Blockade of Angiogenesis by Small Molecule Antagonists to Protease-Activated Receptor-1: Association with Endothelial Cell Growth Suppression and Induction of Apoptosis

Panagiota Zania, Sosanna Kritikou, Christodoulos S. Florides, Michael E. Maragoudakis, and Nikos E. Tsopanoglou

Department of Pharmacology, Medical School, University of Patras, Patras, Greece

Received November 30, 2005; accepted March 31, 2006

ABSTRACT

Many studies support the notion that protease-activated receptor (PAR)-1 plays a pivotal role in angiogenesis. However, direct evidence and understanding the molecular mechanisms involved were limited because PAR-1-specific antagonists have been developed only recently. In the present study, we evaluated the effects of two well characterized PAR-1 antagonists, SCH79797 ([N-(3-cyclopropyl-7-[(4-(1-methylthethyl)phenyl]-methyl)-7H-pyrrolo[3,2-f]quinazoline-1,3-diamine]) and RWJ56110 [(S)-N-[(1S)-3-amino-1-[(phenylmethyl)amino]carbonyl]propyl]-α-[[[(1-[2,6-dichlorophenyl][methyl]-3-[1-(pyrrolidinylmethyl)-1H-indol-6-yl][amino]carbonyl][amino]-3,4-difluoro benzene)propylamine], in the angiogenic cascade. These antagonists suppressed both the basic angiogenesis and that stimulated by thrombin in the chick chorioallantoic membrane model in vivo. PAR-1 antagonists also abrogated tube formation in the in vitro Matrigel system. These inhibitory effects were dose-dependent and well correlated with the inhibitory effects of SCH79797 and RWJ56110 on primary endothelial cell proliferation and on the initiation of apoptosis. PAR-1 blockage resulted in inhibition of endothelial cell growth by increasing the sub-G0/G1 fraction and reducing the percentage of cells in the S phase. Consistent with this, PAR-1 antagonists reduced incorporation of [3H]thymidine in endothelial cells and blocked the phosphorylation of extracellular signal-regulated kinases in a fashion depending specifically on PAR-1 activation. Analysis by annexin V/propidium iodide staining and poly(ADP-ribose) polymerase cleavage revealed that PAR-1 blockage increased apoptotic cell death by a mechanism involving caspases. These results provide further evidence that PAR-1 is a key receptor that mediates angiogenesis and suggest PAR-1 as target for developing antiangiogenic agents with potential therapeutic application in cancer and other angiogenesis-related diseases.

Proteinase-activated receptors (PARs) are a family of G protein-coupled receptors activated by the proteolytic cleavage of their N-terminal extracellular domain, exposing a new amino terminal sequence that functions as a tethered ligand to activate the receptors (Ossovskaya and Bunnett, 2004). PAR-1, the first member of this family to be cloned and the first receptor for which this unique mechanism of activation was described previously (Vu et al., 1991), has been shown to respond to a select group of serine protease that include thrombin, plasmin (Kuliopulos et al., 1999), factor Xα (Riewald et al., 2001), activated protein C (Riewald et al., 2002), and matrix metalloproteinase-1 (Boire et al., 2005). Synthetic peptides (thrombin receptor agonist peptides) of five or more amino acid residues with the identical sequence as the new amino terminus of PAR-1 are also able to activate the receptor (Ossovskaya and Bunnett, 2004).

Interest in PAR-1 was initiated from its involvement in thrombin-induced activation of platelets. Subsequently, PAR-1 activation was found to mediate several processes in vascular biology, inflammation, and tissue remodeling in normal development (Coughlin, 2005). Recently, we have shown that thrombin is a potent angiogenic factor, and this effect is independent of fibrin formation and dependent on

