Urokinase plasminogen activator induces smooth muscle cell migration: key role of growth factor-like domain

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Received 17 July 1997

Abstract **We defined the role of urokinase plasminogen activator (uPA) and its growth factor-like domain (GFD) in stimulating smooth muscle cell (SMC) migration. Recombinant uPA (r-uPA) stimulated migration approximately 3-fold whilst the recombinant uPA mutant containing an altered GFD (r-uPAmut) was ineffective. Both uPA variants bound to the same high affinity receptor in a competitive manner. FGF-2- and PDGF-BB-induced migration was also dependent on uPA, their effects being antagonized by addition of a uPA-neutralizing antibody or the r-uPAmut. Thus r-uPA is chemotactic for SMC and stimulation of cell migration by PDGF-BB and FGF-2 is dependent on uPA. The GFD of uPA is essential for its chemotactic effects.**

Key words: Urokinase; Growth factor; Smooth muscle cell migration

1. Introduction

Migration of vascular smooth muscle cells (SMC) is fundamental to the development of diffuse intimal thickenings, atherosclerotic lesions [1], and vessel wall remodelling [2,3]. SMC migration is regulated by vascular-derived growth factors [4–7], which also appear to control the ability of migrating cells to produce various proteolytic enzymes [7–9]. Increase of proteolytic activity within the vessel wall promotes atherosclerotic plaque instability and thrombolysis at the luminal surface of atherosclerotic lesions [10]. Plasminogen activators (PA), which release plasmin, possessing the broad substrate specificity, have crucial roles in such processes [4,11]. Urokinase PA (uPA), interacting with its receptor (uPAR), either alone or complexed with PA inhibitor (PAI), induces highly regulated proteolysis associated with cell migration [12] and also has the potential to participate in cell signalling processes required for migration [13–15]. In this study we demonstrate that uPA is highly chemotactic for SMC and the ability of FGF-2 to induce migration can be attributed to the chemotactic activity of uPA; PDGF-induced cell migration also exhibits a dependency on uPA. Chemotactic activity of uPA is highly dependent on the growth factor-like domain (GFD) and antagonism of uPA at its receptor by a recombinant uPA containing a mutated growth factor-like domain (r-uPAmut) prevents this ability of uPA to induce SMC to migrate.

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Abbreviations: PDGF BB, platelet-derived growth factor BB; FGF-2, basic fibroblast growth factor; uPA, urokinase plasminogen activator; uPAmut, uPA mutant; GFD, growth factor-like domain of uPA; uPAR, receptor of uPA; PAI-1, plasminogen activator inhibitor type 1; SMC, smooth muscle cell(s)

2. Materials and methods

2.1. Materials

Chemicals and products for cell culture were purchased either from Sigma (St. Louis, MO), Pharmacia LKB Biotechnology (Uppsala, Sweden), Merk (Darmstadt, Germany) or Serva (Heidelberg, Germany). $Na^{125}I$ was obtained from Amersham International (Little Chalfont, GB). PDGF-BB and FGF-2 were purchased from Sigma.

