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Protein kinase C (PKC) dependent induction of tissue factor (TF) by mesangial cells in response to inflammatory mediators and release during apoptosis

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1 In inflammatory kidney diseases procoagulatory activity (PCA) becomes evident. Glomerular fibrin deposits and capillary microthrombi are histopathological hallmarks in most forms of glomerulonephritis.

2 Therefore in this study the expression of tissue factor (TF) as the main inducer of thrombogenesis was examined in cultured human mesangial cells (MC) in response to proinflammatory stimuli such as interleukin-1 (IL-1β), tumour necrosis factor alpha (TNF-α) and lipopolysaccharide (LPS). Also main signalling pathways were investigated.

3 IL-1β, TNF-α and LPS induced TF in MC in a time and dose dependent manner on mRNA and protein levels. Highest activity was found after 12 h of stimulation. Induction of TF was completely blockable by BAPTA-AM, a chelator of intracellular $[Ca^{2+}]_i$ as well as calphostin, a protein kinase C (PKC) inhibitor. Activation of the protein kinase A (PKA) pathway had no influence on basal TF expression, but down-regulated cytokine-induced TF. The PKA blocker, KT5720, increased TF formation significantly. Since TF exerts its activity primarily on the surface of cells and after release of encrypted receptors we further tested TF activity in MC supernatants. IL-1β did not significantly increase TF activity in supernatants of intact cells. However, when MC were rendered apoptotic by oxidative metabolites, IL-1β treated MC released highly stimulated TF activity into the supernatants, suggesting that a paracrine activation of the coagulatory cascade can take place under such conditions.

4 Inflammatory mediators up-regulate TF expression in MC by a PKC dependent pathway whereas PKA can serve as a negative feed-back link. Apoptosis of inflammatory MC may trigger to spread PCA.

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Keywords: Apoptosis; tissue factor; mesangial cell; IL-1β; PKC

Abbreviations: Calph, Calphostin C; CM, culture medium; DOG, 1,2-dioctanoyl-sn-glycerol; FCS, fetal calf serum; HMC, human mesangial cells; HUS, haemolytic uremic syndrome; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; MC, transfected mesangial cells; mRNA, messenger ribonucleic acid; PCA, procoagulatory activity; PKA, protein kinase A; RT–PCR, reverse transcription-polymerase chain reaction; TF, tissue factor; TNF-α, tumour necrosis factor-alpha

Introduction

Glomerular fibrin deposition frequently occurs in many inflammatory kidney diseases and is a typical histopathological manifestation e.g. in crescentic glomerulonephritis or lupus nephritis (Grandaliano *et al*., 2000). Fibrin may exert detrimental effects by direct cytotoxicity, by altering glomerular haemodynamics or by attracting leukocytes. In immune glomerulonephritis fibrin formation can depend on activation of glomerular fibrinolytic activity (Yamamoto *et al*., 1998). In mercuric chloride-induced autoimmune glomerulonephritis massive fibrin deposits are induced by glomerular PCA (Kanfer, 1989). Mesangial cells (MC) themselves have been proposed to contribute to PCA. After stimulation of MC with IL-1β TF gene expression was upregulated (Grandaliano *et al*., 2000). This was a hint for a direct involvement of stimulated glomerular cells by proinflammatory cytokines leading to release of PCA (Grandaliano *et al*., 2000). A recently published study showed that MC remarkably express factor V after stimulation with TNF-α (Ono, *et al*., 2001). Furthermore, it has been proposed that coagulation and fibrinolytic activity in the transplant setting are linked to each other (Faulk *et al*., 1989; Heidenreich *et al*., 1999).

Immunosuppressive regimens have been accused to exert these effects since it has been shown that OKT3-induced coagulopathy is at least in part a result of increased TF expression of monocytes after treatment with OKT-3 (Pradier *et al*., 1996). It has been shown that renal allograft recipients have higher TF levels in blood plasma when compared to healthy volunteers although there was no different TF activity in both groups (Malyszko *et al*., 1999). TF is the major

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initiator of the blood coagulation process. Besides thrombogenesis, TF is involved in angiogenesis and metastasis development. It is present on the surface of cells but is not fully active unless the cells are injured (Camerer *et al*., 1996; Lwaleed, 1999). The interaction of FVIIa and TF has a high affinity with a dramatic enhancement of the catalytic function of FVIIa following the activation of factors IX and X (Nemerson, 1988). TF also functions as a receptor for its ligand FVIIa mediating intracellular processes either by interactions of the TF cytoplasmic domain with the cytoskeleton or by supporting the VIIa-protease-dependent signalling. (Hansen *et al*., 2001; Ruf & Edgington, 1994; Ruf *et al*., 1991). It has been shown that receptor ligand binding of TF/FVIIa induces phospholipase C dependent intracellular calcium fluxes in monocyte-derived macrophages and has direct proinflammatory effects by inducing production of reactive oxygen species (Cunningham *et al*., 1999; Petersen *et al*., 2000; Ruf & Mueller, 1999).

In the glomerulus PCA can be derived from infiltrating leukocytes which are found in many forms of glomerulonephritis or during transplant rejection besides from intrinsic sources (Grau *et al*., 2001; Terry & Callahan, 1996; Tipping *et al*., 1995). Monocytes/macrophages exhibit pronounced PCA when stimulated by endotoxin, TNF-α and a variety of other immune mediators (Al-Saady *et al*., 1999; Yamabe *et al*., 1993). In the present study the role of human MC in generating TF and TF activity under inflammatory conditions and its regulatory pathways were studied.