ABBREVIATIONS: PAR, protease-activated receptor; VEGF, vascular endothelial growth factor; SCH79797, N-3-cyclopropyl-7-[(4-(1-methylthethyl)phenyl]methyl]-7H-pyrrolo[3,2-f]quinazoline-1,3-diamine; RWJ56110, (S)-N-[(1S)-3-amino-1-[(phenylmethyl)amino]carbonyl]propyl]-α-[[[(1-[2,6-dichlorophenyl][methyl]-3-[1-(pyrrolidinylmethyl)-1H-indol-6-yl][amino]carbonyl][amino]-3,4-difluorobenzene)propylamine]; DMSO, dimethyl sulfoxide; bFGF, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; CAM, chorioallantoic membrane; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; PARP, poly(ADP-ribose) polymerase; FITC, fluorescein isothiocyanate; EGFR, epidermal growth factor receptor; PI, propidium iodide.
PAR-1 activation (Tsopanoglou and Maragoudakis, 2004). The proangiogenic action of thrombin was shown to have synergistic effect with vascular endothelial growth factor (VEGF), the specific endothelial cell angiogenic factor. PAR-1 activators up-regulate the expression of VEGF receptors of endothelial cells and potentiate the mitogenic effects of VEGF on endothelial cells (Tsopanoglou and Maragoudakis, 1999). The importance of thrombin and its receptor in embryonic development and angiogenesis is also supported by the findings of Griffin et al. (2001), who showed that the expression of PAR-1 by endothelial cells rescues the fetal vessel fragility and bleeding of mouse embryo engineered to lack PAR-1. More recently, Ma et al. (2005) have shown that PAR-1 activation in human platelets stimulates VEGF release and leads to suppression of endostatin, which is a potent endogenous inhibitor of angiogenesis. These findings point to a central role of PAR-1 in the angiogenic cascade.

Despite the presence of functional PAR-1 on the surface of many cell types, there are formidable obstacles in understanding its role in health and disease. A major difficulty was that effective PAR-1 antagonists were not available. PAR-1-deficient mice were used in elucidating the roles of PAR-1, but mice are not always the optimal species to model human diseases. Another complication is the apparent redundancy of the PAR system. Cells express several PARs that are activated by the same protease, and several proteases can activate the same receptor. To date, few PAR-1-targeting agents have been developed and tested in limited disease models such as thrombosis, intestinal inflammatory disorders, and experimental liver fibrosis (Deren et al., 2003; Fiorucci et al., 2004; Vergnolle et al., 2004). The effect of PAR-1 antagonists on angiogenesis has not been reported. In the present study, we show that SCH79797 and RWJ56110, two well characterized specific and potent PAR-1 antagonists (Andrade-Gordon et al., 1999; Ahn et al., 2000), are capable of blocking angiogenesis in the chick embryo angiogenesis model in vivo and in the Matrigel in vitro system. We also show that these PAR-1 antagonists abrogate endothelial cell growth by induction of apoptosis.

Materials and Methods

Materials and Cell Culture. The selective PAR-1 antagonist SCH79797 was purchased from Torcis Bioscience (Annonym, UK), and RWJ56110 was kindly provided by Johnson & Johnson Pharmaceutical Research & Development (Raritan, NJ). Both antagonists were dissolved in DMSO. Human α-thrombin was kindly provided by Dr. J. Fenton II (New York State Department of Health, Albany, NY). VEGF and basic fibroblast growth factor (bFGF) were obtained from Chemicon International Inc. (Temecula, CA). [3H]Thymidine and [14C]Proline were purchased from ICN Biomedicals, Inc (Irvine, CA). Fresh fertilized chicken eggs were obtained locally (Ioannina, Greece) and were kept at 10°C until incubation at 37°C. DNA synthesis was stopped by removing the radioactive proline. At day 11, the eggs were flooded with 10% buffered formalin, the plastic pellets were removed, and the eggs were kept at 37°C until dissection. A large area around the pellet was removed and placed on a glass slide. Representative specimens were mounted on a stereoscope and photographed.