2.2. Recombinant pro-uPA-mutant and pro-uPA

Preparation of the r-uPA containing an altered growth factor-like domain (r-uPAmut) involved transformation of *E. coli* (K-12JM109 strain) with a pUABC plasmid (4.11 kbp) containing an *Xba*I-*Bgl*I-fragment (1.39 kbp) with an origin of replication, promotor and operator sequences of the *lac*-operon from the pUC19 vector; the *Bgl*I-*Pst*I-fragment (1.28 kbp), containing β-lactamase from the pUC18 vector and *Bgl*I-*Xba*I-cDNA fragment (1.44 kbp) of human urokinase gene. The r-uPAmut is distinct from the wild type of pro-uPA (r-uPA) with the first N-terminal 24 amino acids, **SNELHQVPSNCDC-LNGGTCVSNKY**, replaced by **ITPSLHACRSTLD** [16]. The pUABC plasmid encoding r-uPAmut consisted of pUC18 and pUC19 vectors and human urokinase cDNA fragments *Sca*I-*Eco*RI (0.42 kbp), *Eco*RI-*Eco*RI (0.42 kbp) and *Eco*RI-*Pst*I (0.6 kbp), obtained by immuno- and oligonucleotide screening of cDNA HL 1011b library on the base of gt11 (CLONTECH Laboratories, Inc.). Its construction included digestion of pUC18 with *Eco*RI and *Pst*I, followed by ligation of the fragments with the *Eco*RI-*Pst*I-fragment (0.6 kbp) of the urokinase cDNA using bacteriophage T4 DNA-ligase. Subsequently the plasmid pUC18_uro1039–1642 was isolated from the *E. coli* transformed cells. The pUC19_uro204–623 plasmid was prepared by digestion of pUC19 with *Xba*I, followed by Klenow fragment DNA polymerase reaction, cutting with *Eco*RI and ligation with the *Sca*I-*Eco*RI-fragment (0.42 kbp) of the uPA cDNA. Construction of the pUC19_uro204_623/1039–1642 plasmid was achieved through cleavage of pUC18_uro1039–1642 and pUC19_uro204–623 with *Eco*RI and *Bgl*I, followed by ligation of the resultant 0.77 kbp and 1.1 kbp fragments of the pUC18_uro1039–1642 plasmid with the 1.8 kbp fragment of the pUC19_uro204–623 plasmid. The resultant pUC19_uro204–623/1039–1642 plasmid was then digested with *Eco*RI, dephosphorylated and ligated with the uPA (*Eco*RI-*Eco*RI; 0.42 kbp) cDNA fragment. Its structure was confirmed by *Bam*HI and *Bam*II digestion. The plasmid containing wild type of pro-uPA (r-uPA) cDNA was similarly constructed using the urokinase *Taq*I-*Eco*RI (0.47 kbp) cDNA fragment and ligation with the following two oligonucleotides: AATTCGAACGAACTGCATCAGGTTCC-GT and CGACGGAACCTGATGCAGTTCGTTCG. Recombinant uPA and r-uPAmut primary structures were confirmed by DNA sequencing and N-terminal amino acid sequencing of peptides, purified by methods including denaturation with urea, reconstitution and affinity chromatography. Their purity was greater than 90% and following activation by plasmin, their activities ranged between 1–1.2×10^5 U/mg protein.

PII S0014-5793(97)00993-9

2.3. Preparation of anti-uPA antibodies

The anti-uPA polyclonal antibodies were prepared by immunization of the rabbits with human urinary urokinase, purified as described previously [17]. The anti-uPA IgG exhibited a high affinity for uPA and recognized all forms of uPA, including urinary human uPA, human recombinant uPA variants, rat and bovine uPA, but not tissue plasminogen activator or plasminogen.

2.4. Cell culture

Rat aortic medial SMC isolated as described previously [18], were cultured at 37°C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM/FCS), containing 4 mM glutamine, 20 mM HEPES (pH 7.3), 100 U/ml penicillin, 100 U/ml streptomycin and 10% FCS. Immunohistochemical staining confirmed that all first passage SMC expressed smooth muscle α-actin but not Factor VIII. SMC were used between passage 8 and 15.

