Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 463-469

γ-INTERFERON COUNTERACTS INTERLEUKIN-1α STIMULATED EXPRESSION OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN HUMAN ENDOTHELIAL CELLS *in vitro*

Johann Wojta[1], Hans Zoellner[2], Marisa Gallicchio[1], John A. Hamilton[2] and Katherine McGrath[1]

[1] Dept. Diag. Haematol., The Royal Melbourne Hospital, Parkville 3050, Australia

[2] Dept. Medicine, University of Melbourne, Parkville 3050, Australia

Received August 28, 1992

The effect of γ-interferon (γ-IFN) on the interleukin-1α (IL-1α) induced stimulation of urokinase-type plasminogen activator (u-PA) expression in human foreskin microvascular endothelial cells (HFMEC) and in human umbilical vein endothelial cells (HUVEC) was investigated. When γ-IFN and IL-1α were added to the cells simultaneously, γ-IFN inhibited the IL-1α induced increase in u-PA antigen production in both HFMEC and HUVEC in a dose dependent fashion, with a maximum inhibitory effect achieved between 2.0 and 20.0U/ml of γ-IFN. Pretreatment of HFMEC with γ-IFN for 1 hour before addition of IL-1α resulted in a significant reduction in u-PA synthesis. However, when HFMEC were pretreated for 8 hours with γ-IFN before the addition of IL-1α the reduction in u-PA production was even more significant. When γ-IFN was added to HFMEC 1 hour after IL-1α, a significant inhibition in u-PA synthesis was seen. In contrast only a slight inhibition in IL-1α induced u-PA production was seen when γ-IFN was added to the cells 8 hours after IL-1α. γ-IFN also inhibited significantly the IL-1α induced increase in u-PA specific mRNA in HUVEC and HFMEC.

Endothelial cells (EC) play a key role in regulating fibrinolysis. Human endothelial cells express both types of plasminogen activators (PA's), the urokinase-type PA (u-PA) and the tissue-type PA (t-PA), as well as their inhibitor, plasminogen activator inhibitor type-1 (PAI-1) (1). The balance between PA's and PAI-1 is regulated by a wide variety of stimuli (2, 3). t-PA seems to be mainly responsible for intravascular clot lysis, whereas processes requiring extracellular proteolysis such as angiogenesis and invasion and metastasis seem to be regulated by u-PA produced by EC or tumor cells, respectively (4, 5).

u-PA expression in human EC is upregulated by cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (6). However, relatively little is known about the downregulation of u-PA in EC. In addition, in most of the studies investigating the effect of cytokines on the fibrinolytic system of EC only individual cytokines have been used. By using TNF-α and γ-IFN in combination it has recently been demonstrated that TNF-α-induced upregulation of u-PA in human umbilical vein EC (HUVEC) was reversed by γ-interferon (γ-IFN) (7). Furthermore γ-IFN has been shown to enhance EC activation by TNF-α but not by IL-1 (8). However, TNF-α and IL-1 show striking similarity in many of their effects on EC (9). For example in EC both cytokines increase the expression of adhesion molecules and affect the fibrinolytic system by increasing the expression of u-PA and PAI-1 (6, 10-12). It was therefore the aim of this study to investigate if γ-IFN would also

0006-291X/92 $4.00

inhibit the IL-1 induced increase in u-PA expression in human EC of micro- and macrovascular origin.

MATERIALS AND METHODS:

