Regenerative and Proinflammatory Effects of Thrombin on Human Proximal Tubular Cells

GIUSEPPE GRANDALIANO, RAFFAELLA MONNO, ELENA RANIERI, LORETO GESUALDO, and FRANCESCO P. SCHENA, WITH THE TECHNICAL ASSISTANCE OF CARMELA MARTINO AND MICHELE URSI

Division of Nephrology/Department of Emergency and Transplantation, University of Bari, Italy.

Abstract. Interstitial fibrin deposition is a common histologic feature of tubulointerstitial diseases, which suggests that the coagulation system is activated. Thrombin, generated during the activation of the coagulation cascade, is a powerful activating factor for different cell types. Although proximal tubular cells are potential targets for this coagulation factor, no information is available on the effect of thrombin on these cells. Thus, the expression of protease-activated receptor-1 (PAR-1), the main thrombin receptor, was investigated in cells. Thus, the expression of protease-activated receptor-1 (PAR-1) in human proximal tubular cells (hPTC) in vivo and in vitro. A diffuse expression of PAR-1 was observed by immunohistochemistry along the basolateral membrane of PTC in normal human kidney. This observation was confirmed in vitro in cultured hPTC. Because tubular damage and monocyte infiltration are hallmarks of tubulointerstitial injury, the effect of thrombin on DNA synthesis and monocyte chemotactic peptide-1 (MCP-1) gene and protein expression was evaluated in cultured hPTC. Thrombin induced a significant and dose-dependent increase in thymidine uptake and a striking upregulation of MCP-1 mRNA expression and protein release into the supernatant. Although PAR-1 is a G protein-coupled receptor, its activation in hPTC, as in other cell systems, resulted in a transient increase in cellular levels of tyrosine-phosphorylated proteins. An increased level of tyrosine-phosphorylated c-src suggested the activation of this cytoplasmic tyrosine kinase in response to thrombin and its potential role in thrombin-induced protein-tyrosine phosphorylation. Interestingly, thrombin-induced DNA synthesis and MCP-1 gene expression were completely blocked by genistein, a specific tyrosine kinase inhibitor, but not by its inactive analogue daidzein, demonstrating a central role for tyrosine kinase activation in the thrombin effects on hPTC. Moreover, the specific src inhibitor PP1 abolished the thrombin effect on DNA synthesis. In conclusion, thrombin might represent a powerful regenerative and proinflammatory stimulus for hPTC in acute and chronic tubulointerstitial diseases.

Fibrin deposition in the peritubular capillaries and along the tubular basement membrane is commonly observed in several renal diseases characterized by tubular and/or interstitial damage, such as ischemic tubular necrosis, obstructive nephropathy, experimental lupus nephritis, and acute as well as chronic renal allograft rejection (1–6). This observation indirectly suggests the activation of the coagulation cascade at the interstitial level in the development of acute and chronic renal damage. There is an increasing body of evidence supporting an involvement of the coagulation system in the pathogenesis of glomerular lesions both in human and in experimental glomerulonephritides, whereas the consequences of the coagulation cascade activation in interstitial diseases have not received much attention (7–9).

The activation of the coagulation cascade leads to the partial proteolysis of prothrombin with subsequent local thrombin generation (10). This serine protease may then be accumulated in its active form within the fibrin clots and released locally over a prolonged period of time (11). Thrombin, besides its physiologic action in the clotting cascade, is known to modulate a variety of cell functions through the interaction with specific cell surface receptors (12,13). All of the known thrombin receptors belong to the protease-activated receptor (PAR) family and are characterized by a peculiar proteolytic mechanism of activation (14–16). Indeed, receptor activation occurs when thrombin cleaves the extracellular domain of the receptor exposing a tethered ligand (14). Among the receptors of the PAR family, thrombin can interact specifically with PAR-1, -3, and -4 (14–16). Of these three signaling receptor proteins, however, only PAR-1 has been shown to be expressed in the kidney (14,17). Although proximal tubular cells (PTC) may represent a potential target for thrombin, no information is available on the presence of PAR-1 on their surface as well as on their activation by thrombin.