Methods

Reagents

Calphostin C, 1.2-dioctanoyl-sn-glycerol (DOG), thapsigargin, BAPTA-AM, KT 5720, 8-bromo-cyclo-adenosinmonophosphat (8-br-cAMP) and TNF-α were obtained from Calbiochem (Bad Soden, Germany), IL-1β from Boehringer Mannheim (Mannheim, Germany). Reverse transcriptase was purchased from Stratagene (Heidelberg, Germany), Taq DNA polymerase from Gibco BRL (Karlsruhe, Germany), dNTPs from New England Biolabs (Beverly, MA, U.S.A.), pd(N)6 from Boehringer Mannheim (Mannheim, Germany) and agarose from AGS (Heidelberg, Germany). Bio-Rad protein assay was from BioRad (Munich, Germany), immubind tissue factor ELISA kit and actichrome TF activity assay from American Diagnostica (Pfungstadt, Germany). All other reagents were purchased from Sigma Chemical Co. (Saint Louis, MO, U.S.A.) unless otherwise indicated.

Cell culture

All experiments were performed with an immortalized human mesangial cell line (MC) gifted by B. Banas, Munich (Banas *et al*., 1999). Immortalization was performed by stable transfection with the SV40 large T antigen. The cell line was characterized by indirect immunofluorescence positively staining against fibronectin, smooth muscle actin, β1 integrin α1 and α5 chains, and SV40 large T antigen. MC were negative in indirect immunofluorescence staining for factor VIII, pan-cytokeratin, von Willebrand factor and desmin. With respect to these markers, and by detecting contractile responses to angiotensin II transfected MC showed identity to non-transfected MC. For control most experiments were also performed with MC derived from human kidneys without SV40 transfection (HMC). HMC were freshly isolated from unafflicted parts of kidneys after tumour nephrectomies (Heidenreich *et al*., 1997). MC culture medium (CM) for freshly isolated HMC and immortalized MC consisted of RPMI 1640 supplemented with 1 mM L-glutamine, 50 u ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Seromed, Berlin, Germany) and 1 mM sodium pyruvate. For the first two passages CM of freshly isolated HMC also contained 5 μg ml^{-1} bovine insulin, 5 mg ml^{-1} transferrin and 5 ng ml^{-1} selenit. CM was supplemented with 0.5–10% foetal calf serum (FCS) under normal culture conditions. For experiments HMC between the third and fifth passage were exclusively used after reduction of serum concentration to 0.5% unless otherwise indicated. Control experiments with freshly isolated HMC gave no significant differences to immortalized MC.

Semiquantitative RT–PCR

RNA isolation from MC after stimulation was conducted by RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. Before transcribing into cDNA, DNase (DNase I, RNase-free, Boehringer Mannheim, Germany) digestion was performed. cDNA was synthesized after the addition of 5 μM random primers ($pd(N)_6$, Roche Diagnostics, Mannheim, Germany), 1 mM dNTPs (NEB, Beverly, MA, U.S.A.) and incubation at 37°C with moloney murine leukaemia virus reverse transcriptase (Stratagene, Heidelberg, Germany). Contamination with DNA was excluded by performing PCR from templates incubated without reverse transcriptase. The primers used for PCR amplification were 5′ATG GAT GAT GAT ATC GCC GCG-3′ and 5′-TCT CCA TGT CGT CCC AGT TG-3′ (human β-Actin, 248 bp), as well as 5′-TAT CAC AGC CCT CTT CAC ACA TT-3′ and 5′-CCC CTT CTT TTC ACC ATT CCT A-3′ (human tissue factor, 726 bp). The PCR reaction mixture (40 μl) contained 2 mM $MgCl_2$, 0.2 mM dNTP, 1 μM primers, and 1 U Taq DNA polymerase. Samples were amplified during 30 cycles by 60 s denaturation at 94°C, 30 s annealing at 62°C (hsp70) or 55°C (β-actin) and 60 s elongation in a Peltier thermal cycler (Biometra Uno II Thermocycler, Biometra, Göttingen, Germany).

For semiquantitative PCR, the relation between the expression of β-actin and human tissue factor was analysed. Signal intensity as measured by PCR products was analysed on a 1.5% agarose gel and visualized by ethidum bromide staining. Densitometric quantification of PCR signals was performed by the Bio Image Intelligent Quantifier program (Bio Image, Ann Arbor, MI, U.S.A.).

Detection of TF protein expression by ELISA

Cells were lysated after washing with PBS without Ca/Mg with 2 ml of Triton-X-100 (1% v v^{-1}). Lysates were centrifugated for 60 min at 4°C with $38{,}000 \times g$ and supernatants without nuclei were used for detection of tissue factor protein after storage in −80°C until the assay. Total protein was quantified by the method according to Bradford with reagents from Bio-

Rad (Munich, Germany) [Bradford]. TF antigen of the lysates was measured using the Imubind® TF kit (American diagnostica, Pfungstadt, Germany) according to manufacturers instructions (Xuereb *et al*., 1997). In brief, lysates were incubated in microtiter plates conjugated with a mab against human TF, detecting TF-apoprotein, TF and complexes of TF and factor VII. A biotinylated antibody detected specifically the bound TF. Conjugation with a horseradish-POD converted tetra-methylbenzidine with a specific change of absorption at 450 nm. Results are expressed as nanograms of TF antigen per milligrams of total proteins (ng mg^{-1}).