2.5. Chemotaxis assay

Chemotactic assays were performed as previously described [19] using a micro-Boyden chamber apparatus (Neuroprobe Inc., Cabin John, MD). Briefly, confluent SMC, serum-deprived for 24 h in DMEM containing 0.1% BSA (DMEM/0.1%BSA), were briefly subjected to trypsin/EDTA, washed and resuspended in DMEM/0.1%BSA. Between 3×10^4 and 5×10^4 SMC were then placed into the 50 μl upper wells of the Boyden chamber fitted with a PVP-free 8 μm pore membrane (Nuclepore Corp., Palo Alto, CA), coated with collagen type I (100 μg/ml) (Vitrogen 100, Celtrix Pharmaceuticals Inc., Santa Clara, CA). Growth factors or the different forms of uPA in DMEM/0.1% BSA were added to in the lower chambers. In some instances (see Section 3: Results) the suspended SMC were preincubated with either the polyclonal uPA antibody (120 μg/ml), r-uPA or r-uPAmut for 1 h at 37°C prior to their placement into the upper wells of the Boyden Chamber. Cells in the Boyden chamber were incubated for 4 h at 37°C in the CO_2-incubator. The SMC which migrated through the pores of the membrane then were fixed in methanol, and stained with Dif Quick stain (Baxter) (the non-migrated cells were scraped off before staining). The membranes were then scanned using the 'Deskscan' and 'NIH Image' programs. Intensities from such scans represent the number of migrated SMC and are reported as peak area of the scanned field of stained SMC, expressed as percentage of control, i.e. migrated SMC not exposed to growth factors or the uPA variants.

2.6. ^{125}I-r-uPA binding to SMC

Recombinant uPA and r-uPAmut were iodinated using $Na^{125}I$ and Iodogen at room temperature [20]. The SMC were incubated at 4°C with shaking for 3 h with ^{125}I-r-uPA or ^{125}I-r-uPAmut either without or with 100-fold excess of cold r-uPA or r-uPAmut for determination of the non-specific binding. In the competitive binding studies varying concentrations of 'cold' uPA or uPAmut (see Section 3: Results) were added together with either 3 nM ^{125}I-r-uPA or ^{125}I-r-uPAmut. With the incubation completed, cells were washed with ice-cold PBS/0.1%BSA (×5) and lysed. Radioactivity in the cell lysates was measured using a gamma counter.

Table 1
Increases in SMC migration, stimulated by r-uPA in the absence and presence of the r-uPAmut

Recombinant uPA (nM)	SMC migration (fold stimulation)	
	Recombinant uPAmut	
	0 nM	100 nM
0	1.00	0.82 ± 0.05*
20	1.81 ± 0.06	1.29 ± 0.07*
200	1.92 ± 0.11	1.54 ± 0.1*
500	2.07 ± 0.15	1.93 ± 0.17

SMC migration was determined in the micro Boyden chamber as described in the Methods in Section 2 and represents the means ± S.E.M. of 4 to 5 experiments. Fold stimulation represents increases in the number of migrating SMC above that observed in DMEM/0.1%. *$p < 0.05$ from SMC migration in the absence of r-uPAmut.

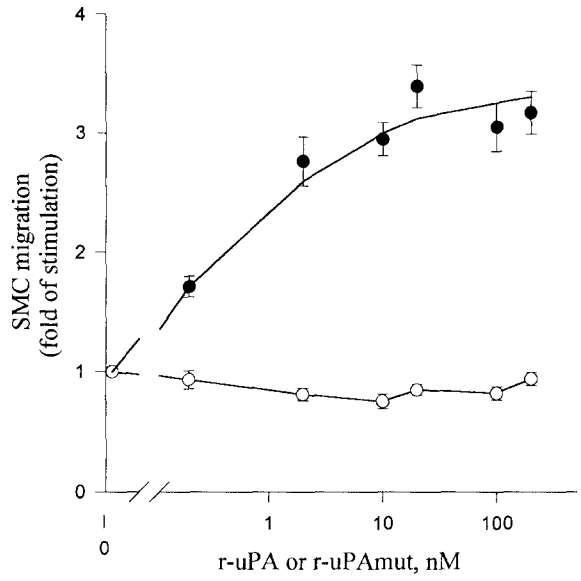

Fig. 1. Concentration-dependent effects of r-uPA (●) and r-uPAmut (○) on SMC migration. SMC were exposed to concentration gradients of r-uPA and r-uPAmut in a micro Boyden chamber and SMC migrated through the 8 μm pores of the membrane measured 4 h later. Results are expressed as fold stimulation relative to SMC migration in the absence of the uPA variants and are mean ± S.E.M. of 4 or 5 experiments.

