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Inducible Nitric Oxide and Prostacyclin Productions Are Differently Controlled by Extracellular Matrix and Cell Density in Human Vascular Endothelial Cells

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Abstract Both cell-matrix and cell-cell interactions are important regulators of the function of most human cells. In this study we investigated how these interactions controlled the production of vasodilators nitric oxide (NO), and prostacyclin (PGI_2), in freshly isolated human umbilical vein endothelial cells (HUVECs). On the reconstituted extracellular matrix (ECM) Matrigel freshly isolated HUVECs treated with interleukin-1β, lipopolysaccharide, and interferon-γ, produced more NO, but less PGI_2, than on gelatin substratum. High cell density was essential for inducibility of NO production in cells plated on gelatin substratum, but not on ECM. In cells plated on gelatin substratum at low cell density, which mimicked conventional HUVEC culturing conditions, both inducible NO production and the inducible NO synthase (iNOS) mRNA levels, detected by competitive RT-PCR, were low. However, inducible PGI_2 production remained high in these cells. Highest inducible NO productions were observed in HUVECs that presumably had best maintained their original differentiated phenotype. Thus our data imply that the inducible NO and PGI_2 productions of freshly isolated HUVECs were differently controlled by the extracellular matrix and cell density. Our data suggest that both cell-matrix and cell-cell interactions may have a strong influence on the proinflammatory cytokine responses of human vascular endothelial cells. J. Cell. Biochem. 64:538–546.

Key words: cytokines; lipopolysaccharide; interleukin-1β; interferon-γ; ECM; Matrigel; PGI_2, iNOS; HUVEC

Nitric oxide (NO) and prostacyclin (PGI_2), which are powerful vasodilators released by the vascular endothelium, are normally produced by the calcium-dependent NO synthase (NOS) and the cyclo-oxygenase (COX)-1 of the vascular endothelial cells [Vane et al., 1990; Moncada et al., 1991; Knowles and Moncada, 1994]. Inflammatory mediators, such as proinflammatory cytokines and bacterial lipopolysaccharides (LPS), induce the expression of the inducible NO synthase (iNOS) in several cell types, and large amounts of NO are produced locally [Nathan, 1992; Bredt and Snyder, 1994]. In these conditions also PGI_2 synthesis may be stimulated, and an inducible form of cyclo-oxygenase (COX-2) expressed [Hla et al., 1993].

The regulation of inducible NO production in human is still poorly understood, and although murine macrophages are easily induced to produce NO and express iNOS mRNA, inducibility of NO production in human monocytes/macrophages remains controversial [Albina, 1995]. The proinflammatory cytokines induced NO production in cultured murine microvascular endothelial cells [Kilbourn and Belloni, 1990; Singh et al., 1996], but not in cultured human umbilical vein endothelial cells (HUVECs) [Rosenkranz-Weiss et al., 1994]. This has raised a question, whether human vascular endothelial cells induce NO production, and what mechanisms control inducible NO and PGI_2 productions in these cells.

We have recently shown that freshly isolated HUVECs treated with calcium ionophore A23187 produced large amounts of NO, suggesting that the calcium-dependent endothelial NOS system was functional in freshly isolated HUVECs [Orpana et al., 1996]. We have also observed ample induction of NO production and iNOS mRNA expression in freshly isolated HUVECs, which have been plated immediately

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after isolation on the reconstituted extracellular matrix (ECM), Matrigel, and then treated with tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ [Orpana et al., 1994; Orpana et al., manuscript submitted].

In several in vitro cell models it has been shown, that some of the specialized cell functions are rapidly lost when cells are removed from their natural in vivo environment. Plating immediately after isolation on reconstituted ECM [Schuez et al., 1988; Li et al., 1987] or at high cell density [Ben-Ze'ev et al., 1988; Streuli et al., 1991] are cell culture conditions that aid in maintaining the original phenotype of hepatocytes or epithelial cells during in vitro cell culturing. In the present investigation we focused on the control of the proinflammatory cytokine-induced production of NO and PGI_2 by the extracellular matrix and cell density in freshly isolated HUVECs.

METHODS

Cell Culture

Endothelial cells were isolated from the umbilical cords by a modified collagenase dispersion method of Jaffe et al. [1973]. The lumens of three to four fresh umbilical cord veins were rinsed with phosphate buffered saline (PBS), filled with solution containing collagenase type IV (0.3 g/l, Sigma Chemicals, St. Louis, MO) in Hank's Balanced Salt Solution (Sigma), and incubated at 37°C for 10 min. The collagenase solution was removed, and veins flushed with serum-free medium (SFαMEM), consisting of minimum essential medium-alpha (Gibco BRL, Grand Island, NY), containing bovine serum albumin (fraction V, 5 g/l, Sigma), 2.5 mmol/l, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid (HEPES), pH 7.4, and gentamicin (50 mg/l) (Gibco) [Orpana et al., 1996]. The cells were collected (100 × g, 10 min), washed with SFαMEM, and suspended in SFαMEM supplemented with 2% fetal calf serum (Bioclear, Berks, UK). The immunocytochemical detection of the von Willebrand factor demonstrated negligible contamination of the cell suspension by other cells than erythrocytes, which were removed at the washing steps.