Detection of TF activity by a chromogenic assay

In supernatants of cell cultures and cell lysates activity of TF was measured by actichrome® TF activity kit (American diagnostica, Pfungstadt, Germany) according to manufacturers instructions (Carson, 1987). In brief, samples were coincubated with factor VII and spectrozyme fVIIa. TF-FVIIa complex cleaves spectrozyme fVIIa as a highly specific chromogenic substrate releasing a paranitroanilin-chromophore with a specific change of absorption at 405 nm. Results are expressed in picomoles per litre (pM) of peptidyl activity of lipidated TF cleaving the spectrozyme fVIIa complex.

Induction and determination of apoptosis by flow cytometry

Mesangial cell apoptosis was induced by incubation with hydrogen superoxide in a concentration of 10 μM for 24 h. Apoptosis was determined by flow cytometry by propidium iodide (PI) staining of nuclei which is based on the principle that after DNA fragmentation permeabilized cells exhibit a reduced chromatin stainability and accessibility to fluorochromes (Heidenreich *et al*., 1997; Saleh *et al*., 2000). Mesangial cells were washed in PBS, fixed with 4% paraformadlehyde and permeabilized with 0.1% saponin. For staining, PI (5 μg ml^{-1}) was applied for 30 min before cells were washed again in sodium azide. Cells were analysed on a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.) for a total of 10,000 events.

Statistical analysis

Results are given as means $\pm$ s.d. For statistical analysis, Mann–Whitney *U* test and for paired comparisons Wilcoxon signed rank test or student's *t*-test was performed. $P<0.05$ was considered statistically significant. All experiments were performed at least five times.

Results

Time- and dose dependent regulation of TF mRNA, TF protein and TF activity in response to IL-1β

As shown in Figure 1, TF mRNA expression was induced by IL-1β in a time- and dose-dependent fashion. IL-1β in a concentration of 25 u ml^{-1} increased TF-mRNA significantly with a maximum at 12 h and returned to base line levels after 30 h shown by a semiquantitative RT–PCR in comparison to β-actin (Figure 1A). TF protein and TF activity similarly

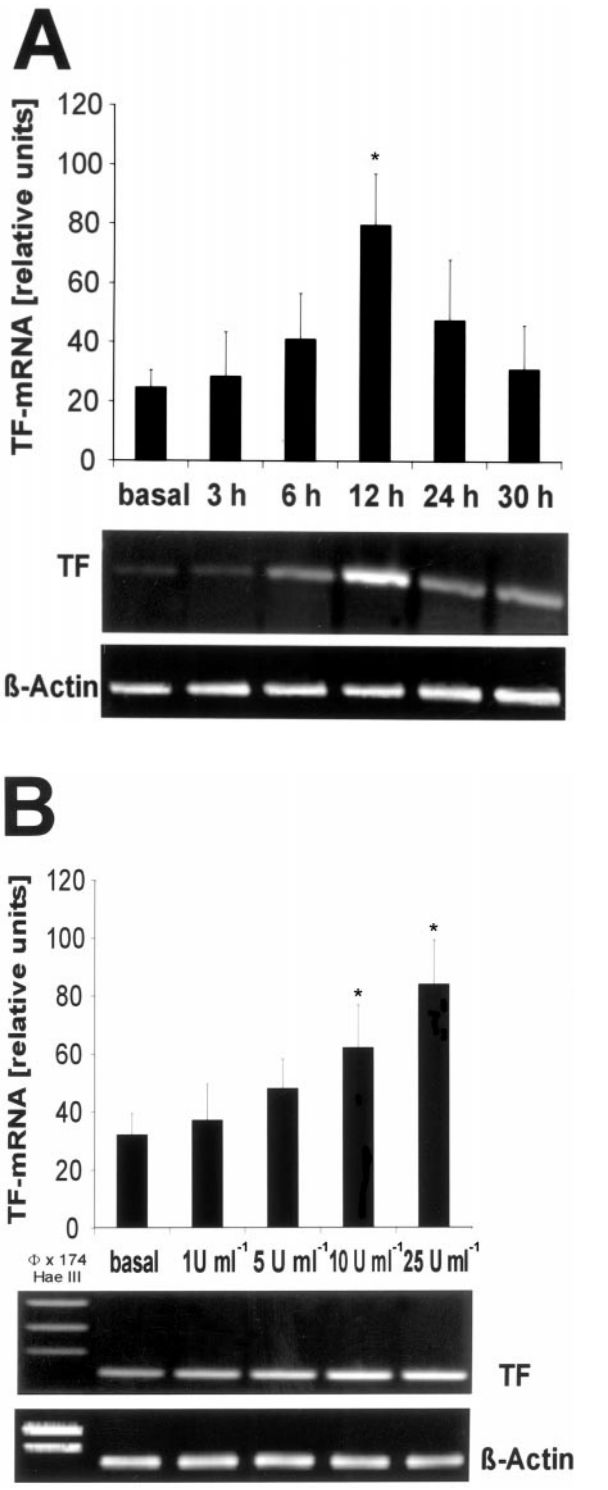

Figure 1 Time (A) and dose (B) dependent effects of IL-1β on TF-mRNA expression by MC. MC were synchronized and limited in growth by reducing serum concentration of culture medium as described under Methods before treated with IL-1β for indicated hours. TF mRNA expession in MC lysates was detected by a semiquantitative RT–PCR in comparison to β-actin. IL-1β in a concentration of 25 u ml^{-1} induced TF-mRNA with a peak after 12 h time dependently (A). Increasing concentrations of IL-1β after incubation of 12 h led to dose-dependent increase of TF mRNA expression (B). $*P<0.05$.