3. Results

3.1. Recombinant uPA, uPAR and SMC migration

Recombinant uPA elicited a dose-dependent chemotactic response in SMC with maximal (~3-fold) increases at approximately 10 nM ($p < 0.01$); half maximal effects occurred at approximately 5 nM (Fig. 1). Addition of excess anti-uPA antibody inhibited the r-uPA-induced, but not the basal level of SMC migration (data not shown).

^{125}I-r-uPA bound to the SMC with high affinity, similar to its EC_{50} for inducing SMC migration. Scatchard analysis of the binding isotherms indicated an affinity of binding (K_d) averaging 7.2 ± 2.1 nM. Binding sites appeared to be of a single type, averaging 3×10^5 sites/cell (Fig. 2). Recombinant ^{125}I-uPAmut also bound to the uPAR on SMC ($K_d \sim 16.3 \pm 1.6$ nM; $B_{max} \sim 3\times10^5$ sites/cell) (data not shown). In addition, r-uPAmut competed with ^{125}I-r-uPA for binding to uPAR as well as r-uPA competed with bound ^{125}I-r-uPAmut, confirming interactions of r-uPA and r-uPAmut with the same uPAR on SMC (Fig. 2).

However, in contrast to r-uPA, the r-uPAmut (1–100 nM) did not induce the SMC to migrate in the Boyden Chamber (Fig. 1). To determine whether the r-uPAmut might be acting as a uPAR antagonist, we examined its ability to inhibit r-uPA-induced SMC migration. As shown in Table 1, the r-uPAmut (100 nM) inhibited SMC migration stimulated by the r-uPA in a competitive manner. Thus, binding of ligand to uPAR is not sufficient for activation of SMC migration and the intact structure of 1–24 amino acids in N-terminus of the uPA GFD is essential for chemotactic stimulation of SMC.