Cell Incubations

Two ways to vary cell density were used. In the first set of experiments, the cells were plated on tissue culture plates having different plating areas. To study the effects of cell-matrix interactions at high cell density, similar cell aliquots were plated on 24-well culture plates (Nunclon, Roskilde, Denmark), coated with either the reconstituted ECM, Matrigel (Collaborative Biomedical Products, Bedford, MA), or standard 0.2% gelatin solution in PBS (Merck, Darmstadt, Germany). Similar cell aliquots were also plated on gelatin-coated six-well culture plates (Nunclon) to study the effects of cell density. This last condition mimics conventional HUVEC culturing. The cells were incubated at 37°C in 5% CO_2 in air. After 24 h unattached cells were removed by washing with fresh culture medium, and 48 h later, experiments with proinflammatory cytokines were performed as described below. The plating areas were 1.9 and 9.6 cm^2, and the incubation volumes 1 ml and 3 ml, for the 24- and six-well plates, respectively.

In the second set of experiments, cell suspensions were serially diluted (1:2), and aliquots plated on ECM or gelatin-coated wells of 24-well plates. The progressive reduction of plating density was thus achieved, while all other experimental variables remained unchanged. This model gives a wide range of cell densities on both ECM and gelatin substrata.

Supplementation of the culture medium with only 2% fetal calf serum without additional endothelial growth factors minimized proliferative signaling of the culture medium, and in preliminary studies we observed no increase in cell number, when freshly isolated HUVECs were cultured in these conditions on gelatin substratum, although cell viability was fully maintained, as assessed by the activity of the mitochondrial dehydrogenases (cell proliferation assay II [XTT], Boehringer Mannheim, Mannheim, Germany) essentially as described by Orpana et al. [1996].

Experiments With Proinflammatory Cytokines

The culture medium was removed and the cells were washed with fresh SFαMEM, and then treated with interleukin (IL)-1β (10 kU/l, Immugenex, Los Angles, CA), LPS from *Salmonella typhimurium* (10 mg/l, Sigma) and interferon (IFN)-γ (200 μg/l, Sigma) in SFαMEM. The incubation volumes were 0.8 ml and 2 ml for the 24- and six-well plates, respectively. After 48 h, the culture media were collected for NO and PGI_2 measurements, and total RNA was prepared from the cells for the quantitative reverse transcriptase-polymerase chain reac-

tion (RT-PCR) analysis of iNOS mRNA levels. In experiments, in which the effect of cell plating density was studied with progressive cell dilutions, incubations with cytokines lasted for 72 h to allow accumulation of sufficient amounts of nitrite and nitrate (see below) even in the most diluted cell suspensions. This could be done, as preliminary studies demonstrated, that after induction treatments, inducible NO production was detectable after 24 h, and accumulation of nitrite and nitrate continued for up to 72 h.

Determinations

The NO production was measured as combined accumulation of nitrite and nitrate into the culture medium, as described previously in our study on the calcium-dependent NO production of freshly isolated HUVECs [Orpana et al., 1996]. Production of PGI_2 was measured as accumulation of the hydrolysis metabolite 6-keto-$PGF_{1\alpha}$ in the culture medium using a specific radioimmunoassay as described in [Mäkilä et al., 1983].

RT-PCR

Total cellular RNA was isolated, and 1 µg was reverse transcribed to complementary DNA (cDNA) with 10 IU AMV reverse transcriptase (RT) (Promega, Madison, MA) using random hexamer primers (Pharmacia, Uppsala, Sweden) in the presence of 20 U RNasin (Promega). The cDNA synthase reactions were performed at 42°C for 1 h, after which the 80 µl of TE buffer (10 mmol/l Tris-HCl, pH 8.0, 1 mmol/l ethylenediaminetetraacetic acid [EDTA] was added, and the diluted cDNAs were stored at −20°C.