peaked after 12 h (data not shown). Studying dose-dependency, 25 u ml^{-1} IL-1β induced the highest TF responses on mRNA level (Figure 1B). TF protein was significantly increased after incubation with 10 u ml^{-1} IL-1β (31$\pm$4 ng mg^{-1}) and 25 u ml^{-1} IL-1β (39$\pm$3 ng mg^{-1}) for 12 h (Figure 2A). A relevant up-regulation of TF activity in cell lysates was already found after incubation with 5 u ml^{-1} IL-1β (4480$\pm$491 pM) for 12 h and higher concentrations further potentiated the formation (Figure 2B).

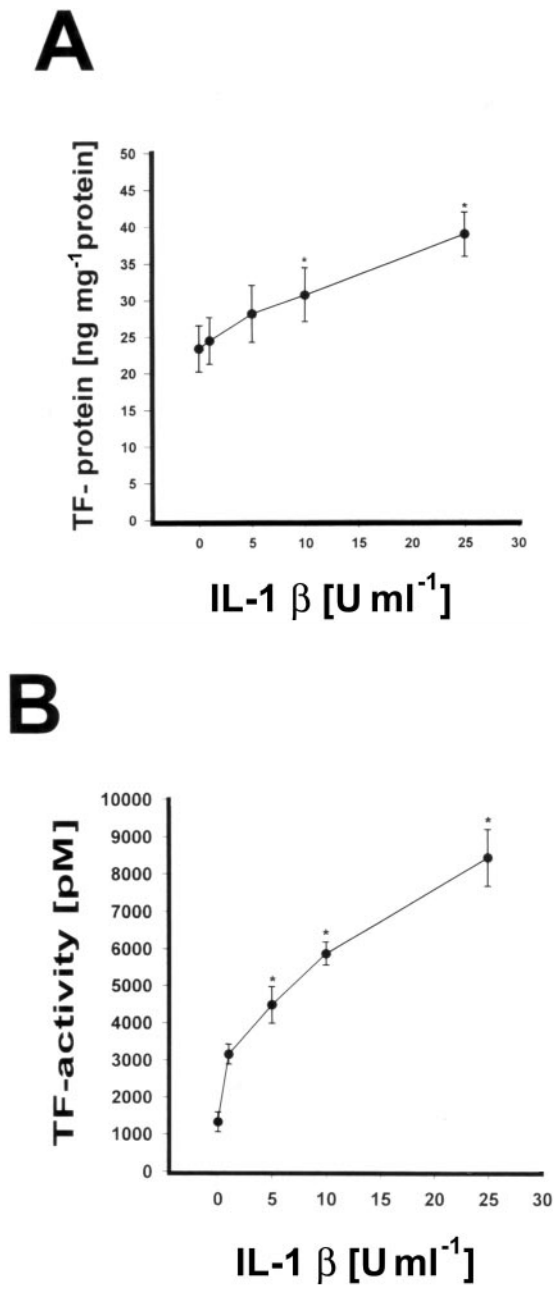

Figure 2 TF-protein expression (A) and TF-activity (B) in MC lysates after stimulation with IL-1β. MC lysates were investigated for TF-protein expression by ELISA by chromogenic assay as described under Methods. Significant increase of TF protein was found after incubation with 10 u ml^{-1} IL-1β (30.8$\pm$3.7 ng mg^{-1}) and 25 u ml^{-1} IL-1β (38.98$\pm$3.06 ng mg^{-1}) (A). Significant increase of TF activity was already seen after incubation with 5 u ml^{-1} IL-1β (4480$\pm$491 pM) followed by 5874$\pm$314 pM TF activity after stimulation with 10 u ml^{-1} IL-1β and 8451$\pm$762 pM TF activity after stimulation with 25 u ml^{-1} IL-1β (2B). *$P<0.05$.

Inflammatory mediators LPS and TNF-α and TF generation

Dose- and time dependent induction of TF was also found after stimulation with LPS (10 μg ml^{-1}) and TNFα (100 ng ml^{-1}) (Figure 3). These data are shown in Figure 3 for mRNA expression after 12 h. Additionally, proliferating MC, cultured in 10% FCS where studied which showed a significantly higher expression of TF mRNA as compared to resting cells (Figure 3).

HMC and MC do not exert different characteristics

Comparison of HMC and MC shown in Table 1 proved that no significant differences were found between freshly isolated HMC and transfected MC when cells were analysed for TF mRNA expression, TF protein determination and TF activity under base-line conditions or after IL-1β and LPS treatment.