Cell culture: Human foreskin microvascular EC (HFMEC) were isolated according to the method of Jackson et al. (13). Briefly, specimens of human neonatal foreskin were cut into 5mm cubes and incubated for 40 minutes at 37°C in 0.3% trypsin, 1% EDTA in Hanks balanced salt solution (HBSS). Thereafter, the tissue was washed three times with HBSS and microvascular segments were squeezed from the cut edges by downward pressure with the flat side of a scalpel blade. The suspension was pushed through a 100µl nylon mesh and centrifuged for 5 min at 200g. The cells were resuspended in 80µl of HBSS containing 5% supplemented calf serum (SCS) (Hyclone; Logan, UT) and incubated with 20µl of Dynabeads (10^8 beads/ml) (Dynal; Norway) coated with *Ulex europaeus* agglutinin (UEA) (Sigma; St. Louis , MO) for 10 min at 4°C with end over end rotation. The beads were collected with a magnetic particle concentrator (MPC) (Dynal; Norway) and the supernatant was discarded. Thereafter, EC bound to the beads were washed five times by resuspending with 2ml of HBSS containing 5% SCS, mixing by end over end rotation for 1 min followed by separation using the MPC for 1 min. Thereafter, the EC were resuspended in M199 ccontaining 20% SCS, 50µg/ml endothelial cell growth supplement (ECGS), prepared as described by Maciag et al. (14) and 5U/ml heparin (Fisons, Australia) and plated in petri dishes (100mm) (Costar; Cambridge,MA) coated with gelatin (Biorad; Richmond, CA). After 5 to 8 days HFMEC reached confluence and were subculturded using a split ratio of 1:3. HUVEC were isolated by mild collagenase treatment following the method of Gimbrone et al. (15). The cells were confirmed as endothelial by their "cobblestone" morphology, positive staining with anti-von Willebrand Factor VIII antibodies and uptake of immunofluorescent labelled acetylated LDL as described (16). All EC used in this study were between passge 2 and 4.

Cytokines: Recombinant human γ-IFN (rh γ-IFN), expressed in *E. coli*, was a gift from Dr. E. Hochuli, Hoffmann-La Roche, Switzerland, and had a specific activity of $2.7x10^7$U/mg. Recombinant human IL-1 α (rh IL-1α), expressed in *E. coli*, was a gift from Dr. P. Lomedico, Hoffmann-La Roche, Nutley, NJ, and had a specific activity of $2.5x10^7$U/mg. Recombinant human TNF-α (rh TNF-α), expressed in *E. coli* , was obtained from Boehringer- Ingelheim, Sydney, and had a specific activity of $6x10^7$U/mg.

Cytokine treatment: EC, grown to confluence in 6 or 24 well dishes (Costar; Cambridge, MA) were rinsed twice with HBSS containing 10mM Hepes. Thereafter, M199 containing 5µg/ml insulin, 5µg/ml transferrin and 5ng/ml sodium selenite (M199-ITS) without or with variuos concentrations of rh γ-IFN, rh IL-1α or rh TNF-α or combinations of rh γ-IFN and rh IL-1α or rh γ-IFN and rh TNF-α, respectively, was added to the cells. At time points indicated conditioned media (CM) of such treated cells were collected, centrifuged at 300xg and stored at -70°C. At the same time points, the EC monolayers were rinsed with HBSS and lysed with phosphate buffered saline, pH=7.4, (PBS) containing 0.1% Triton X-100. Cell lysates (CL) were centrifuged and stored as described above.

Assays for u-PA, t-PA and PAI-1 antigen: u-PA, t-PA and PAI-1 antigen, respectively, in CM and CL was determined by specific ELISA's, employing monoclonal antibodies specific for the respective antigens (16-18).

Northern blot analysis: Northern blot analysis was performed using HFMEC and HUVEC, respectively, grown in 6 well plates with each well having a surface area of $9.6cm^2$. Total cellular RNA from triplicates was extracted and pooled by the guanidine isothiocyanate/cesium chloride method (19). Agarose gel electrophoresis was used to size fractionate RNA, using a formaldehyde containing 1.4% (w/v) agarose gel. RNA was transferred from the gel to a Bio Trace HP membrane (Gelman Sciences; Ann Arbor, MI). Hybridisations were performed overnight, using cDNA fragment probes encoding u-PA (kindly provided by Dr. R. Miskin, Rehovot) and the "housekeeping" gene GAPDH (kindly provided by Dr. O. Bernard, Melbourne). Probes were labelled by random priming with ^{32}P ATP (Amersham; Australia) and added to a 50% (v/v) formamide hybridisation buffer to achieve a minimum specific activity of $2x10^6$ cpm/ml. Following hybridisation, membranes were washed in reducing concentrations of SSC to a final concentration 0.1xSSC/0.1%SDS (w/v) at 65°C. Autoradiography was performed using Kodak XAR-5 film at -70°C. Films were scanned using an LKB 2202 Ultro Scan Laser Densitometer (LKB; Sweden) in order to quantify differences in mRNA expression.