Tubular damage and monocyte infiltration are two of the histopathologic hallmarks of acute and chronic tubulointerstitial injury (18,19). Tubular necrosis and atrophy, especially in the setting of acute tubulointerstitial damage, are potentially reversible, although the mechanisms responsible for the regenerative response are still poorly understood (18). Monocyte interstitial infiltration is a key step in the pathogenesis of
tubulointerstitial damage and monocye chemotactic peptide (MCP-1), expressed by tubular cells, may be involved in the monocye influx into the interstitial tissue (19,20).

In the present study, PAR-1 expression by human PTC was investigated in vivo and in vitro. Moreover, the thrombin effect on human PTC mitogenesis and MCP-1 gene and protein expression was evaluated.

Materials and Methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM)/F12, trypsin, penicillin, streptomycin were obtained from Maccania Brunelli (Milan, Italy). Fetal bovine serum (FBS), l-glutamine, sodium pyruvate, non-essential amino acids, insulin, transferrin, and selenium were from Sigma Cell Culture (Milan, Italy). Bovine thrombin, genestin, daizaeim, herbimycin A, prostatadolin E1, hydrocortisone, and T3 were from Sigma Chemical Co. (Milan, Italy). Epidermal growth factor (EGF), PP1, and PP3 were obtained from Calbiochem (La Jolla, CA). The monoclonal antibody directed against the extracellular domain of human PAR-1 was kindly provided by Dr. L. F. Brass (Thomas Jefferson University, Philadelphia, PA). Polyclonal rabbit anti-human src antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The monoclonal anti-phosphotyroine antibody Py20 was obtained from Upstate Biotechnology (Lake Placid, NY). The horseradish-peroxidase-conjugated sheep anti-mouse and sheep anti-rabbit antibodies were obtained from Amersham (Buckinghamshire, United Kingdom). [32P]dCTP and [methyl-3H]-thymidine were purified from Amersham (Buckinghamshire, United Kingdom). The DNA fragment was labeled by random priming using [32P]dCTP and [methyl-3H]-thymidine were purchased from Amersham. All other chemicals were reagent grade.

Cell Isolation and Culture

HK2, an immortalized PTC line from normal adult human kidney (21), was obtained from American Type Culture Collection (Manassas, VA). Cells were grown to confluence in DMEM/F12 medium supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 5 pg/ml T3, 5 ng/ml hydrocortisone, 5 pg/ml prostatadolin E1, and 10 ng/ml EGF. For passage, confluent cells were washed with phosphate-buffered saline (PBS), removed with 0.05% trypsin/0.02% ethylenediaminetetra-acetic acid in PBS, and plated in DMEM/F12 medium.

Immunohistochemistry and Immunocytochemistry

Apparently normal kidney portions from 10 patients undergoing nephrectomy for renal cell carcinoma and cultured HK2 cells plated on 8-well multilister slides (ICN, Aurora, OH) were used to investigate PAR-1 protein expression. The detection of this thrombin receptor was performed on frozen 4-μm-thick kidney sections and on subconfluent HK2, fixed in 4% paraformdehyde, using specific mouse monoclonal anti-human PAR-1 antibody directed against an epitope (corresponding to PAR-1 residues 51 to 64) in the N terminus of the receptor that is retained after PAR-1 cleavage at 1:200 dilution (17). HK2 cells were serum-starved overnight and incubated in serum-free medium with or without thrombin (5 U/ml) for 15 min, before fixation. The anti-PAR-1 antibody recognizes the extracellular domain of the inactive as well as of active PAR-1. Immobilized mouse antibodies were detected by the immunoenaline phosphatase (APAAP) method with affinity-purified rabbit anti-mouse IgG (Dako, Glostrup, Denmark) and APAAP complex (1:50 dilution; Dako), following a two-step technique as described previously (19). Alkaline phosphatase was developed with New Fuchsin (Sigma). Negative controls were performed by omitting the primary or secondary antibodies, and using nonimmune mouse or rabbit antiserum as first layer.