3.2. FGF-2- and PDGF-BB-induced chemotaxis are dependent on uPA-uPAR interactions

PDGF-BB is a potent chemoattractant whose effects on

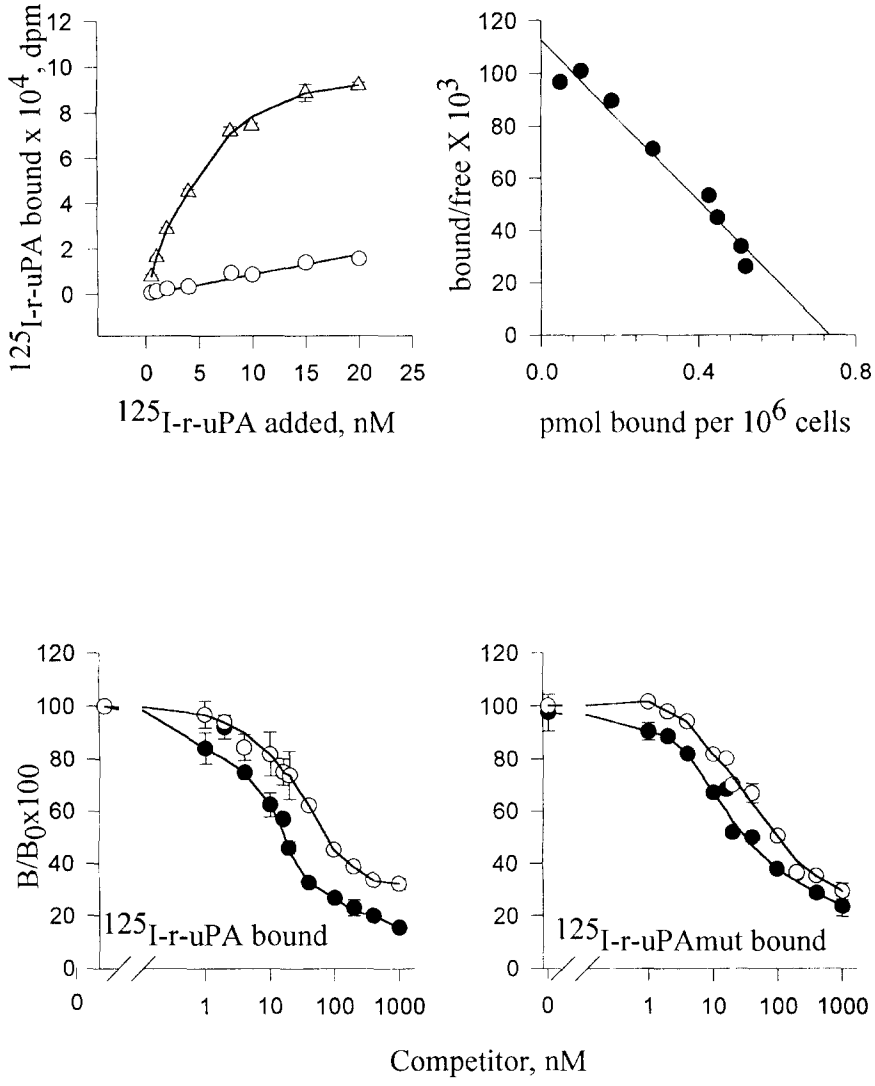

Fig. 2. Binding characteristics of r-u-PA and r-uPAmut to rat SMC. Upper panels: Concentration-dependent ^{125}I-r-uPA binding to SMC at 4°C in the absence (△) and presence of 100-fold molar excess of r-uPA (○) during a 3 h incubation (left). Scatchard plot of ^{125}I-r-uPA specific binding to the SMC (right). Values are given as the mean of triplicate determinations and represent the counts of ^{125}I-r-uPA with specific radioactivity 7.25×10^5 dpm/pmol, bound per 10^6 cells. Lower panels: Competitive binding between 3 nM ^{125}I-r-uPA and varying concentrations of r-uPA (●) or r-uPAmut (○) to SMC at 4°C (left). The right panel represents competition between 3 nM ^{125}I-r-uPAmut bound to the SMC and r-uPA (●) or r-uPAmut (○). All results are the means ± S.E.M. of at least 4 experiments. B_0 indicates total binding in the absence of unlabeled competitor and was 5% and 2.5% of total counts added, for ^{125}I-r-uPA or ^{125}I-r-uPAmut, respectively. B indicates ^{125}I-r-uPA or ^{125}I-r-uPAmut binding in the presence of a given concentration of unlabelled competitor. Nonspecific binding values were 10.8 ± 2.7% of total binding for ^{125}I-r-uPA bound and 21.3 ± 2.1% for ^{125}I-r-uPAmut bound.

SMC are considered to be dependent on FGF-2 [21]. Since endothelial cell migration induced by FGF-2 appears dependent on plasminogen activator synthesis [22,14], we also investigated the extent to which SMC migration induced by either PDGF-BB or FGF-2 was dependent on uPA. PDGF-BB and FGF-2 (both 50 ng/ml) stimulated SMC migration (Fig. 3). The anti-uPA polyclonal antibody completely inhibited the FGF-2-stimulated SMC migration; SMC migration stimulated by PDGF-BB was inhibited by nearly 50% (Fig. 3). To confirm the specificity of action of the uPA antibody on growth factor-induced cell migration, we compared these inhibitory responses with those of the r-uPAmut. Recombinant uPAmut (10 nM) inhibited the PDGF-BB-stimulated cell migration to the same extent as did the polyclonal anti-uPA antibody (Fig. 3).

4. Discussion

In this study we have demonstrated that SMC migration is critically dependent on the interaction between urokinase receptors on SMC and growth factor-like domain (GFD) of uPA. Our studies also indicate that PDGF-BB and FGF-2 induce SMC migration dependent on uPA production and its interactions with SMC surface uPAR.