Statistical analysis: Data were compared statistically by using a student's t-test for paired observations.

RESULTS:

As can be seen from Fig. 1, panel A, the production of u-PA antigen by HFMEC was not influenced when such cells were incubated with increasing concentrations of rh γ-IFN. When HFMEC were incubated with 100.0U/ml rh IL-1α, u-PA antigen production was stimulated up to five-fold. Higher concentrations of rh IL-1α did not result in a further increase in u-PA antigen (data not shown). When rh IL-1α was added to the cells at the above concentration together with increasing concentrations of rh γ-IFN, u-PA antigen decreased significantly in a dose dependent manner with maximum effects (approximately 55% reduction) achieved between 2.0 and 20.0U/ml of rh γ-IFN. It is, noteworthy, however, that u-PA antigen levels were not reduced to control levels in IL-1α stimulated cells, even in the presence of 200.0 U/ml rh γ-IFN.

Similar results were obtained when HUVEC were incubated with rh IL-1α or γ-IFN or a combination of both (Fig. 1, panel B). u-PA antigen levels in unstimulated HUVEC, however, were below the detection limit of the ELISA used (<0.2ng/ml). No u-PA antigen was detected in CL of either cell type tested.

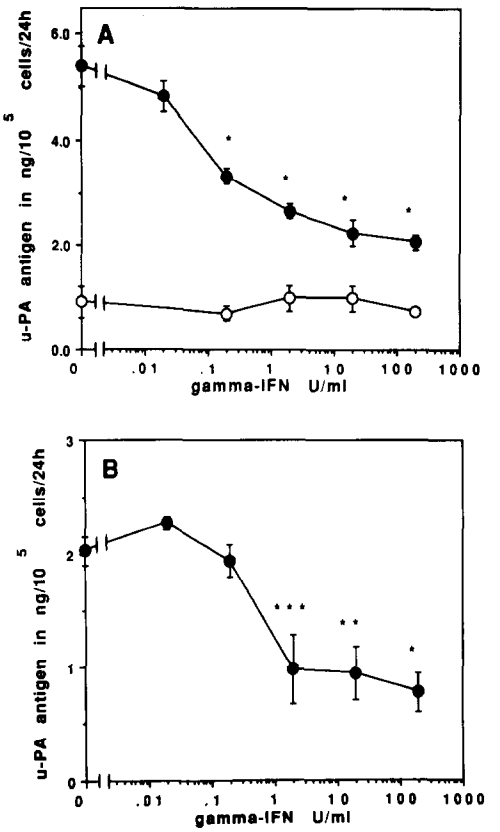

Figure 1: Influence of γ-IFN on IL-1α induced-increase in u-PA antigen produced by HFMEC (panel A) or HUVEC (panel B). Confluent monolayers of HFMEC or HUVEC, respectively, were incubated for 24 hrs without or with various concentrations of rh γ-IFN in the absence (open symbols) or presence of rh IL-1α at a concentration of 100.0U/ml (full symbols). No u-PA antigen was detectable in the conditioned media (CM) of HUVEC in the absence of rh IL-1α (lower detection limit of the ELISA used: 0.2ng/ml). CM was collected and processed, and u-PA antigen in the samples was determined as outlined under Materials and Methods. Values are given as ng/10^5 cells/24hrs and represent means±S. D. of three independent wells.
* $p<0.001$, ** $p<0.005$, *** $p<0.01$ (as compared to IL-1α treated cells).