3H-Thymidine Incorporation

DNA synthesis was measured as the amount of [methyl-3H]-thymidine incorporated into TCA-precipitable material, as described previously (22). Briefly, HK2 were plated in 24-well dishes at a density of 4 × 10^4 cells/well, grown to confluence, and made quiescent by being placed in serum-free medium for 48 h. The cell monolayer was then incubated with thrombin at the indicated concentrations for 24 h at 37°C. In separate sets of experiments, cells were preincubated with genestin (25 μM), daizaeim (25 μM), PP1 (25 μM), and PP3 (25 μM) for 18 h before adding thrombin. At the end of the incubation period, cells were pulsed for 4 h with 1.0 μCi/ml 3H-thymidine. The medium was then removed, the cells were washed twice in ice-cold 5% TCA, and then incubated in 5% TCA for 5 min. The monolayer was solubilized by adding 0.75 ml of 0.25N NaOH in 0.1% sodium dodecyl sulfate (SDS). Half-milliliter aliquots were then neutralized and counted in scintillation fluid using a beta counter.

In parallel experiments, cell proliferation was determined by direct cell counting after 24 and 48 h of incubation with thrombin (5 U/ml), as described previously (23).

RNA Isolation and Northern Blot Analysis

HK2 cells were plated in 75-mm2 flasks and cultured as described above. After reaching confluence, cells were serum-starved for 48 h and then incubated for the indicated time periods with thrombin (5 U/ml). In separate sets of experiments, cells were preincubated with genestin (25 μM), daizaeim (25 μM), PP1 (25 μM), and PP3 (25 μM) for 18 h before adding thrombin. At the end of incubation, cells were lysed with 4 M guanidinium isothiocyanate containing 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 mM β-mercaptoethanol. Total RNA was isolated by the single-step method, using phenol and chloroform/isooamyl alcohol (24).

MCP-1 gene expression was studied by Northern blotting, as described previously (24). Briefly, electrophoresis of 20 μg of total RNA from each experimental condition was carried out in 1% agarose gel with 2.2 M formaldehyde. The RNA was then transferred overnight onto a nylon membrane (Schleicher & Schuell, Dassel, Germany). The membrane was washed with ethidium bromide to evaluate the 28S and 18S ribosomal bands and prehybridized at 42°C for 2 h in 50% formamide, 0.5% SDS, 5× SSC, and 0.1 mg/ml salmon sperm DNA. A 0.7-kb fragment of the baboon MCP-1 cDNA was used as a probe (25). The DNA fragment was labeled by random priming using a commercially available kit (Amersham) and [32P]dCTP (specific activity, 3000 Ci/mmol). The probe (10^6 cpm/ml) was added to 10 ml of prehybridization solution, and the blots were hybridized for 16 h at 42°C. The membranes were then washed once in 2× SSC, 0.1% SDS at room temperature for 5 min, once in the same buffer at 55°C for 30 min, and in 1× SSC, 0.1% SDS at 55°C for an additional 30 min. After drying, membranes were exposed to a Kodak X-OMAT film with intensifying screens at −70°C.

Enzyme-Linked Immunosorbent Assay

HK2 cells plated in 6-well dishes and grown to 70% confluence were serum-starved for 24 h and then incubated for 24 h in serum-free medium with or without thrombin (5 U/ml). At the end of the incubation, the supernatant was harvested, centrifuged for 10 min at 1000 × g to remove the cell debris, and stored at −80°C until used. MCP-1 measurement in the supernatant was performed using a commercial human MCP-1 enzyme-linked immunosorbent assay (ELISA)
kit (Quantikine; R&D Systems, Abingdon, United Kingdom). This is a multiple sandwich solid-phase enzyme immunoassay that uses a specific monoclonal antibody raised against human MCP-1. The sensitivity of the ELISA is 5 pg/ml. The MCP-1 concentration of the unknown samples was determined by interpolation into a standard curve developed with known amounts of recombinant human MCP-1 protein. MCP-1 protein concentration was normalized to cell counts.