Regulation of TF is dependent on PKC and [Ca^{2+}]$_i$

Figure 4 shows that TF is up-regulated by IL-1β using a PKC dependent pathway at mRNA level (Figure 4A) and protein level (Figure 4B). Calphostin C (1 μM), a selective PKC inhibitor completely abrogated the induction of TF by

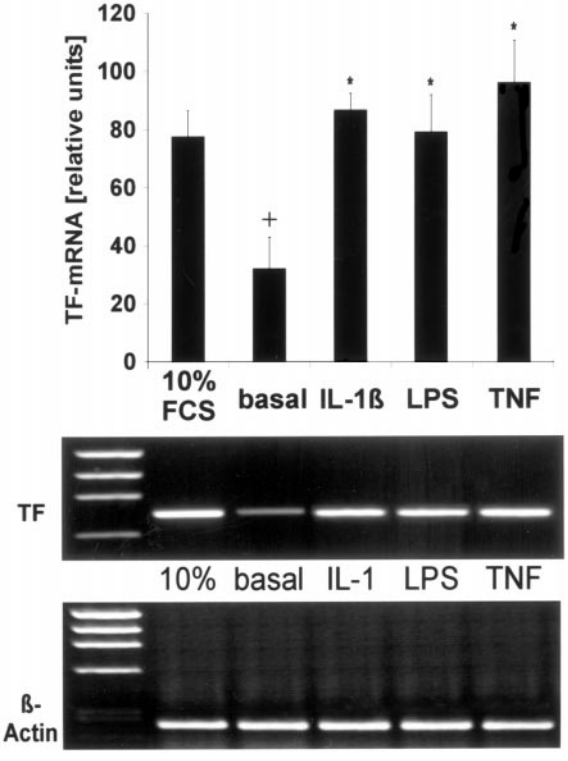

Figure 3 Comparison of effects on TF mRNA expression in MC by different proinflammatory stimuli. LPS (10 μg ml^{-1}) and TNFα (100 ng ml^{-1}) showed similar induction of TF RNA expression in comparison to IL-1 after 12 h in resting MC. Proliferating MC cultured in 10% FCS (first lane), expressed as high levels of TF mRNA as did stimulated resting cells. *$P<0.05$ as compared to basal; $^{+}P<0.05$ as compared to 10% FCS.

Table 1 Comparison of human mesangial cells (HMC) with transfected mesangial cells (MC) under different conditions. Significant differences between both cell types were not found. Data are given as means ± s.d.

	HMC			*MC*		
	Basal	*IL-1β* (25 U ml^{-1})	*LPS* (10 μg ml^{-1})	*Basal*	*IL-1β* (25 U ml^{-1})	*LPS* (10 μg ml^{-1})
TF-mRNA relative units	27 ± 5.6	84 ± 10.3	90 ± 11.1	32 ± 7.3	84 ± 15.4	79 ± 12.8
TF (ng mg^{-1} protein)	27.1 ± 4.3	37.8 ± 4.8	44.3 ± 3.3	22.7 ± 2.6	39.0 ± 3.1	37.4 ± 3.4
TF-activity (pM)	150 ± 10	780 ± 40	840 ± 160	148 ± 30	856 ± 137	607 ± 129

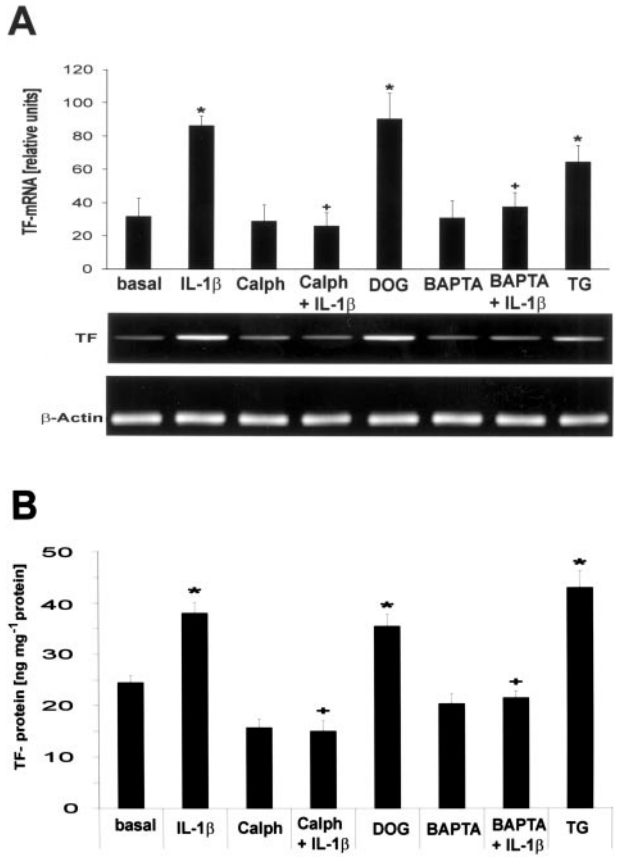

Figure 4 Blocking of PKC reduced IL-1β induced TF mRNA (A) and TF protein expression to base line (B) while increase of $[Ca^{2+}]_i$ enhanced TF. Incubation of MC with Calphostin C (Calph) (1 μM), a selective protein kinase C inhibitor completely abrogated the induction of TF mRNA expression (A) and protein (B) by IL-1β indicating that TF expression by IL-1 is mediated *via* a PKC dependent pathway. TF was enhanced by direct stimulation of PKC by 1,2-dioctanoyl-sn-glycerol (DOG) (1 μM), a cell-permeable activator of PKC, which induced TF (90.9 ± 15 relative units, 35.5 ± 2.3 ng mg^{-1}) similarly to IL-1β (86.6 ± 5.7 relative units, 38.0 ± 2.1 ng mg^{-1} protein). Preincubation with the intracellular Ca^{2+}-chelator BAPTA-AM (BAPTA) (10 μM) for 30 min significantly reduced induction of TF by IL-1β (31.4 ± 10.1 relative units, 20.4 ± 2.0 ng mg^{-1} protein). Increasing $[Ca^{2+}]_i$ by thapsigargin upregulated TF expression (TG, 100 nM, 65.1 ± 9.8 relative units, 43.1 ± 3.2 ng mg^{-1} protein) similarly as did IL-1β and DOG. $^*P<0.05$ as compared to basal; $^+P<0.05$ as compared to IL-1β.