Our findings that cultured SMC from rat aorta bind r-uPA with high affinity in a saturable and reversible manner are consistent with earlier studies on cultured bovine aortic SMC [20]. High affinity binding of uPA to this receptor on the SMC appear to be slightly reduced by replacement of amino acids 1–24 for 13 foreign within the uPA GFD. The analysis of competition isotherms for uPA and uPAmut and

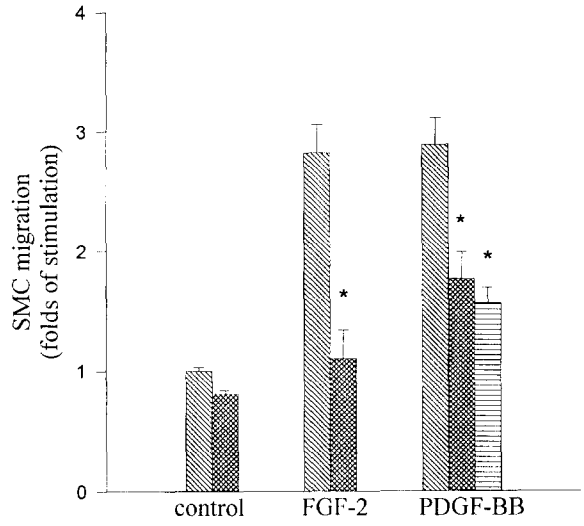

Fig. 3. Dependency of the stimulation of SMC migration by FGF-2 and PDGF-BB on uPA. SMC suspensions were pre-incubated either without (hatched bars) or with the anti-uPA antibody (cross-hatched bars) or 10 nM r-uPAmut (horizontally lined bars) for 1 h prior to placement in the micro Boyden chamber. SMC migration was then measured over a 4 h period in the absence (control) and presence of FGF-2 (50 ng/ml) or PDGF-BB (50 ng/ml) as described in the Methods (see Section 2). $*p < 0.05$ from SMC migration in the absence of the antibody of r-uPAmut.

numbers of binding sites for these uPA-variants (Fig. 2) indicates that uPA and uPAmut can interact with the same specific receptor. However, in contrast to r-uPA, modification within the GFD of uPAmut abolished its chemotactic activity, suggesting the antagonistic properties of this uPA-mutant.

Occupancy of uPAR by uPA not only influences proteolysis but also initiates intracellular signalling events promoting cell migration. Proteolytically inactive uPA-forms or N-terminal fragment of uPA activated protein kinase C, serine phosphorylation [23] and tyrosine kinases [24,25] inducing cell migration. One may suggest that GFD modification in the uPAmut could attenuate its ability to initiate signalling events required for cell migration.

Interactions between uPA and uPAR also appear to be essential for the efficient stimulation of SMC migration by FGF-2 and PDGF-BB. Although these two growth factors appear to stimulate cell migration to similar levels as does uPA, additional mechanisms are likely to be involved. Growth factors are known to upregulate uPAR number on cultured bovine SMC and endothelial cells [20,26] and influence β1-integrin receptors required for cell migration on collagen [19]. Since the r-uPAmut attenuates PDGF-BB-induced migration it is possible that intracellular signals initiated by the interaction between uPA and uPAR are also involved.

In conclusion our studies indicate that uPA-uPAR interaction appeared to be important for the growth factors-dependent SMC migration and uPA can stimulate SMC migration by mechanisms critically dependent on its GFD. Structural modification of 1–24 region within the uPA GFD leads to the loss of its chemotactic properties with no significant changes in receptor binding characteristics.

Acknowledgements: This collaboration was made possible under the agreement of the Russian and Australian Governments for exchange in Medicine and Public Health. This work is also supported by the Russian Fundamental Research Foundation grant 96-04-50714.