**Western Blot**

HK2 were plated in 60-mm² Petri dishes and grown to confluence in DMEM/F12 medium supplemented with 10% FBS. The cells were incubated for 48 h in serum-free medium and then exposed to thrombin (5 U/ml) for the indicated time periods. At the end of the treatment, the cell monolayer was rapidly rinsed twice with ice-cold PBS and lysed in 100 μl of RIPA buffer (1 mM phenylmethylsulfonyl fluoride, 5 mM ethylenediaminetetra-acetic acid, 1 mM sodium orthovanadate, 150 mM sodium chloride, 8 μg/ml leupeptin, 1.5% Nonidet P-40, and 20 mM Tris-HCl, pH 7.4). The lysates were set on ice for 30 min and centrifuged at 10,000 g for 5 min. The supernatants were collected and stored at −80°C until used. Aliquots containing 7.5 μg of proteins from each lysate were subjected to SDS-polyacrylamide gel electrophoresis on a 7.5% gel under reducing conditions and then electrotransferred onto nitrocellulose membrane (Hybond™C; Amersham). The filter was blocked overnight at room temperature with 2% bovine serum albumin in PBS containing 0.1% Tween 20 (TBS) and incubated with monoclonal anti-phosphotyrosine antibody at room temperature for 4 h. The membranes were washed twice in TBS and incubated for 2 h at room temperature with horseradish peroxidase-conjugated sheep anti-mouse IgG at 1:1500 dilution in TBS. The membranes were washed three times at room temperature in TBS and then once with 0.1% SDS in PBS. The ECL enhanced chemiluminescence system (Amersham) was used for detection.

**Immunoprecipitation**

Confluent HK2 cells in 60-mm² culture dishes were placed in serum-free medium for 48 h. Thrombin (5 U/ml) was then added for the indicated time periods. Cells were washed twice with ice-cold PBS and lysed in situ with RIPA buffer for 30 min at 4°C. The cell lysate was centrifuged at 10,000 × g for 30 min at 4°C. One hundred microliters of protein from the supernatant was first incubated with anti-phosphotyrosine antibodies for 2 h on a rocking platform at 4°C and then with agarose-linked protein A for 2 h at 4°C. The immunobeads were washed twice with RIPA buffer and twice with 0.5 mM LiCl, 0.1 mM Tris-HCl, pH 7.5, 1 mM sodium orthovanadate. The beads were then resuspended in sample buffer and boiled. The immunoprecipitated proteins were separated by electrophoresis on a 7.5% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked as described previously and incubated with rabbit anti-src antibody (1:1000) for 4 h at room temperature, washed, and incubated with horseradish peroxidase-conjugated mouse anti-rabbit IgG (1:1500). The ECL system was used for detection of the horseradish peroxidase-coupled antibodies.

**Statistical Analyses**

Data are presented as mean ± SD and compared by ANOVA. P < 0.05 was considered significant.

**Results**

PAR-1 expression has been demonstrated in vivo and in vitro in glomerular cells, whereas no information is available on its presence in tubular cells (17,25–27). To address this issue, PAR-1 protein expression was investigated in normal kidney sections by immunohistochemistry, using a specific monoclonal antibody that recognizes the extracellular domain of this thrombin receptor. Although glomerular cells represented the main site of PAR-1 expression within the kidney, PTC were also specifically and significantly stained (Figure 1, A through C). Indeed, all of the glomeruli and 30 to 40% of the cortical tubular sections were positive for PAR-1. The new fuchsin deposits within PTC were mainly localized to the basolateral membrane, although in a few tubular sections an apical expression was also observed (Figure 1, A through C). Proximal tubular sections were recognized by cell morphology and immunohistochemistry (Tamm–Horsfall-negative, aquaporin 2-positive sections; data not shown). To confirm this observation, PAR-1 protein expression was studied in vitro, by immunocytochemistry, in an immortalized and well characterized human PTC line (21). PAR-1 protein was strongly expressed by untreated cultured human PTC (hPTC) (Figure 2A). Moreover, as shown previously in mesangial and endothelial cells (28,29), also in hPTC, incubation with thrombin (5 U/ml) induced an almost complete downregulation of PAR-1-specific staining within 15 min (Figure 2B).

Thrombin is a powerful mitogen for several cell types in culture (26,27,30). It is conceivable that thrombin, frequently activated in the setting of acute and potentially reversible tubular damage, may represent a regenerative stimulus for hPTC. To support this hypothesis, the effect of thrombin on hPTC DNA synthesis was investigated. As shown in Figure 3, the serine protease induced a dose-dependent increase in tritiated thymidine uptake that reached statistical significance at 0.05 U/ml and peaked at 5 U/ml. The proliferative effect of thrombin was further confirmed by direct cell counting. Indeed, incubation with this protease, at the concentration of 5 U/ml, caused a statistically significant increase in PTC number after 24 and 48 h (Figure 4).