The PCR was performed with Dynazyme II DNA Polymerase (Finnzymes, Espoo, Finland), using the diluted cDNA reaction as template. The iNOS sense primer was 5′-TACATGCAGAATGAATAC-3′, and the antisense primer, 5′-CTGATCAATGTCATGAGC-3′. These primers amplify a 594 bp from the human iNOS cDNA [Geller et al., 1993]. To verify the quantity and quality of the RNA template and the RT reaction, the 540 bp segment of cyclophilin mRNA [Pellegrini et al., 1992] was PCR amplified from equal amounts of the same cDNA template. Denaturation was performed at 94°C for 2 min, and the amplification was performed by denaturing at 94°C for 50 sec, annealing at 63°C for 50 s, and extension at 72°C for 70 s for 35 cycles. The samples were tested that generation of PCR product was dependent on reverse transcription reaction.

Preparation of the Template DNAs for Competitive RT-PCR

The iNOS PCR primers were originally designed so that they flanked a unique *Sac* I restriction site. The template for the competitive PCR was generated by ligation of a 234 bp Φx 174 DNA *Hae* III fragment into the linearized *Sac* I site of the cloned iNOS PCR product. Prior to ligation, the ends of the linearized plasmid DNA were treated with T4 DNA polymerase (Promega) and 250 µmol/L dNTPs, and dephosphorylated with shrimp alkaline phosphatase (USB, Cleveland, OH). The clones containing the additional 234 bp were identified by direct colony PCR, as the size of the iNOS competitor PCR product was 824 bp.

Identically, a 194 bp *Hae* III fragment from Φx 174 DNA was ligated into the linearized unique *Ssp* I site of the cloned cyclophilin PCR product to prepare cyclophilin competitor DNA.

Competitive RT-PCR Assay

The principle of the competitive PCR assay used for quantitation of the mRNA levels of iNOS and the control gene cyclophilin is presented in Figure 1. The PCR reactions supplemented with a constant amount of sample cDNA and increasing concentrations of the competitor DNA as template were resolved on agarose gels. The ratios between the intensities of the PCR bands amplified from the competitor DNA and sample cDNA were calculated for each sample. The amount of iNOS mRNA in the sample cDNA was taken as the calculated competitor DNA concentration when the ratio of their digitized band intensities were equal.

In this assay, a PCR reaction master mix, sufficient for 20 individual PCR reactions was prepared, and supplemented with an aliquot of the cDNA reaction of interest. Equimolar amounts of the cyclophilin and iNOS competitor plasmid DNAs, usually at 500 nmol/l of each, were combined, and a 10-step (1:4) dilution series of this competitor DNA was prepared in TE buffer. Two µl of these competitor DNA dilutions were transferred into corresponding PCR reaction tubes in duplicate. Primers for iNOS were added to one of these duplicates, while the other one was used for cyclophilin PCR. Then the PCR master mix already contain-

