1/2 phosphorylation. Therefore, ILK is down-regulated in COL I and this induce an increase in ERK 1/2 activation which finally allow to ERK 1/2 to inhibit the expression of P-selectin and to increase the MCP-1 expression. The mechanism involved in the ILK inhibition of ERK activity have not been studied in the present work but could be the same that are described in the paper by Wang et al. [42] where it is showed that Glycogen Synthase Kinase-3 is a negative regulator of ERK in human colon cancer cells lines.

Considering together the presented results, the following mechanism could be proposed. In pathological conditions, collagen type I could appear at the subendothelial space and came into contact with endothelial cells. This interaction would promote the decrease in ILK activity and this would induce an increase in ERK 1/2 activity. As a consequence, the P-selectin content is diminished and, by contrast, the MCP-1 content is increased. The final effect is a lower recruitment of PMN and a higher adhesion of PBMC.

To our knowledge, this is the first time that the modulation of interactions between endothelial cells and leukocytes by ECM components is reported. A more profound study will be necessary to analyse the intracellular mechanisms implied in this phenomenon and its biological relevance.

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