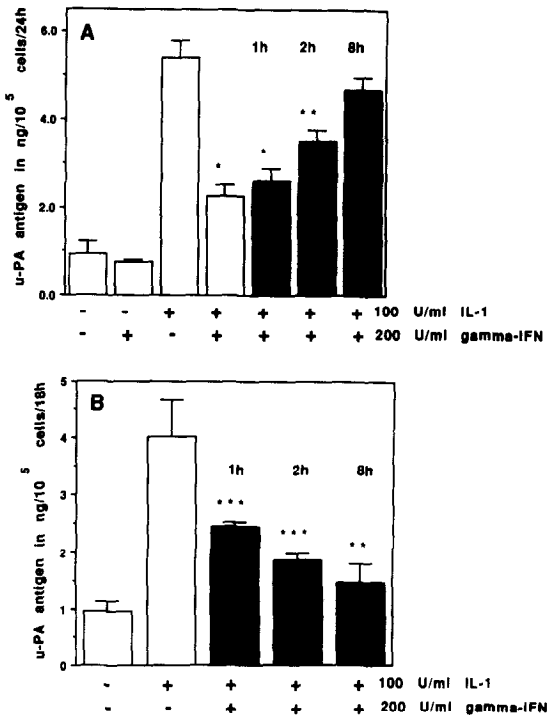

Figure 2: Influence of delayed addition of γ-IFN on IL-1α-induced increase in u-PA antigen produced by HFMEC (panel A). Confluent monolayers of HFMEC were incubated for 24 hrs without or with rh γ-IFN (200.0U/ml) in the presence or absence of rh IL-1α (100.0U/ml) (open bars). To some of the cells rh γ-IFN (200.0U/ml) was added 1 hr, 2hrs or 8hrs after the addition of rh IL-1α (100.0U/ml) (full bars). CM of these cells were harvested and processed as described in the Materials and Methods section. Influence of pretreatment of HFMEC with γ-IFN on IL-1α-induced increase in u-PA antigen produced by HFMEC (panel B). Confluent monolayers of HFMEC were incubated for 8hrs with control medium (M199-ITS) (open bars) or with rh γ-IFN (200.0U/ml) for 1hr, 2hrs or 8hrs, respectively (full bars). Thereafter, the monolayers were rinsed with HBSS and rh IL-1α (100.0U/ml) or M199-ITS without any addition was added to the cells. After 18 hrs CM of these cells were harvested and processed as desribed in the Materials and Methods section.

u-PA antigen in the samples was determined as outlined under Materials and Methods. Values are given as ng/10^5 cells/24hrs (panel A) or 18hrs (panel B) and represent means±S. D. of three independent wells.

* $p<0.001$, ** $p<0.005$, *** $p<0.02$ (as compared to IL-1α treated cells).

When rh γ-IFN (200.0U/ml) was added to HFMEC 1 hour after rh IL-1α (100.0U/ml) a significant reduction in u-PA antigen was still seen (IL-1α: 5.4±0.4ng/10^5 cells/24 hrs, IL-1α+γ-IFN: 2.6±0.3ng/10^5 cells/24 hrs; $p<0.001$). When γ-IFN was added to the cells 2 hours after IL-1α the reduction in u-PA antigen was still significant (IL-1α+γ-IFN: 3.5±0.3ng/10^5 cells/24 hrs; $p<0.005$). However, when γ-IFN was added to the cells 8 hours after the addition of IL-1α, no significant reduction in u-PA antigen was observed (IL-1α+γ-IFN: 4.7±0.3ng/10^5 cells/24 hrs) (Fig. 2, panel A).

In a separate experiment HFMEC were pretreated for 1, 2 or 8 hours with rh γ-IFN (200.0U/ml). Thereafter the cells were rinsed twice with HBSS and fresh medium containing rh IL-1α (100U/ml) was added. IL-1α at the same concentration or control medium (M199-ITS) without any additions was added to untreated HFMEC. CM from these cells were collected after 18 hours. As can be seen from Fig. 2, Panel B, pretreatment of HFMEC with γ-IFN for the respective time periods resulted in a significant reduction in u-PA antigen (IL-1α without pretreatment with γ-IFN:

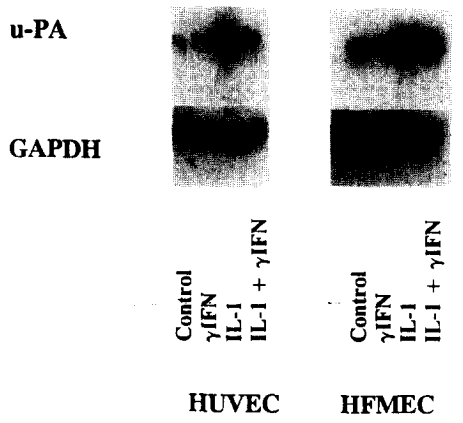

Figure 3: Influence of γ-IFN on IL-1α-induced increase in u-PA specific mRNA in HFMEC and HUVEC. (Northern analysis). HFMEC or HUVEC were incubated for 8 hrs without or with rh γ-IFN (200.0U/ml), or rh IL-1α (100.0U/ml) or a combination of both cytokines at the respective concentration. Northern blots of RNA from these cells were hybridised to cDNA probes for u-PA and GAPDH as outlined in the Materials and Methods section.

4.0±0.4 ng/10^5 cells/18hrs, IL-1α after 1 hr pretreatment with γ-IFN: 2.5±0.1ng/10^5 cells/18hrs; $p<0.02$, IL-1α after 2 hrs pretreatment with γ-IFN: 1.9±0.3ng/10^5 cells/18hrs; $p<0.02$, IL-1α after 8 hrs pretreatment with γ-IFN: 1.5±0.2ng/10^5 cells/18hrs: $p<0.005$).

As can be seen from Fig. 3, the reduction of IL-1α stimulated u-PA expression by γ-IFN was also seen on the level of specific mRNA expression in both HFMEC and HUVEC, as demonstrated by Northern analysis. In the presence of rh IL-1α (100.0U/ml), u-PA mRNA increased up to five-fold in HFMEC and up to ten-fold in HUVEC, respectively, as determined by densitometry. This increase in u-PA mRNA was reduced almost to control values, when HFMEC or HUVEC, respectively, were treated with rh IL-1α (100.0U/ml) and rh γ-IFN (200.0U/ml) simultaneously.

The effect of γ-IFN on IL-1α stimulated u-PA expression was unlikely to be due to lipopolysaccharide (LPS) contamination of the cytokines used, since it was observed in the absence, as well as in the presence of polymyxin B (data not shown).

As described by Niedbala and Stein Picarella (7) γ-IFN also inhibited the TNF-α-induced stimulation of u-PA expression in HFMEC and HUVEC (data not shown).

Table 1: Influence of γ-IFN on IL-1α-induced effects on u-PA, PAI-1 and t-PA production by HFMEC and HUVEC

	u-PA		PAI-1		t-PA	
	HFMEC	HUVEC	HFMEC	HUVEC	HFMEC	HUVEC
control	1.6±0.4	<0.2	404±51	378±44	1.5±0.1	2.2±0.2
200.0U/ml γ-IFN	1.2±0.1	<0.2	371±69	270±31	1.1±0.3	2.2±0.2
100.0U/ml IL-1α	6.9±1.5	3.8±0.3	679±83	820±89	0.9±0.3	1.7±0.1
200.0U/ml γ-IFN +100.0U/ml IL-1α	2.9±0.3#	1.8±0.5#	571±40	692±31	1.0±0.1	1.9±0.3

Confluent monolayers of HFMEC or HUVEC were incubated for 24 hrs without or with rh γ-IFN (200.0U/ml), or rh IL-1α (100.0U/ml) or a combination of both cytokines at the respective concentration. CM of these cells were harvested, processed and u-PA, PAI-1 and t-PA antigen was determined as described in the Materials and Methods section. u-PA, PAI-1 and t-PA antigen values are given as ng/10^5 cells/24hrs and represent means±S. D. of three independent wells.
# $p<0.01$ (as compared to IL-1α treated cells).