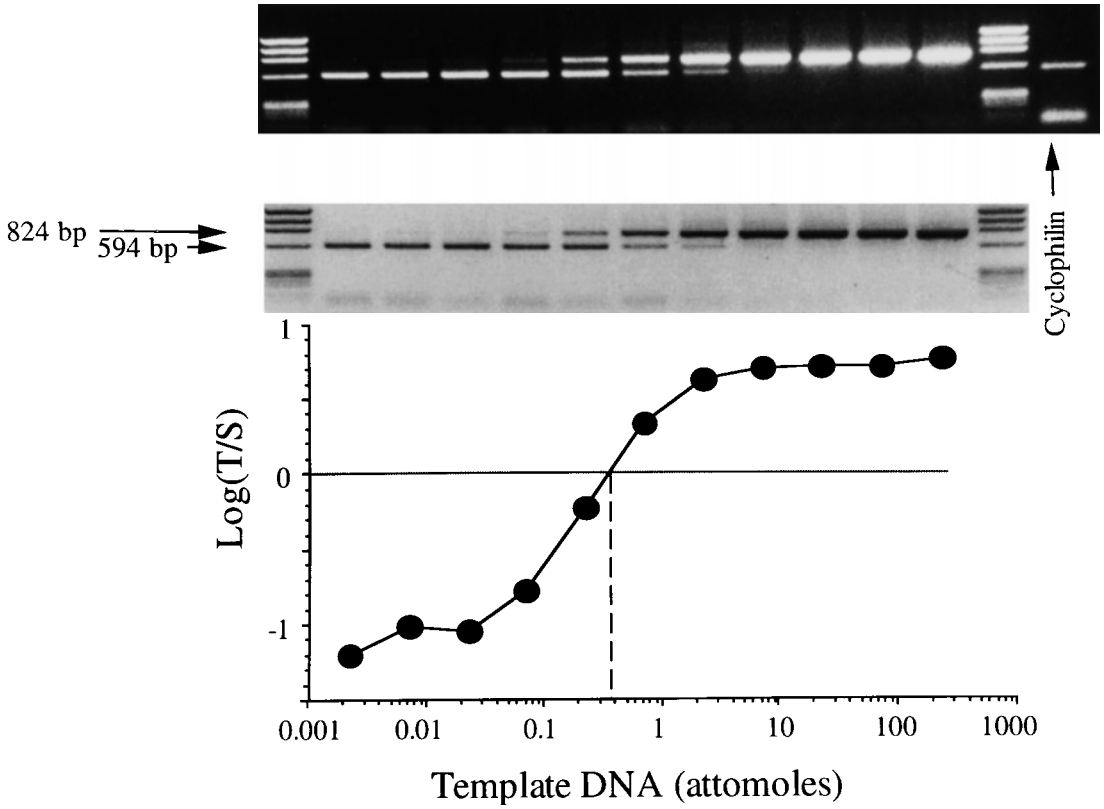

Fig. 1. Competitive RT-PCR assay for detection of iNOS mRNA levels. The constant amount of sample cDNA, prepared from proinflammatory cytokine-treated HUVECs expressing iNOS mRNA, together with increasing amounts of the competitor DNA (1:4 dilution series) is PCR amplified, resolved on agarose gels, and photographed scanned, and digitized. The ratios between relative intensities of the PCR products obtained from competitor template DNA (T) (824 bp), and the sample (S) cDNAs (594 bp) are calculated. The concentration of competitor DNA that equals to the sample cDNA concentration was estimated based on these ratios.

ing sample cDNA was added, and PCR amplification was performed as described above, but the denaturation time at 94°C, the annealing time at 55°C, and the extension time at 72°C were all 50 s. Both iNOS and cyclophilin competitive PCR amplifications were performed simultaneously. PCR products were resolved on 2% agarose gels run in TBE buffer containing ethidium bromide, or stained with CYBR Green I (FMC BioProducts, Rockland, ME). The agarose gels were photographed with instant Polaroid system, and the pictures digitized with a standard flat bed scanner (ScanMaker II). The band intensities were quantitated using NIH Image program (version 1.56).

Data Analysis

ANOVA with Fisher's PLSD and paired *t*-test were used for comparisons.

RESULTS

On conventional gelatin substratum freshly isolated HUVECs flattened, migrated, and at highest plating densities, showed the typical cobble stone morphology of HUVECs (Fig. 2A,B). When plated on ECM, the cells remained in cell aggregates and maintained rounded, compact, morphology at all cell densities (Fig. 2C,D). The cells were identified as endothelial cells with immunocytochemical staining for von Willebrand factor.

Treatment with IL-1β, LPS, and IFN-γ induced NO production in freshly isolated HUVECs, and on gelatin substratum significantly less NO was produced than on ECM (Fig. 3). Unstimulated HUVECs did not produce detectable amounts of NO (not shown). When the cell density was decreased by plating similar aliquots of cells on five times larger surface

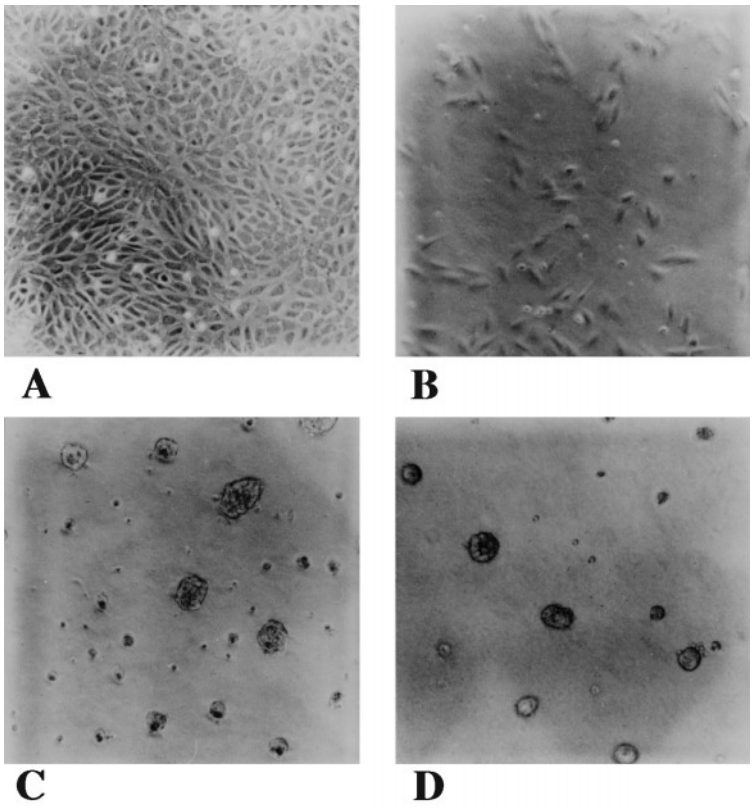

Fig. 2. Effect of plating substratum and cell density on the morphology of freshly isolated HUVECs. Freshly isolated human umbilical vein endothelial cells were plated on 24-well plates coated with gelatin substratum (**A, B**), or with the reconstituted extracellular matrix (ECM) Matrigel (**C, D**), at high plating density (A, C) or at low plating density (B, D).