IL-1β without exerting an influence on resting MC. 1,2-dioctanoyl-sn-glycerol (DOG) (1 μM), a cell-permeable activator of PKC, by itself induced TF (90.9 ± 15 relative units, 35.5 ± 2.3 ng mg^{-1} protein) similarly as did IL-1β. Preincubation with the intraellular Ca^{2+}-chelator BAPTA-AM (10 μM) for 30 min significantly reduced induction of TF by IL-1β (31.4 ± 10.1 relative units, 20.4 ± 2.0 ng mg^{-1} protein) whereas BAPTA-AM application alone did not alter base-line expression of TF. Increasing intracellular $[Ca^{2+}]_i$ by the Ca^{2+}-ATPase inhibitor thapsigargin (100 nM) significantly enhanced TF expression similarly as did IL-1β and DOG (65.1 ± 9.8 relative units, 43.1 ± 3.2 ng mg^{-1} protein). Compounds which increased TF and blocked IL-1β induced formation exerted their effects on mRNA and protein levels much in parallel.

PKA is involved in TF expression as a negative regulator

Figure 5 demonstrates that stimulated TF formation was associated with downregulation of PKA. The cell permeable cAMP analogue, 8-bromo-cAMP (0.5 mM), as well as the activator of adenylate cyclase, forskolin (10 μM), which both induce PKA activity were not able to alter basal TF expression by MC but reversed IL-1β dependent increase to base-line. This was true for TF mRNA levels and protein amounts (Figure 5A,B). The selective intracellular blocker of PKA, KT 5720 (5 μM), significantly increased basal and more markedly IL-1β dependent TF expression suggesting that PKA is involved in TF expression by MC as an intrinsic inhibitor.

Induction of apoptosis leads to an increased release of TF activity

Treatment of MC with hydrogen superoxide (10 μM) for 24 h induced apoptosis which was quantified by flow cytometry detecting DNA stainability of chromatin by PI as shown in Figure 6. Rates of necrotic cells were always below 3% as detected by trypan blue exclusion. Basal apoptotic rate was 5.0 ± 2.5% without a significant affection by IL-1β (4.5 ± 2.8%). Treatment with hydrogen superoxide led to a significant increase of apoptotic cells (17.7 ± 3.0%) without affection by IL-1β (16.1 ± 3.6) (Figure 6A). Detection of TF-activity in supernatants of hydrogen superoxide treated MC (Figure 6B) gave higher amounts of TF-activity (83 ± 13.4 pM) in comparison to basal (41 ± 10.9 pM) or IL-1β-stimulated (54 ± 14.8 pM) conditions. IL-1β stimulated MC that were rendered apoptotic by hydrogen superoxide exerted a highly potentiated release of TF activity into culture supernatants (150 ± 13.2 pM). Necrotic MC that were destructed by repeated thawing and freezing missed this burst of TF activity (data not shown).

Discussion

Prothrombotic states occur in numerous human and experimental renal diseases and a lot of evidence indicates