IL-1α stimulated PAI-1 expression was also reduced by concomitant incubation of HFMEC or HUVEC, respectively, with rh IL-1α (100.0U/ml) and rh γ-IFN (200.0U/ml). This reduction, however, was moderate when compared to the effect of γ-IFN on IL-1α-induced stimulation of u-PA expression (see Table 1). t-PA expression was not affected by such treatment (Table 1).

DISCUSSION:

Human EC in culture produce only low levels of u-PA. However, u-PA expression can increase dramatically with passage (20). Furthermore, cytokines like IL-1 or TNF-α, or growth factors like basic fibroblast growth factor (bFGF), have been shown to increase significantly u-PA expression in EC (4, 6). u-PA is thought to be involved mainly in extracellular, spatially controlled proteolysis, via binding to its receptor, which is present on the surface of many cell types, including EC (21, 22). This concept is supported by the fact that u-PA expression correlates with migration in EC (23). On the other hand, relatively little is known about the factors downregulating u-PA expression in EC. Furthermore most of the studies investigating the regulation of the plasminogen activating system of EC by growth factors or cytokines have used only individual cytokines or growth factors. By using TNF-α and γ-IFN in combination, it has been shown recently that u-PA upregulation in HUVEC caused by TNF-α was inhibited by γ-IFN (7). γ-IFN has been also shown to act in combination with TNF-α to reorganise EC monolayers and to enhance EC activation by TNF-α but not by IL-1 (8,24). In many of their effects, however, TNF-α and IL-1 show a striking similarity (9).

In this report we present evidence that γ-IFN also counteracts the IL-1α-induced upregulation of u-PA antigen in human EC of micro- and macrovascular origin. This effect was dose dependent, with a maximum effect achieved between 2.0 and 20.0U/ml of γ-IFN. Simultaneous incubation of HFMEC or HUVEC, respectively, with both γ-IFN and IL-1α resulted in a significant reduction in the levels of u-PA specific mRNA, when compared to levels of u-PA mRNA in the respective cells treated with IL-1α alone. In experiments in which EC were treated with IL-1α, followed by the delayed addition of γ-IFN, it was observed that γ-IFN was able to counteract the IL-1α-induced stimulation in u-PA expression, when added to the cells one or two hours after IL-1α but not 8 hours after IL-1α. This suggests that the presence of γ-IFN is required during early ligand/receptor signal transducing events. Pretreatment of EC with γ-IFN for eight hours made the cells refractory to IL-1α stimulation of u-PA expression when such pretreated cells were then exposed to IL-1α for 18 hours in the absence of γ-IFN. These results are similar to the data published by Niedbala and Stein Picarella, demonstrating the inhibition of TNF-α stimulated u-PA expression in EC by γ-IFN (7). This similarity could suggest a common mechanism by which γ-IFN counteracts the stimulation of u-PA expression in EC brought about by TNF-α as well as by IL-1α.

The IL-1α-stimulated increase in PAI-1 antigen was only slightly decreased by γ-IFN, whereas t-PA expression was not affected in the presence of γ-IFN.

In conclusion, our data provide evidence that γ-IFN, in addition to its known inhibitory effect on the TNF-α-induced stimulation of u-PA expression (7), can counteract the IL-1α-stimulated increase in u-PA expression in human EC of micro- and macrovascular origin. Thus, the concomitant presence of γ-IFN and IL-1α during inflammatory responses could contribute to the modulation of processes requiring EC-mediated extracellular proteolysis by u-PA such as EC migration, neovascularisation and wound healing.

ACKNOWLEDGMENTS:

The authors wish to thank Dr. B. R. Binder (Vienna) for the monoclonal antibodies and the respective standard preparations used in the ELISA's for u-PA, t-PA and PAI-1, Dr. R. Miskin (Rehovot) for the u-PA probe, Dr. O. Bernard (Melbourne) for the GAPDH probe, the staff of the Delivery Suite in The St. Vincent's Private Hospital and The Freemason's Daycare Hospital for collecting foreskin specimens and the staff of the Delivery Suite of The Essendon and District Memorial Hospital for collecting umbilical cords for the preparation of the respective endothelial cells. This work was supported by a grant from The National Heart Foundation of Australia and by a grant from The Austrian Fund for the Promotion of Scientific Research (P7617 MED) to Dr. Wojta and by a Program Grant from The National Heart Foundation of Australia to John A. Hamilton.