area (on gelatin-coated six-well plates) the amount of NO produced further decreased (Fig. 3). The total RNA was prepared from these cells, and iNOS mRNA levels of HUVECs plated at high density on ECM, or at low density on gelatin substratum were compared using competitive RT-PCR assay. In Figure 4 are collected the most informative lanes from the total of 40 individual PCR reactions. The amount of cDNA template used in the analysis was initially tested to give equal cyclophilin cDNA amplification. As shown in Figure 4, cDNA prepared from cells plated at high density on ECM, efficiently competed with a 16-fold higher iNOS competitor DNA concentration than did cDNA prepared from the same cells plated on gelatin substratum at low density. Cyclophilin competitive RT-PCR assay verified that the levels of this cDNA were comparable, and actually slightly higher in the sample from the cells plated on gelatin substratum at low density (Fig. 4). Essentially similar results were obtained in other experiments.

Treatment of freshly isolated HUVECs with IL-1β, LPS, and IFN-γ stimulated PGI_2 production by 12.1 ± 3.6-fold compared with untreated cells (mean ± SE from seven experiments). The plating matrix did not significantly affect Il-1β, LPS, and IFN-γ-induced PGI_2 production of cells plated at high density, and PGI_2 production of cells plated on gelatin substratum at low density was highest (Fig. 3).

In the cell dilution experiments, the progressive decrease in the number of cells plated on ECM resulted in corresponding decrease in NO production induced by IL-1β, LPS, and IFN-γ (Fig. 5). On the other hand, when identical aliquots of the cells were plated on gelatin substratum, the inducibility of NO production was gradually lost (Fig. 4). This was seen in the slopes of the cell dilution curves presented in Figure 5, which were significantly different [*ECM:* $\log_2(NO_2 + NO_3) = 4.96 - 0.83$ ($\underline{-0.96 - -0.70}$)* $\log_2$ (Dilution factor), $r^2 = 0.89$; *Gelatin:* $\log_2 (NO_2 + NO_3) = 4.20 - 1.47$ ($\underline{-1.72 - -1.23}$)* $\log_2$ (Dilution factor), $r^2 = 0.88$, under-