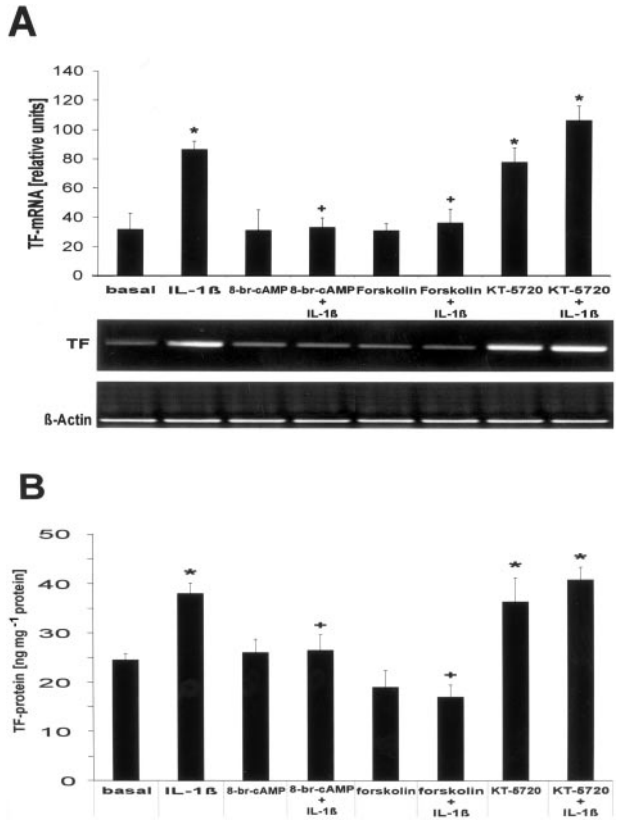

Figure 5 PKA and increases of cAMP reversed IL-1β induced TF mRNA (A) and TF protein expression (B) while blocking of PKA enhanced TF. 8-bromo-cAMP (8-br-cAMP, 0.5 mM), a cell permeable cAMP analogue which induces PKA activity reversed the induction of TF by IL-1β to basal levels (33.5 ± 6.4 relative units, 26.5 ± 2.5 ng mg^{-1} protein). So did forskolin (10 μM), a cell permeable activator of adenylate cyclase which also induces PKA *via* release of cAMP (36.6 ± 9.3 relative units, 16.9 ± 2.6 ng mg^{-1} protein). Blocking of PKA by stimulation with KT5720 (5 μM), a cell permeable specific inhibitor of PKA increased TF mRNA (78.0 ± 9.6 relative units) and protein expression (36.3 ± 4.9 ng mg^{-1} protein) without exerting additive effects when applied together with IL-1β (106.6 ± 9.6 relative units, 40.7 ± 2.5 ng mg^{-1} protein). $^{*}P<0.05$ as compared to basal; $^{+}P<0.05$ as compared to IL-1β.

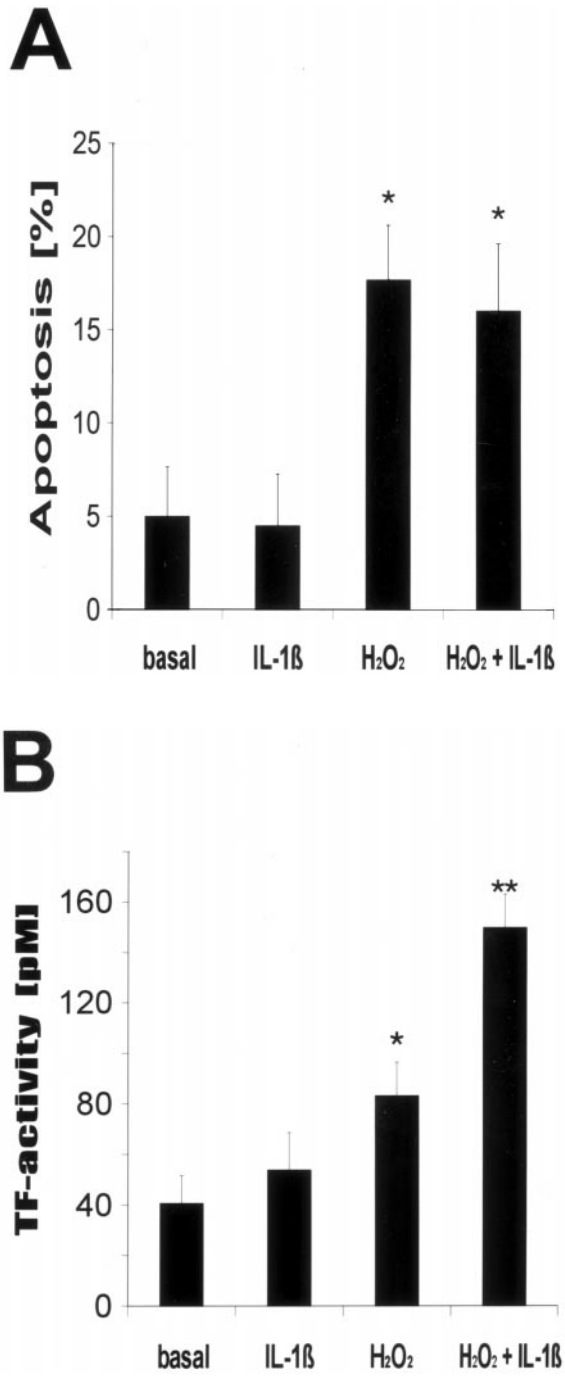

Figure 6 Induction of apoptosis in MC by hydrogen superoxide (A) led to a significant increase of release of TF into cell culture supernatants which was additionally augmented by IL-1β (B). Treatment of MC with hydrogen superoxide (10 μM) for 24 h induced apoptosis ($17.7\pm3.0\%$) without or with additional application of IL-1β (16.1 ± 3.6) as determined by flow cytometry. Basal apoptotic rate was low ($5.0\pm2.5\%$) without significant affection by IL-1β ($4.5\pm2.8\%$) (A). Detection of TF-activity in supernatants of MC proved that cells undergoing apoptosis released a significantly higher amount of TF-activity (83 ± 13.4 pM) in comparison to basal (41 ± 10.9 pM) or IL-1β-stimulated (54 ± 14.8 pM) conditions. TF release was potentiated in MC treated with H_2O_2 and IL-1β. (B). $^{*}P<0.05$ as compared to basal; $^{**}P<0.05$ as compared to H_2O_2.