Matrigel Tube Assay. Matrigel is a mixture of basement membrane components extracted from the Englebreth-Holm-Swarm tumor, and it has been demonstrated that endothelial cells attach, migrate, and assemble to form tube-like structures resembling capillaries within 18 h. The tube formation assay was performed as described previously (Grant et al., 1989). In brief, Matrigel was used to coat 24-well plates (0.25 ml/well). After polymerization of Matrigel at 37°C for 1 h, indicated concentrations of PAR-1 antagonists were added in 0.5 ml of serum-free medium. Cells (40,000 cells/well) were then added suspended in 0.5 ml of medium containing fetal bovine serum (FBS) making 4 or 10% final concentration in FBS. After 18 h of incubation, the medium was removed, and the cells were fixed and stained. Thereafter, the tube-like structures formed were quantitated in triplicates with a computerized digital image analyzer. Each experiment was repeated at least twice. Results are given as mean percentage change of control ± S.E. Statistical analysis was performed with Student’s t test.

Cell Proliferation Assay. Cell proliferation was evaluated by, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) assay. In brief, endothelial cells (10,000/well) were seeded in 24-well tissue culture plates previously coated with 1% gelatin (Sigma) and incubated with growth medium for 6 h. Cells were then treated with the indicated concentrations of PAR-1 antagonists for 1 to 4 days. After 24, 48, 72, or 96 h of exposure to compounds (SCH79797 or RWJ56110), MTT (50 μl) solution (5 mg/ml) was added to each well to incubated for 3 h at 37°C. The blue formazan crystals were solubilized by addition of DMSO (200 μl). Absorbance at 450 nm was recorded using a 96-well plate reader. Results are expressed as mean ± S.E. of number from cells and presented as fold increased of cell number at the beginning of experiments. Each experiment included three wells in each condition tested and was repeated at least twice. Statistical analysis was performed with Student’s t test.

[3H]Thymidine Incorporation Assay. HUVECs were seeded into gelatin-coated 24-well tissue culture plates and incubated with growth medium until 50 to 60% confluence. After changing the medium to 4% FBS, cells were treated with the indicated concentration of PAR-1 antagonists for 18 h. In some experiments, HUVECs were seeded sparsely into a gelatin-coated well and incubated with growth medium until confluence. After washes, confluent cells were then treated with PAR-1 antagonists for 18 h in medium containing 4% FBS. All cells were pulsed with 0.5 μCi/ml [3H]Thymidine for an additional 6 h. DNA synthesis was stopped by removing the radioactive media, washing the cells with PBS, and fixing them with ice-cold methanol (300 μl/well). Cells were then washed twice with 5% trichloroacetic acid, and the acid-insoluble fractions were lysed in 0.5 N NaOH (300 μl/well). The radioactivity was determined in a liquid scintillation counter. Each experiment included three wells in each condition tested and was repeated at least twice. Results are
expressed as mean change of control ± S.E. Statistical analysis was performed with Student’s t test.

**Western Blot Analysis.** Endothelial cells were cultured in 35-mm tissue culture plates. After reaching confluence, cells were growth factor-starved and subsequently stimulated for 10 min with thrombin or bFGF or VEGF or epidermal growth factor (EGF). In experiments with PAR-1 antagonists, endothelial cells were pretreated with the indicated concentrations of SCH79797 or RWJ56110 for 15 min and subsequently were stimulated by growth factors. Attached cells were lysed with Laemmli sample lysis buffer, and lysates were resolved with 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies using the following antibodies concentration: antiphospho p42/44 mitogen-activated protein kinases (MAPKs; Erk1/2) (1:3000; New England Biolabs, Beverly, MA) that detects p42 and p44 MAPK only when catalytically activated by phosphorylation at Thr 202 and Tyr 204, anti-p42/44 MAPK (1:3000; New England Biolabs). Membranes were then probed with secondary antibodies horseradish peroxidase-conjugated, and proteins were visualized by chemiluminescent detection.

For analysis of poly(ADP-ribose) polymerase (PARP), endothelial cells were cultured in gelatin-coated 60-mm tissue culture plates. After reaching 50 to 60% confluence, cells were treated with the indicated concentrations of PAR-1 antagonists for 24 h or 4 days or staurosporine for 10 h. Attached and suspended cells were lysed with RIPA lysis buffer, and pooled lysates were resolved with 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies using the following concentration: mouse anti-PARP monoclonal (1:3000; BD Biosciences, San Jose, CA) that detects both the 116-kDa intact and 85-kDa cleaved forms of PARP, anti-α-tubulin monoclonal (1:2000; Sigma). Membranes were then probed with secondary antibodies horseradish peroxidase-conjugated, and proteins were visualized by chemiluminescent detection.