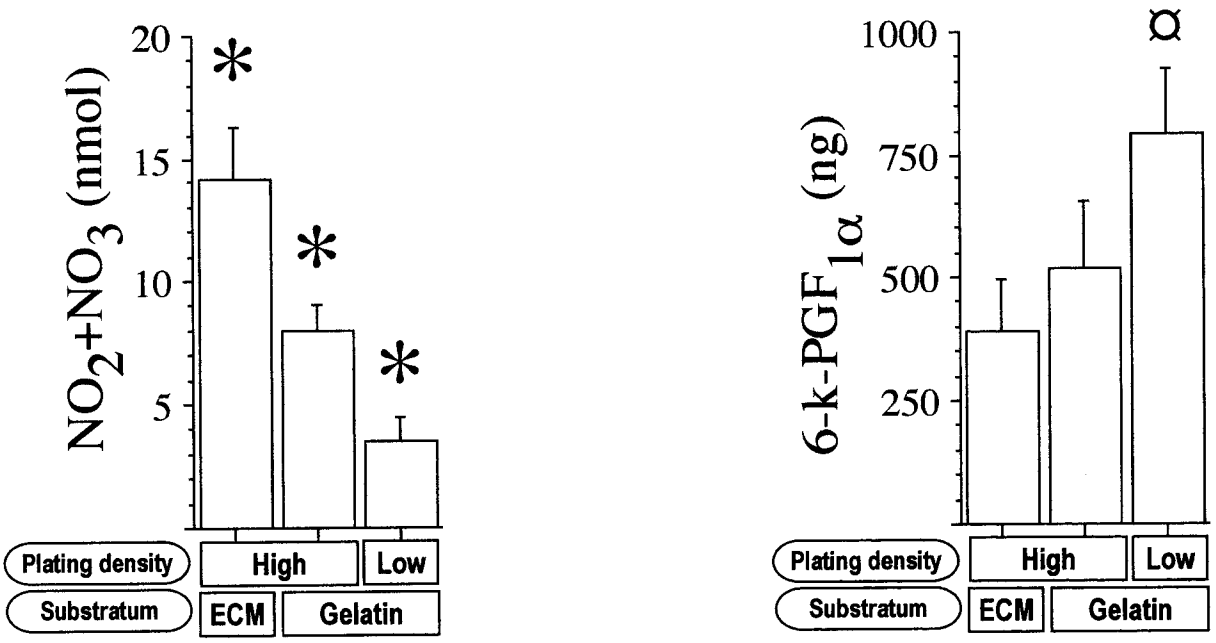

Fig. 3. Effect of plating substratum and cell density on inducible NO or PGI_2 production. Freshly isolated human umbilical vein endothelial cells were plated on the reconstituted extracellular matrix Matrigel (ECM) or gelatin coated 24-well plates (high plating density) or on gelatin-coated six-well plates (low plating density) for 72 h, and then treated for 48 h with interleukin-1β, lipopolysaccharideand inteferon-γ. The nitric oxide (NO) production was measured as accumulated nitrate + nitrite, and prostacyclin production was measured as 6-keto $PGF_{1\alpha}$ in the culture medium. Mean ± SE from six experiments, $^*P < 0.01$, vs. other two groups, $¤P < 0.01$, high vs. low plating density, paired *t*-test.

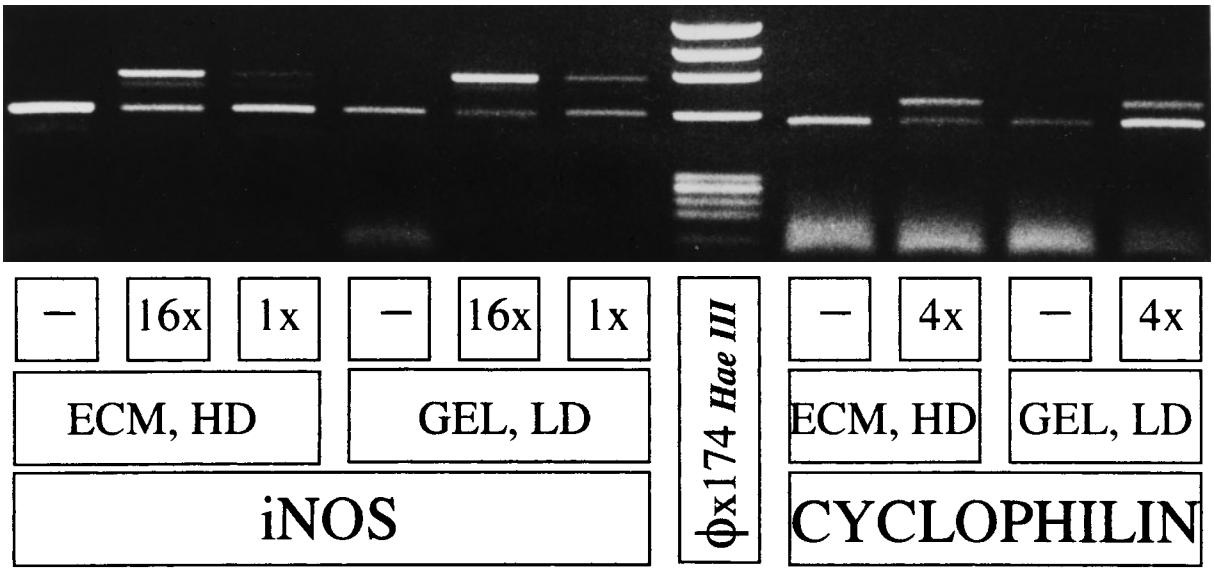

Fig. 4. Effect of plating substratum and cell plating density on iNOS mRNA levels. Total RNA was prepared from cells used in experiments presented in Figure 3. Competitive PCR analysis was performed for estimation of iNOS and cyclophilin cDNA levels. The most informative data are gathered to a single gel. The relative amount of competitor DNAs (1×, 4×, or 16×) PCR amplified with iNOS or cyclophilin cDNAs from the freshly isolated human umbilical vein endothelial cells plated either on the reconstituted extracellular matrix Matrigel-coated 24-well plates at high density (ECM, HD) or on gelatin-coated six-well plates at low density (GEL, LD). The *Hae* III-digested Φ174 DNA was used as molecular weight standard.