References

[1] Stary, H.C. (1989) Arteriosclerosis 9, (Suppl. I) 119–132.
[2] Mulvany, M.J. (1995) J. Hum. Hypertens. 9, 479–485.
[3] Forrester, J.S., Fishbein, M., Helfant, R. and Fagin, J. (1991) J. Am. Coll. Cardiol. 17, 758–769.
[4] Clowes, A.W., Clowes, M.M., Au, Y.P.T., Reidy, M.A. and Belin, D. (1990) Circ. Res. 67, 61–67.
[5] Raghunath, P.N., Tomaszewski, J.E., Brady, S.T., Caron, R.J., Okada, S.S. and Barnathan, E.S. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 1432–1443.
[6] Lupu, F., Heim, D.A., Bachmann, F., Hurni, M., Kakkar, V.V. and Kruithoff, E.K.O. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 1444–1455.
[7] Zempo, N., Koyama, N., Kenagy, R., Lea, H.J. and Clowes, A.W. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 28–33.
[8] Zempo, N., Kenagy, R.D., Au, Y.P.T., Bendeck, M., Clowes, M.M., Reidy, M.A. and Clowes, A.W. (1994) J. Vasc. Surg. 20, 209–217.
[9] Bendeck, M.P., Zempo, N., Clowes, A.W., Galardy, R.E. and Reidy, M.A. (1994) Circ. Res. 75, 535–539.
[10] Schwartz, R.S., Murphy, J.G., Edwards, W.D., Camrud, A.R., Vlietstra, R.E. and Holmes, D.R. (1990) Circulation 82, 2190–2200.
[11] Reidy, M., Irvin, C. and Lindner, V. (1996) Circ. Res. 78, 405–414.
[12] Stefansson, S. and Lawrence, D.A. (1996) Nature 383, 441–443.
[13] Del Rosso, M., Anchini, E., Pedersen, N., Blasi, F., Fibbi, G., Pucci, M. and Ruggerio, M. (1993) Biochem. Biophys. Res. Commun. 190, 347–352.
[14] Odekon, L.E., Sato, Y. and Rifkin, D. (1992) J. Cell Biol. 150, 258–263.
[15] Gyetko, M.R., Todd III, R.F., Wilkinson, C.C. and Sitrin, R.G. (1994) J. Clin. Invest. 93, 1380–1387.
[16] Belogurov, A.A., Bibilashvily, R.Sh., Gorunova, L.E., Delver, E.P., Domkin, V.V., Shevelev, A.Ya. and Ujakov, A.A. (1993) Patent SU No. 1692151 A1.
[17] Yakubov, L.Z., Kratasuk, G.A., Sinitsyn, V.V., Domogatskii, S.P., Rohlin, O.V. and Smirnov, V.N. (1986) Patent SU No. 1384614 A1.
[18] Neylon, C.B., Nickashin, A., Little, P.J., Tkachuk, V.A. and Bobik, A. (1992) J. Biol. Chem. 267, 7295–7302.
[19] Skinner, M.P., Raines, E.W. and Ross, R. (1994) Am. J. Pathol. 145, 1070–1081.
[20] Reuning, U. and Bang, N.U. (1992) Arterioscler. Thromb. 12, 1161–1170.
[21] Bilato, C., Pauly, R.R., Melillo, G., Monticone, R., Gorelick-Feldman, D., Gluzband, Y.A., Sollott, S.J., Ziman, B., Lakatta, E.G. and Crow, M.T. (1995) J. Clin. Invest. 96, 1905–1915.
[22] Sato, Y. and Rifkin, D.B. (1988) J. Cell Biol. 107, 1199–1205.
[23] Busso, N., Masur, S.K., Lazega, D., Waxman, D. and Ossowski, L. (1994) J. Cell Biol. 126, 259–270.
[24] Blasi, F. (1996) Semin. Thromb. Hemost. 22, 513–516.
[25] Resnati, M., Guttinger, M., Valcamonica, S., Sidenius, N., Blasi, F. and Fazioli, F. (1996) EMBO J. 15, 1572–1582.
[26] Koolwijk, P., van Erk, M.G.M., de Vree, W.J.A., Vermeer, M.A., Weich, H.A., Hanemaaijer, R. and van Hinsbergh, V.W.M. (1996) J. Cell Biol. 132, 1177–1188.