**Assessment of Apoptosis by Flow-Cytometry Analysis.** HUVECs were grown in gelatin-coated 100-mm tissue culture plates until approximately 50% confluence. In some experiments, HUVECs were seeded sparsely into gelatin-coated plates and incubated until confluency. In cell cycle analysis, cells were treated in the absence (control) or presence of SCH79797 (3 μM) or RWJ56110 (30 μM) for 24 h. After treatment, attached cells were collected by trypsinization, washed, and fixed in methanol for 1 h at –20°C. Fixed cells were then incubated with RNase (100 μg/ml; Sigma) for 30 min and stained with propidium iodide (50 μg/ml; Sigma) for 20 min at 4°C in the dark. Flow cytometry was performed on a fluorescence-activated cell sorting flow cytometer (EPICS XL-MCL; Beckman Coulter, Fullerton, CA). The propidium iodide-stained cell populations in sub-G1/G0, G1, S, and G2/M phases were represented by distinct and quantified peaks in the fluorescence histograms obtained using the WinMDI logiciel program. Experiments were run in triplicate and repeated twice. Results are expressed as mean percentage of cell population in each phase ± S.E. Statistical analysis was performed with Student’s t test.

To assess cell death specific to apoptosis, we used the annexin V-FITC assay kit (BD Biosciences PharMingen, San Diego, CA). Endothelial cells were treated with the indicated concentrations of both PAR-1 antagonists for 96 h or with 3 μM SCH79797 for 12, 24, 48, and 72 h. After treatment, attached cells were collected by trypsinization, pooled with suspended cells, and washed in PBS. Cells (1 × 10⁷) were then resuspended in 100 μl of the kit reaction buffer containing propidium iodide and annexin V-FITC, according to the manufacturer’s instructions. After mixing, cells were incubated for 15 min in the dark at room temperature and analyzed on the fluorescence-activated cell sorting flow cytometer within 1 h after staining.

**Results**

**PAR-1 Antagonists Inhibit Angiogenesis in Vivo.** We have used the in vivo model of chick chorioallantoic membrane system of angiogenesis and collagenous protein biosynthesis as a biochemical index of angiogenesis (Maragoudakis et al., 1995). As shown in Fig. 1A, PAR-1 antagonists, SCH79797 and RWJ56110, were very potent antiangiogenic agents. SCH79797 and RWJ56110, at concentrations of 30 and 100 μg/pellet, respectively, completely blocked the formation of new vessels. This inhibitory effect was dose-dependent and not toxic for the chick embryo, even at as high concentration as 300 μg/pellet. The antiangiogenic effect of PAR-1 antagonists was evident both for basic angiogenesis and that stimulated by thrombin (Fig. 1B).

**PAR-1 Antagonists Inhibit Capillary-Like Structure Formation in Matrigel In Vitro.** Within 18 h, control wells containing endothelial cells plated on Matrigel form a network of capillary-like structures, which is used by many investigators as reliable angiogenesis model. When...
SCH79797 and RWJ56110 were present, there was a significant inhibitory effect on the rate and extent of tube formation (Fig. 2A). At concentrations ranging from 0.1 to 3 μM for SCH79797 and 1 to 30 μM for RWJ56110, both compounds caused a dose-dependent inhibition of tube formation by endothelial cells plated on either medium containing 4 or 10% serum (Fig. 2B).

PAR-1 Antagonists Inhibit Growth of Endothelial Cells. To investigate the effect of PAR-1 antagonists on endothelial cell proliferation, HUVECs were plated on gelatin-coated 24-well plates and grown with complete culture medium for 96 h in the absence or presence of various concentrations of SCH79797 and RWJ56110. On each of 4 consecutive days, cells were washed and fixed, and cell growth was determined indirectly by MTT assays. As shown in Fig. 3A, endothelial cell number doubled every 18 to 26 h over the 96-h period. In the presence of SCH79797-selective PAR-1 antagonist, the rate of endothelial cell growth was significantly decreased (Fig. 3A). HUVECs growth was essentially blocked by SCH79797 at 3 μM. This inhibitory effect of SCH79797 was dose-dependent with half-maximal inhibitory concentration at approximately 0.3 μM. Similar results were also obtained using the RWJ56110-specific PAR-1 antagonist with half-maximal inhibitory concentration of 10 μM (data not shown).