Interstitial monocyte infiltration is a histopathologic hallmark of acute and chronic tubulointerstitial disease (19). MCP-1 is a specific and powerful chemotactic factor for monocytes, and its expression is strikingly upregulated during the development of tubulointerstitial damage (20,31). The expression of this chemokine has been demonstrated to be induced by thrombin in vascular smooth muscle and in endothelial and mesangial cells, but not in epithelial cells (25,32,33). Thus, the effect of thrombin on MCP-1 gene expression was investigated in cultured hPTC. Thrombin at the dose that maximally stimulated DNA synthesis caused a marked upregulation of MCP-1 mRNA abundance that was evident already at 3 h and was still present after 24 h (Figure 5). To determine whether the increased MCP-1 mRNA levels correlated with an increased translation, the MCP-1 protein concentration was evaluated, by ELISA, in the supernatant of serum-starved hPTC after 24 h of incubation in serum-free medium in the presence and in the absence of thrombin (5 U/ml). As shown in Figure 6, thrombin induced a statistically significant increase in the MCP-1 protein released into cell supernatant.

Tyrosine phosphorylation of growth factor receptors plays...
an important role in their mitogenic and cell-activating effect (34). In the past few years, different G protein-coupled receptor agonists, including thrombin, have been shown to induce tyrosine phosphorylation of several cellular proteins (13,34). To investigate the effect of thrombin on tyrosine phosphorylation of cellular proteins in hPTC, equal amounts of protein from unstimulated and thrombin-stimulated cells were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting, using a specific anti-phosphotyrosine monoclonal antibody. Thrombin, at the dose that maximally stimulated DNA synthesis, caused a transient increase in the cellular levels of tyrosine-phosphorylated proteins, with the most prominent phosphorylated bands of 60, 70, and 90 kD (Figure 7). This early cellular effect of thrombin has been shown in different in vitro systems to be dependent on the activation of cytoplasmic and/or receptor tyrosine kinases. In platelets, thrombin stimulation induced a strong and rapid activation of c-src, a ubiquitous cytoplasmic tyrosine kinase, whereas in fibroblasts, keratinocytes, and COS-7 cells, thrombin has been shown to cross-activate the EGF receptor, a transmembrane tyrosine kinase (35,36). To investigate whether thrombin activates c-src in hPTC, the state of tyrosine phosphorylation of this enzyme was investigated as indirect evidence of its activation. For this purpose, cell lysates from unstimulated and stimulated PTC were immunoprecipitated with anti-phosphotyrosine antibodies and blotted with anti-src antibody. As shown in Figure 8, thrombin induced a time-dependent increase in tyrosine-phosphorylated c-src that peaked at 30 min. PTC express in vivo and in vitro the EGF receptor (37). Thus, the phosphorylation of this receptor in response to thrombin was also evaluated, but no EGF receptor tyrosine phosphorylation was observed upon thrombin stimulation (data not shown).

To determine the role of the early tyrosine kinase activation in thrombin-induced DNA synthesis and MCP-1 gene expression, the effect of a specific tyrosine kinase inhibitor, genistein, on these two cellular responses was evaluated. Genistein at a concentration of 25 μM, a dose that completely blocks tyrosine phosphorylation, abolished completely thrombin-induced DNA synthesis as well as MCP-1 expression, whereas its inactive analogue daidzein was unable to influence both thrombin effects (Figures 9 and 10). The central role of tyrosine kinase activation in thrombin-elicited DNA synthesis was further confirmed using a second specific tyrosine kinase inhibitor, herbimycin A, with a mechanism of action different from genistein (Figures 9 and 10). Herbimycin A inhibited thymidine incorporation and MCP-1 expression induced by thrombin to the same extent as genistein. To better define the role of src activation in thrombin-induced DNA synthesis, the effect of a specific src inhibitor, PP1, was investigated. Preincubation of PTC with PP1 at the concentration of 25 μM significantly inhibited the increase in DNA synthesis caused by thrombin, whereas PP3, the inactive analogue of PP1, at the same molar concentration, was unable to influence the thrombin proliferative effect (Figure 11).