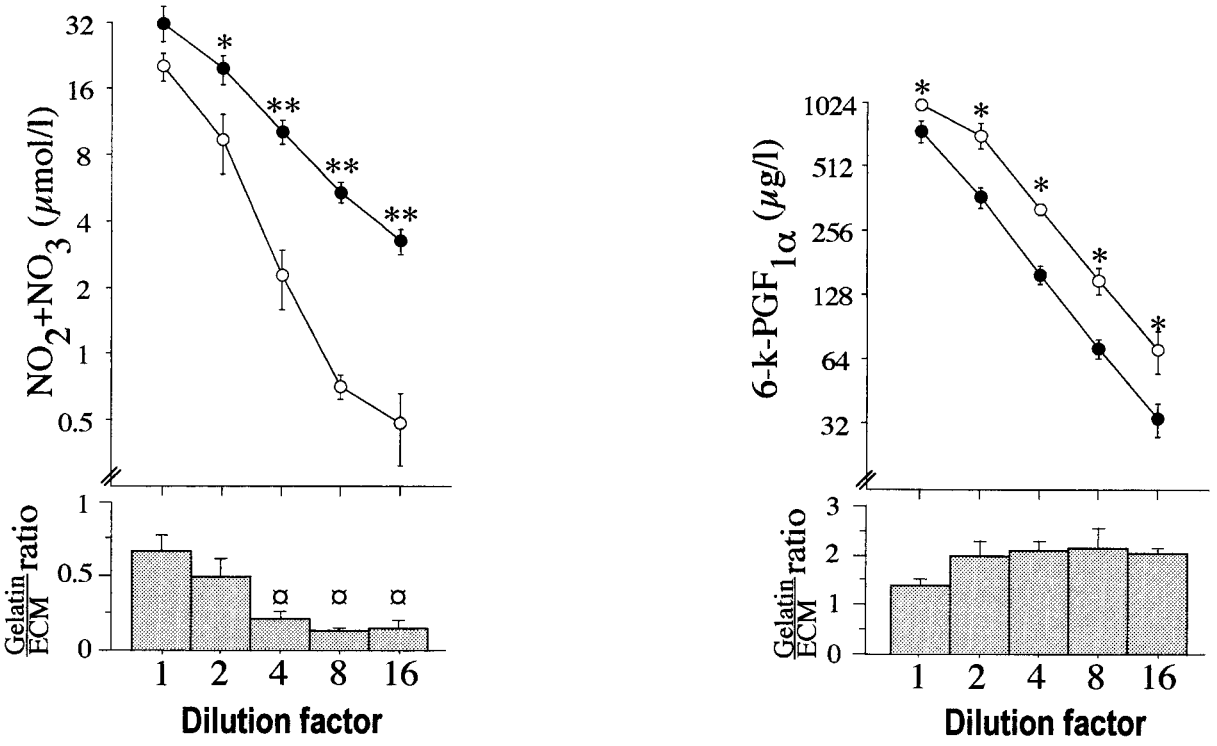

Fig. 5. Effect of plating substratum and gradually decreasing cell plating density on inducible nitric oxide and prostacyclin productions. **Top:** Serial (1:2) dilutions of freshly isolated human umbilical vein endothelial cells were plated on the reconstituted intracellular matrix (ECM), Matrigel (closed circles) or on gelatin substratum (open circles) for 72 h, and then treated for 72 h with interleukin-1β, lipopolysaccharide, and interferon-γ. Production of NO and prostacyclin was measured as described in legend to Figure 3. Mean ± SE from five experiments, $^{**}P < 0.01$, $^{*}P < 0.05$, paired *t*-test, ECM vs. gelatin. **Bottom:** The calculated gelatin/ECM ratios, $^{¤}P < 0.01$ vs. [Dilution factor = 1], paired *t*-test.

lined = 95% confidence interval]. In this presentation, the slope value should be exactly −1.0, if dilution of the cell suspension by half directly halved the production. The fall in the gelatin/ECM ratios calculated in each experiment also demonstrate the gradual loss of inducibility of NO production with decreasing cell density on gelatin substratum (Fig. 5).

Opposite to NO production, PGI_2 production was higher in cells plated on gelatin substratum, but the dilution curves remained parallel and the calculated gelatin/ECM ratios remained unchanged (Fig. 5), clearly demonstrating that cell density as such was not a similarly important regulatory factor for PGI_2 production, as it was for NO production.

DISCUSSION

We have recently shown that when freshly isolated HUVECs were treated with calcium ionophore A23187, they produced large amounts of NO, suggesting that the calcium-dependent endothelial NOS system was functional in freshly isolated HUVECs [Orpana et al., 1996]. We have also observed induction of NO production and iNOS mRNA expression in freshly isolated HUVECs plated on ECM, after treatment with TNF-α, IL-1β, LPS, and IFN-γ, while iNOS mRNA was not expressed in untreated control cells [Orpana et al., 1994, submitted]. It has been difficult to study either calcium-dependent or inducible NO production in cultured HUVECs, because the amount of NO produced by these cells is very low.