The ability of PAR-1 antagonists to inhibit DNA synthesis of endothelial cells was assessed in thymidine incorporation assays. Endothelial cells, which were in fast-growing state (50–60% confluence), were cultured in medium containing 4% FBS for 18 h in the presence or absence of PAR-1 antag-
onists and subsequently were pulsed with \(^{3}H\)thymidine for an additional 6 h. As shown in Fig. 3B, SCH79797 and RWJ56110 reduced the DNA synthesis in HUVECs in a dose-dependent manner. However, when the DNA synthesis experiments were repeated with cells that were in the quiescent state (100% confluent), the inhibitory effect of PAR-1 antagonists was much less pronounced (data not shown).

Several studies have shown that the activation of PAR-1 by thrombin or agonist peptides results in MAPK activation. The MAPK (Erk1/2, p42/44) cascade is usually mitogenic since cell cycle progression has been shown to depend on sustained activation of the Erk signal transduction pathway (Roovers and Assoian, 2003). Considering the antimitogenic and antiproliferative effects of PAR-1 antagonists, we examined a possible inhibition of Erk1/2 activity by SCH79797 and RWJ56110 in endothelial cells. As shown in Fig. 4A, SCH79797 inhibits thrombin-induced Erk1/2 activation in a concentration-dependent manner (0.1–3 \( \mu M \)). Similar inhibitory effect was evident using RWJ56110 (Fig. 4B). This effect was specific for thrombin-mediated activation since PAR-1 antagonists did not have any effect on bFGF-, VEGF-, or EGF-induced Erk1/2 phosphorylation (Fig. 4C; data not shown). However, when endothelial cells were stimulated by FBS (final concentration 4%), the presence of both PAR-1 antagonists reduced partially the activated levels of Erk1/2, providing evidence that the serum contains proteases that are able to activate PAR-1 (Fig. 4D).

**Growth Inhibition of Endothelial Cells Is Associated with Induction of Apoptosis.** Flow-cytometric cell cycle analysis was performed to determine whether the results of the proliferation and thymidine incorporation were a reflection of cytostatic or cytotoxic effects due to cell cycle arrest or apoptosis. HUVECs at approximately 50% confluence were treated with 3 \( \mu M \) SCH79797 or 30 \( \mu M \) RWJ56110. After 24 h of drug treatment, cells were fixed and resuspended in propidium iodide, and DNA content was measured in comparison with untreated cells. The sub-G\(_0\)/G\(_1\) fraction (subdiploid region on the DNA content histogram) was used as a measure of the percentage of apoptotic cells. SCH79797 induced a 19-fold increase in the subdiploid endothelial cell population (Fig. 5). In addition, SCH79797 reduced the percentage of cells in the S phase (DNA synthesis) by more than 4-fold. This is consistent with the results of the effect of PAR-1 antagonists on endothelial cell proliferation and thymidine

**Fig. 4.** Effect of PAR-1 antagonists on MAPK activation in endothelial cells. A, serum-starved HUVECs were pretreated with the indicated concentrations of SCH79797 or vehicle (DMSO) for 10 min. B, cells were pretreated with the indicated concentrations of RWJ56110 or vehicle (DMSO) for 10 min. C, cells were pretreated with the indicated concentrations of SCH79797 or RWJ56110 or vehicle (DMSO) for 15 min and then were stimulated with bFGF for 10 min. D, cells were pretreated with SCH79797 (SCH; 3 \( \mu M \)) or RWJ56110 (RWJ; 10 \( \mu M \)) or vehicle (DMSO) for 15 min and then were stimulated with thrombin or bFGF for 10 min. Cell lysates were probed with antiphospho Erk1/2-specific antibody. To determine total protein level, membranes were probed with Erk1/2 antibody. Representative membranes are shown. Similar results were obtained in three independent experiments.
obtained when endothelial cells were treated with 30 μM SCH79797 (3 μM) or RWJ56110 (30 μM) under normal culture conditions for 24 h. Cells were harvested with trypsin, stained with propidium iodide, and analyzed with a flow cytometer. The mean ± S.E. of percentage of cells in sub-G0/G1, G1, S, and G2/M phases of the cell cycle are shown, as determined from the histogram of propidium iodide-stained cells. The data presented in Fig. 6A revealed that SCH79797 increased the percentage of endothelial cells in early apoptosis. In addition, a recent study by Arisato et al. (2003) demonstrated that the cytotoxicity observed is due at least in part to caspase activation.