Discussion
The potential role of the coagulation system in glomerular diseases has been investigated extensively over the past 30 yr (7–9). The accumulated evidence suggests that the activation
of the clotting cascade, revealed by fibrin deposition, may play a significant role in the development of several glomerular lesions (7–9). Although the local priming of the clotting cascade within the tubulointerstitium has been suggested by several studies (1–6), the potential involvement of the coagulation system in the pathogenesis of tubulointerstitial damage has been underestimated.

The present study demonstrated for the first time that PTC express both in vivo and in vitro PAR-1, and, therefore, may be considered potential targets for the modulatory action of thrombin. Interestingly, the thrombin receptor was localized mainly at the basolateral level. This localization, facing the interstitial space, may be pathogenically relevant. Indeed, fibrin deposits, the potential sites of thrombin accumulation, are mainly described in the peritubular capillaries and along the tubular basement membrane (1–6). In addition, in only a few tubular sections was luminal expression of PAR-1 observed. Although there are no reports on the presence of thrombin within the intratubular protein casts in proteinuric glomerular diseases, we hypothesize that thrombin generated within the glomerular tuft may reach the urinary space and activate the PTC by interacting with PAR-1 present at the luminal level.
The interaction of thrombin with the PAR-1 present on PTC leads to an increase in DNA synthesis and MCP-1 gene and protein expression. Both of these responses may be relevant in the setting of acute as well as chronic tubulointerstitial damage. Most of the acute conditions associated with interstitial fibrin deposition are characterized by potentially reversible tubular damage (2,4,6). Acute renal ischemia was the first pathologic condition in which this association was demonstrated (2). The ligation of the renal artery causes tubular necrosis and the intrarenal activation of the coagulation system with the subsequent extensive deposition of fibrin within the interstitial space (2,38). In this scenario, the ability of thrombin to act as a "growth factor" for PTC and stimulate a regenerative response may represent the first step toward the potential recovery, once the ischemic injury has been removed.

The ability of thrombin to induce MCP-1 gene and protein expression could represent a key event in the development of acute allograft rejection and in the progression of chronic rejection. Indeed, both acute and chronic renal graft rejection are characterized by a diffuse monocytic infiltrate (39). Monocytes, once recruited within the interstitial space, may represent a reservoir of cytokines and growth factors that can prime and maintain the activation of resident cells (40). Although monocytes may play a pivotal role in the pathogenesis of interstitial

Figure 5. Thrombin-stimulated monocyte chemotactic peptide-1 (MCP-1) gene expression in hPTC. Quiescent confluent PTC were stimulated with thrombin (5 U/ml) for the indicated time periods. The cells were then harvested and total RNA was isolated as described in Material and Methods. MCP-1 expression was evaluated by Northern blotting (top panel). 28S and 18S ribosomal RNA bands on ethidium bromide-stained gels were used to control the RNA loading (bottom panel). Results are representative of three experiments.

Figure 6. Thrombin-stimulated MCP-1 protein production and release in hPTC. Subconfluent and quiescent PTC were stimulated with thrombin (5 U/ml) for 24 h. At the end of the incubation period, the supernatant was collected and the cells were trypsinized and counted. MCP-1 protein concentration was determined by enzyme-linked immunosorbent assay as described in Materials and Methods and normalized to cell number. Data represent mean ± SD of three different experiments. *P < 0.01 versus control.