REFERENCES:

1. Loskutoff, D. J., and Curriden, S. A. (1990) Ann. NY. Acad. Sci. 598, 238-247.
2. Van Hinsbergh, V. W. M. (1988) Haemost. 18, 307-327.
3. Schleef, R. R., and Loskutoff, D. J. (1988) Haemost. 18, 328-341.
4. Montesano, R., Vassalli, J. D., Baird, A., Guillemin, R., and Orci, L. (1986) Proc. Nat. Acad. Sci. USA 83, 7297-7301.
5. Dano, K. Behrendt, N., Lund, L. R., Ronne, E., Pollanen, J., Salonen, E. M., Stephens, R. W., Tapiovaara, H., and Vaheri, A. (1989) In Cancer and Metastasis: Molecular and cellular biology, host immune responses and perspectives for treatment (V. Schirrmacher, and R. Schwartz-Albiez, eds.), pp. 98-107. Springer Verlag, Berlin.
6. Van Hinsbergh, V. W. M., Van den Berg, E. A., Fiers, W., and Dooijewaards, G. (1990) Blood 75, 1991-1998.
7. Niedbala, M. J., and Stein Picarella, M. (1992) Blood 79, 678-687.
8. Doukas, J., and Pober, J. S. (1990) J. Immunol. 145, 1727-1733.
9. Pober, J. S., and Cotran, R. S. (1990) Physiol. Rev. 70, 427-451.
10. Pober, J. S., Gimbrone, M. A., Lapierre, L. A., Mendrick, D. L., Fiers, W., Rothlein, R., and Springer, T. (1986) J. Immunol. 137, 1893-1896.
11. Pober, J. S., Bevilacqua, M. P., Mendrick, D. L., Lapierre, L. A., Fiers, W. and Gimbrone, M. A. (1986) J. Immunol. 136, 1680-1687.
12. Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi-Russo, G., and Lobb, R. (1989) Cell, 59, 1203-1211.
13. Jackson, C. J., Garbett, P. K., Nissen, B., and Schrieber, L. (1990) J. Cell Sci. 96, 257-262.
14. Maciag, T., Hoover, G. A., Stemerman, M. B., and Wellnstein, R. (1984) In Biology of endothelial cells (E. A. Jaffe, Ed.), pp. 87-96. Martinus Nijhoff Publ., Dortrecht.
15. Gimbrone, M. A., Cotran, R., and Folkman J. J. (1974) J. Cell Biol. 60, 673-684.
16. Wojta, J., Holzer, M., Hufnagl, P., Christ, G., Hoover, R. L., and Binder, B. R. (1991) Amer. J. Pathol. 139, 911-919.
17. Wojta, J., Binder, B. R., Huber, K., and Hoover, R. L. (1989) Thromb. Haemost. 61, 289-293.
18. Wojta, J., Turcu, L., Wagner, O. F., Korninger, C., and Binder, B. R. (1987) J. Lab. Clin. Med. 109, 665-671.
19. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Ruttner, W. J. (1979) Biochemistry 18, 5294-5299.
20. Van Hinsbergh, V. W. M., Binnema, D., Scheffer, M. A., Sprengers, E. D., Kooistra, T., and Rijken, D. C. (1987) Arteriosclerosis 7, 389-400.
21. Blasi, F., Vassalli, J. D., and Dano, K. (1987) J. Cell Biol. 104, 801-804.
22. Miles, L., Levin, E. G., Plesica, J., Collen, D., and Plow, E. F. (1988) Blood 72, 628-635.
23. Pepper, M. S., Vassalli, J. D., Montesano, R., and Orci, L. (1987) J. Cell Biol. 105, 2535-2541.
24. Stolpen, A. H., Guinan, E. C., Fiers, W., and Pober, J. S. (1986) Amer. J. Pathol. 123, 16-24.