Our interest in investigating the role of extracellular matrix and cell density in controlling functions of freshly isolated HUVECs arose from studies performed with hepatocytes and epithelial cells, in which the specialized cell activities were better preserved in cells plated on ECM or at high density, than if the cells were cultured in conventional in vitro conditions [Li et al., 1987; Ben-Ze'ev et al., 1988; Schuez et al., 1988; Streuli et al., 1991]. It is well documented that attachment to the reconstituted ECM, like Matrigel prepared from the natural base-

ment membrane of Engelbrecht-Holm-Swarm tumors [Kleinman et al., 1986], influences the behavior and functions most cell types [Liu et al., 1991; Nathan and Sporn, 1991; Adams and Watt, 1993; Lin and Bissel, 1993; Juliano and Haskill, 1993].

In the present work, freshly isolated HUVECs treated with IL-1β, LPS, and IFN-γ produced large quantities of NO and PGI_2, demonstrating that freshly isolated HUVECs were able to induce NO and PGI_2 productions in vitro even without TNF-α supplementation. We originally used the combination of TNF-α, IL-1β, LPS, and IFN-γ, as this was shown to induce iNOS expression in human hepatocytes [Geller et al., 1993]. However, because the omission of TNF-α did not affect the productions of inducible NO and PGI_2, it was not included in the induction mixture.

The most important finding in the present work was that IL-1β, LPS, and IFN-γ-induced NO and PGI_2 productions were very differently controlled by extracellular matrix and cell density. At high density, cells plated on ECM produced significantly more NO, and the cell-matrix interaction was sufficient in maintaining iNOS inducibility at lower cell plating densities. At high cell density, freshly isolated HUVECs on gelatin substratum produced NO, but decrease in cell density was accompanied by decreasing inducible NO production. The low inducible NO production in cells plated at low density on gelatin substratum, compared with that in similar cells plated at high density on ECM, was associated with lower relative iNOS mRNA levels in these cells. However, the present data only demonstrate that iNOS mRNA levels and inducible NO production behaved similarly in these experimental conditions, and further studies are needed to clarify if this was caused by decreased transcription or lower iNOS mRNA stability. This data also demonstrate the usefulness of competitive RT-PCR for analyzing mRNA levels from samples containing only 1–2 μg of total RNA.

The roles of cell-matrix and cell-cell interactions in controlling functions associated with the differentiated cell phenotype have been widely studied in hepatocyte and epithelial cell in vitro models. Besides plating on reconstituted ECM [Schuetz et al., 1988; Li et al., 1987], freshly isolated hepatocytes and epithelial cells preserved their specialized functions better if the cells were plated at very high cell density [Ben-Ze'ev et al., 1988; Streuli et al., 1991], and in the present study the inducibility of NO production behaved in a very similar way.

On ECM, even at lowest cell densities, freshly isolated HUVECs remained rounded and in cell clusters, and the migration was minimal, while on gelatin substratum these cells actively spread and migrated, suggesting that signalling from ECM prevented freshly isolated HUVECs from changing their phenotype. It is well documented, that cultured HUVECs do not proliferate when plated on Matrigel [Kubota et al., 1988], and in preliminary experiments we observed that the number of freshly isolated HUVECs plated on gelatin substratum in medium supplemented with 2% fetal calf serum did not increase, and their viability remained unchanged. Due to the presence of large quantities of contaminating protein and DNA in Matrigel, we did not attempt to relate NO and PGI_2 productions to cell numbers obtained by measuring cellular protein or DNA content in a well after the incubations. However we have used both the mitochondrial dehydrogenase activity [Orpana et al., 1996] and the cellular DNA content to validate our plating method, and we are certain that the differences observed were not caused by variation in cell numbers. Identical responses to decreasing cell density were observed in experiments where the cell number was kept constant, but the plating area was increased, or the plating area kept constant and the number of cells plated decreased. Inducibility of PGI_2 production appeared to behave in the opposite way compared with NO production, and on ECM cells produced less PGI_2 than on gelatin substratum, especially at low cell densities, although both NO and PGI_2 productions were measured from the same cells.

In conclusion, our data demonstrate that freshly isolated human vascular endothelial cells in vitro responded to treatment with IL-1β, LPS, and IFN-γ by induction of NO, and PGI_2 productions, and that these responses were very differently controlled by the extracellular matrix and cell density. Cell culture conditions, which in other cell types have been shown to aid in maintaining the differentiated phenotype of cells, supported inducible NO production but not inducible PGI_2 production, suggesting that in freshly isolated HUVECs, vasoactive responses to proinflammatory cytokines are under control of complex network of extracellular

signals, among which cell-matrix and cell-cell interactions seem to be important.

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