Figure 7. Thrombin-induced protein tyrosine phosphorylation in hPTC. Confluent quiescent PTC were stimulated with thrombin (5 U/ml) for 10, 30, and 60 min and then lysed in RIPA buffer. Equal amounts of protein from each cell lysate (7.5 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto nitrocellulose filters and probed with mouse monoclonal anti-phosphotyrosine antibody as described in Materials and Methods. Molecular mass markers are on the left. The arrows on the right indicate the main tyrosine-phosphorylated bands.
damage, the mechanisms underlying their influx into the interstitial space are still largely undefined. The local release of chemokines may represent the initial step in this event (19). In the growing chemokine family, MCP-1 represents the most specific and powerful chemotactic and activating factor for monocytes (41). We have recently demonstrated an increased MCP-1 expression at the tubular level in acute transplant rejection that was significantly correlated with monocyte infiltration (31). The infiltrating monocytes represent a major source of tissue factor, and thus powerful inducers of clotting cascade activation (42). The subsequent release of thrombin and its induction of MCP-1 production by PTC may stimulate a further influx and activation of circulating monocytes, closing a positive feedback loop and amplifying the phenomenon. Indeed, in human renal allograft rejection, activated interstitial macrophages are closely associated with fibrin deposits (43). Moreover, the hypothesis of a relevant role for thrombin in the setting of acute dysfunction of the renal allograft is further supported by the recent observation that proximal tubular cells express and produce anti-thrombin III, and the depletion of tubular anti-thrombin in the donor kidney is correlated with the degree of allograft function at 3 d after transplantation (44).

The mechanisms underlying thrombin-induced cell activation are still poorly understood. All of the known thrombin receptors, including PAR-1, belong to the G protein receptor superfamily (14–16). This class of receptors signal inside the cells through the interaction with one or more heterotrimeric G protein(s), leading to the activation of the phospholipase C–protein kinase C pathway on one side and to the modulation of adenylcyclase on the other (12,13,34). Although signaling receptors have always been rigidly divided in tyrosine kinase and G protein-coupled receptors, cross-talk between these two systems frequently occurs in rapidly induced cellular responses (34). In the past 10 yr, an increasing body of evidence suggested the activation of different tyrosine kinases in response to thrombin and the relevance of this phenomenon in thrombin-
induced cell activation (45–47). Interestingly, both cellular responses described in the present study relied on the activation of the same signaling pathway involving protein-tyrosine phosphorylation. Indeed, thrombin stimulated the tyrosine phosphorylation of an array of cellular proteins in cultured hPTC. Although the precise identity of these phosphoproteins remains to be determined, the 60-kD protein most likely represents one of the cytoplasmic tyrosine kinases of the c-src family. In platelets, thrombin has been shown to activate different tyrosine kinases of this family, including c-src, fyn, yes, and lyn (45). These observations were, at least partially, reproduced in other cell types (45). However, recently it has been demonstrated that the serine protease can cross-activate the EGF receptor in several cell lines (36,47). PTC express in vivo and in vitro PAR-1 and represent a potential target for thrombin. This serine protease, interacting with PAR-1 and activating c-src, might represent a regenerative and proinflammatory stimulus for PTC in acute and chronic tubulointerstitial damage.

**Acknowledgments**

This study was supported in part by a Baxter Extramural grant (eight round, 1996–1998), the Associazione per il Progresso Scientifico in Nefrologia e Trapianto (APSNT), the Consiglio Nazionale
delle Ricerche (C.N.R., 98.513.04), the CNR target project on Bio-
technology, the Ministero dell’Università e della Ricerca Scientifica e
Tecnologica (60%, 97.7703), and by a grant from the Istituto Supe-
riore di Sanità. We are grateful to Dr. L. F. Brass (Thomas Jefferson
University, Philadelphia, PA) for providing the anti-PAR-1 antibody.