**Discussion**

Angiogenesis is a complex biological process that is involved in many physiological and pathological situations, such as wound healing and tumor growth. In the present study, we provided direct evidence that PAR-1 is involved in the initiation of the angiogenic cascade. We have shown that selective blockage of PAR-1 by two chemical antagonists, SCH79797 and RWJ56110, inhibited angiogenesis in vivo in the chick embryo angiogenesis model. These PAR-1 antagonists were also able to attenuate the migration and differentiation of endothelial cells in the Matrigel system in vitro. In addition, they suppress in a dose-dependent manner the proliferation of endothelial cells. Although these effects on endothelial cells provide a plausible explanation for the suppression of angiogenesis observed in chick embryo, this cannot rule out the possibility that the antiangiogenic effect of PAR-1 antagonists could be mediated through effects on other cell types, which also express PAR-1. For example, platelets could play a pivotal role by releasing a number of factors that can mediate angiogenesis. PAR-1 activation in human platelets causes stimulation of the release of VEGF and suppression of endostatin release (Ma et al., 2005). In addition, a recent study by Arisato et al. (2003) demonstrated that release of VEGF from human smooth muscle cells could be stimulated by PAR-1 agonists.

Thrombin has been shown to activate PAR-1 in vitro and is considered the endogenous activator of PAR-1 on platelets (Coughlin, 2005). Because thrombin is rapidly inactivated in plasma, its temporal and local effect depends on the presence of tissue factor. Tissue factor activates factor X, which leads to cleavage of circulating prothrombin, thereby generating thrombin. Tissue factor production has been shown to be up-regulated in endothelial cells by serum (Faucette et al., 1993) as well as by the angiogenic factors VEGF and bFGF (Zucker et al., 1998; Kaneko et al., 2003).

In addition, PAR-1 can be activated by other proteases.
from the coagulation cascade such as factor Xa or VIIa and serine proteases that include plasmin and activated protein C (Ossovskaya and Bunnett, 2004). Recently, Boire et al. (2005) have identified matrix metalloprotease-1 to be a novel nonserine protease agonist that cleaves PAR-1 at the proper site for receptor activation and generates PAR-1-dependent intracellular signals. Indeed, matrix metalloprotease-1 is one the dominant matrix metalloprotease secreted by endothelial cells and seems to play an important role during angiogenesis (Fisher et al., 1994; Sato et al., 2000). We have shown that PAR-1 antagonists suppressed basic angiogenesis (in the absence of thrombin). Therefore, in view of these findings, the possibility exists that proteases other than thrombin might be implicated in PAR-1 activation and may initiate angiogenesis. This may also explain the inhibitory effect of PAR-1 antagonists on tube formation, proliferation, DNA synthesis, and MAPK activation by endothelial cells, which were performed in serum-containing medium. In addition, these findings point to the fact that PAR-1 can be activated in endothelial cell cultures. The majority of cells express more than one PAR for the same protease, and several proteases can activate the same PAR. It is possible that PAR-1 acts as a high-gain sensor of various extracellular proteases and allows the cell to react to a proteolytically altered environment. This unique ability to sense proteases can be used by cells for migration and differentiation or growth and survival.

Endothelial cell proliferation was inhibited after exposure of PAR-1 antagonists in a concentration- and time-dependent manner, and this inhibition does not seem to be the result of a nonspecific cytotoxic mechanism. SCH79797 and RWJ56110 treatment of HUVECs inhibited the thrombin-induced activation of MAPK. This effect was specific since

Fig. 6. Effect of PAR-1 antagonists on annexin V/propidium iodide (PI) double staining for apoptosis in endothelial cells. A, HUVECs were treated under normal growth conditions in the presence of vehicle (control, DMSO) or the indicated concentrations of SCH79797 or RWJ56110 for 96 h. B, HUVECs were treated under normal growth conditions in the presence of vehicle (control, DMSO) or SCH79797 (3 μM) for the indicated time intervals. Cells were fixed, stained with annexin V-FITC and PI, and analyzed for early apoptotic cells (annexin V-positive, PI-negative) and late apoptotic or dead cells (annexin V- and PI-positive) by flow cytometry. The corresponding percentages of stained cells are shown. Representative dot plots are shown. Similar results were obtained in three independent experiments.
the levels of activated MAPK triggered by other growth factors such as VEGF, bFGF, or EGF were not affected by PAR-1 antagonists. The ras-Raf-MAPK pathway is one of the major downstream pathways that is initiated as a consequence of PAR-1 activation (Ossovskaya and Bunnett, 2004). MAPKs phosphorylate a large number of targets, which eventually initiate and regulate various cellular processes including proliferation, differentiation, and development. We have seen in HUVECs that PAR-1 activation results in elevated phosphorylated levels of MAPK through a mechanism involving EGFR receptor (EGFR) transactivation (P. Zania and N. E. Tsopanoglou, unpublished data). Previous work has shown that EGFR signaling plays a role in angiogenesis because inhibition of EGFR decreases the production of proangiogenic molecules and inhibits neoangiogenesis (Hirata et al., 2002). It is intriguing that EGFR antagonists readily induce apoptosis in cells dependent solely on EGFR signaling for cell growth and survival (Moyer et al., 1997). It is of interest that PAR-1 antagonists are most likely due to induction of apoptosis.

Activation of PAR-1 has been found to induce or inhibit apoptosis in a variety of cells, depending on the dosage of thrombin or that of synthetic receptor activators (Flynn and Buret, 2004). It has been shown that thrombin or thrombin receptor agonist peptide (SFLLRN) prevent apoptosis of CC139 fibroblast cells and promote survival of prostate cancer cells through inhibition of Bim (Bel-2-interacting mediator of cell death) expression and nuclear factor κB activation, respectively (Chalmers et al., 2003; Tantivejkul et al., 2005). However, Zain et al. (2000) have shown that thrombin has a bimodal effect on PAR-1-dependent growth of melanoma, colon, and prostate carcinomas. Low thrombin concentrations enhance the growth of these cancer cells, whereas high thrombin impairs growth and induces apoptosis. PAR-1-mediated effects after exposure to high concentrations of thrombin may be related to desensitization and down-regulation of PAR-1 by thrombin, and this inactivation of PAR-1 could lead to initiation of apoptosis and inhibition of angiogenesis.

PAR-1 was identified as an oncogene that actively induced the loss of anchorage-dependent growth and focus-forming activity in NIH-3T3 cells (Martin et al., 2001) and has long been proposed to be involved in the invasive and metastatic processes of cancers of the breast, colon, lung, pancreas, prostate, and melanoma (Ossovskaya and Bunnett, 2004). These aforementioned findings and the results from this study point to the pivotal role of PAR-1 activation in tumor biology and angiogenesis. Furthermore, our results validate the concept that PAR-1 antagonists are effective antiangiogenic agents and as such have the potential therapeutic application in cancer and other disease states where angiogenesis is deranged. The fact that PAR-1 antagonists also function as antithrombotic agents (Derian et al., 2003) may present another beneficial action for the thrombosis-prone cancer patient.

Acknowledgments

We thank Claudia Derian (Johnson & Johnson Pharmaceutical Research & Development, Raritan, NJ) for providing RWJ56110. We are especially grateful for the expert technical advice and support in
flow-cytometry experiments of Eleni Thanopoulou and Nickolas Zoubos.

References


Zanias et al.