References
1. Yamamoto K, Loskutoff DJ: The kidneys of mice with autoimmune
disease acquire a hypofibrinolytic/procoagulant state that corre-
lates with the development of glomerulonephritis and tissue
2. Enestrom S, Druid H, Rammer L: Fibrin deposition in the kid-
1988
3. Faulk WP, Gargiulo P, McIntyre JA, Bang NU: Hemostasis and
fibrinolysis in renal transplantation. Semin Thromb Hemostasis
15: 88–98, 1989
MG, Sacks SH: Upregulation of type 1 plasminogen activator
inhibitor mRNA with thrombotic changes in renal grafts. Trans-
plantation 61: 684–689, 1996
5. Wang Y, Pratt JR, Hartley B, Evans B, Zhang L, Sacks SH:
Expression of tissue type plasminogen activator and type 1
plasminogen activator inhibitor and persistent fibrin deposition
6. Wendt T, Zhang YM, Bierhaus A, Kriegsmann J, Deng Y,
Waldherr R, Teske T, Luther T, Funstuk R, Nawroth PP, Stein
G: Tissue factor expression in an animal model of hydropneu-
183–190, 1972
8. Sraer JD, Kanfer A, Rondeau E, Lacave R: Glomerular hemo-
stasis in normal and pathological conditions. Adv Nephrol 17:
27–56, 1988
9. Ono T, Kanatsu K, Doi T, Sekita K, Onoe C, Nagai H, Muso E,
Yoshida H, Tamura T, Kawai C: Relationship of intraglomerular
coagulation and platelet aggregation to glomerular sclerosis.
10. Mann KG, Lundblad RL: Biochemistry of thrombin. In: Ho-
meostasis and Thrombosis, edited by Colman R, Hirsh J, Marder
Selective immobilisation of α-thrombin by surface-bound fibrin.
13. Grand RJ, Turner AS, Graham PW: Cellular consequences of
of a functional thrombin receptor reveals a novel proteolytic
15. Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Tim-
mons C, Tram T, Coughlin SR: Protease-activated receptor 3 is
a second thrombin receptor in humans. Nature 386: 502–506,
1997
16. Xu WF, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching
A, Gilbert T, Davie EW, Foster DC: Cloning and characteriza-
tion of human protease-activated receptor 4. Proc Natl Acad Sci
USA 95: 6642–6646, 1998
17. Xu Y, Zacharias U, Peraldi MN, He CJ, Lu C, Sraer JD, Brass
LF, Rondeau E: Constitutive expression and modulation of the
functional thrombin receptor in the human kidney. Am J Pathol
146: 101–110, 1995
18. Wolf G: Cellular mechanisms of tubule hypertrophy and hyper-
plasia in renal injury. Miner Electrolyte Metab 21: 303–316,
1995
19. Van Goor H, Ding G, Kees-Folts D, Grond J, Schreiner GF,
Diamond JR: Macrophages and renal diseases. Lab Invest 71:
456–464, 1994
20. Grandaliano G, Gesualdo L, Ranieri E, Monno R, Montinaro V,
Marra F, Schena FP: Monocyte chemotactic peptide-1 expression
in acute and chronic human nephritides: A pathogenetic role in
interstitial monocyte recruitment. J Am Soc Nephrol 7: 906–913,
1996
Tork-Kostub B: HK-2: An immortalized proximal tubule epi-
thelial cell line from normal human kidney. Kidney Int 45:
48–57, 1994
22. Grandaliano G, Choudhury GG, Biswas P, Abboud HE: Mi-
togenic signaling of thrombin in mesangial cells: Role of tyrosine
human mesangial cell proliferation: Effect on PDGF β-receptor
by acid guanidinium thiocyanate-phenol-chloroform extraction.
tivity of thrombin: Stimulation of monocyte chemotactic pro-
26. He CJ, Peraldi MN, Adida C, Rebibou JM, Meulders Q, Sraer
JD, Rondeau E: Thrombin signal transduction mechanisms in
human glomerular epithelial cells. J Cell Physiol 150: 475–483,
1992
27. Grandaliano G, Pootic E, Barnes JL, Choudhury GG, Woodruff
K, Abboud HE: Thrombin stimulates platelet-derived growth
factor production in cultured bovine glomerular endothelial cells.
28. Hoxie JA, Ahuja M, Belmonte E, Pizarro S, Parton R, Brass LF:
Internalization and recycling of activated thrombin receptors.
29. Zacharias U, Xu Y, Haege J, Sraer JD, Brass LF, Rondeau E:
Thrombin, phorbol ester, and cAMP regulate thrombin receptor
protein and mRNA expression by different pathways. J Biol
30. Shultz PJ, Knauss TC, Mené P, Abboud HE: Mitogenic signals
for thrombin in mesangial cells: Regulation of phospholipase C
Schena FP: Monocyte chemotactic peptide-1 expression and
monocyte infiltration in acute renal transplant rejection. Tran-
YS, Valente AJ, Abboud HE: Thrombin regulates expression of
monocyte chemoattractant protein-1 in vascular smooth muscle
A: Expression of monocyte chemotactic protein-1 by monocytes
and endothelial cells exposed to thrombin. Am J Pathol 144:
975–985, 1994
34. Pouyssegur J, Seuwen K: Transmembrane receptors and intra-