that coagulation activation is linked to glomerular injury (Kanfer, 1989). Presence of glomerular fibrin deposits, capillary microthrombi and complete glomerular capillary occlusion are histological hallmarks of PCA (Grandaliano *et al*., 2000). In one-third of patients with glomerulonephritis fibrin deposition can be found and even more dramatically renal PCA is observed in acute renal failure due to disseminated intravascular coagulation or in HUS (Gordon & Kwaan, 1999; Mehta *et al*., 2001; Neale *et al*., 1988; Proesmans, 2001). In these entities signs of prothrombotic states may be used as prognostic markers. Fibrin deposits can damage glomerular structures by interrupting blood flow in glomerular capillaries; by direct cytotoxicity to MC and by participating in inflammatory reactions with consecutive attraction of monocytes and macrophages (Grandaliano *et al*., 2000; Tipping *et al*., 1988). Since the latter cells are a rich source of PCA it has been reasoned to what extent intrinsic glomerular cells contribute to activation of coagulation. The presented data clearly indicate that HMC in culture were able to produce TF on mRNA and protein levels. TF as the principal initiator of the extrinsic coagulation casade is a member of the cytokine receptor family and serves as a

receptor or cofactor for coagulation factor VII/VIIa (Bach, 1998; Hölschermann *et al*., 1999; Schecter *et al*., 1997; Watanabe *et al*., 1999). TF is normally found on surfaces of fibroblasts and smooth muscle cells which come in contact to plasmatic coagulation factors after damage of vessels and tissues (Camerer *et al*., 1996). TF participates in cell signalling by two distinct mechanisms: one *via* a proteolytic mechanism where TF interacts with its physiological ligand factor VIIa in the extracellular compartment. Extracellular signalling, e.g., regulates localization of TF to different membrane domains where it can be found on smooth plasmamembrane areas, on cellular processes containing unique cytoskeletal structures or in fine plasmalemma vesicles called carveolae (Ruf & Mueller, 1999). On the other hand, the short cytoplasmic domain of TF participates independently in intracellular signalling of protease function (Prydz *et al*., 2000; Prydz, 1999).

Our work, however, was not conducted to elucidate the consequences of TF upregulation in MC which are probably numerous as expected from work with other cells (Napoleone *et al*., 1997). We could show that inflammatory mediators, e.g. IL-1β, TNF-α and LPS highly stimulated TF expression. A previous study by Wiggins *et al*. (1990) examined TF production by cultured rat MC which similarly found stimulation by TNF-α and LPS. However, this work analysed TF activity merely indirectly by determining PCA in a functional clotting assay and not by direct TF expression on mRNA and protein levels as we did. Our different and more specific analytical methods together with the different origin of cells may be the reason that we could find TF upregulation also by IL-1β which was missed in the former publication. Additionally questions about the pathway used by IL-1 to activate cells have been risen (O'Neill, 1992). Especially the role of protein kinases was a focus of interest. In this field much more data on IL-1 dependent signalling are available for lymphocytes as compared to MC (Knop *et al*., 1998; Lang *et al*., 1998; Micheau & Riedel, 1999). We could show that PKC upregulation was required for TF stimulation in MC. This process was dependent on rises of $[Ca^{2+}]_i$. A similar PKC dependent regulation of cytokine-induced TF transcription has been previously described for endothelial cells (Pettersen *et al*., 1992). In a second step we could demonstrate that PKA acted as a negative intrinsic regulator of TF expression, that means that cAMP increases abolished the cytokine-induced TF upregulation and that the specific PKA antagonist, KT-5720, could elevate TF by itself. Also this cAMP-dependent down-regulation of prothrombotic effects and TF induction has been confirmed in former studies, e.g. using monocytes, and thus seems to reflect a highly conserved principle of PCA regulation acting in distinct cell types (Lyberg, 1983; Ollivier *et al*., 1993). By elucidating these intrinsic pathways of TF regulation pharmacological targets are established by which fibrin deposition may be effectively inhibited in human renal diseases and thus helping to find general treatment modalities.

MC undergoing apoptosis significantly released higher amounts of TF activity as compared to intact cells. Our experiments, however, could not exclude for sure whether released TF overwhelmingly came for apoptotic cells or whether apoptotic MC indirectly stimulated intact cells for TF release. After induction of apoptosis captured TF is delivered more from IL-1β stimulated MC as opposed to resting cells. Since other apoptosis inducing stimuli, e.g. UV-light showed similar effects on TF release, we can assume that putative stimulating effects of hydrogen peroxide were not responsible for release of captured TF. These data indicate that at least two stimuli are required by which MC release high amounts of TF, one inflammatory and one apoptotic signal. A proliferative stimulus may be substituted for the inflamatory as we could show that proliferating MC were equally effective to form TF as cytokine-stimulated cells. Under healthy conditions MC do not proliferate in the kidney. The interrelation between MC growth and apoptosis has been described in different glomerular diseases, e.g. in experimental anti-Thy 1 nephritis, but also in cultured MC (Baker *et al*., 1994; Harrison, 1988; Saleh *et al*., 2000). Although apoptosis may help to down-regulate an enhanced cell number within the glomerulus after a proliferative signal, exactly this sequence of events may be the substratum for TF induction, release and spread of PCA with putative unfavourable consequences (Greeno *et al*., 1996). Lupus nephritis may serve as a good example for the association of apoptosis with prothrombotic states in human renal disease. Systemic lupus is characterized by a general tendency of accelerated cell senescence and apoptotic cell death, and in this disease apoptotic cells have been described within the glomerulus (Berden & van Bruggen, 1997; Kodera *et al*., 1997; Pickering *et al*., 2000). Of special interest, particularly in lupus nephritis microthrombi formation in the glomerular capillaries is a histological hallmark together with or without other prothrombotic factors such as antiphospholipid antibodies (Hughson *et al*., 2001). In summary, our study detected cytokine-dependent TF expression and release in MC together with the involved signal transduction pathways. The findings underline the contribution of intrinsic glomerular cells for PCA and point to a network between MC, inflammatory cytokine releasing cells and apoptotic events in renal diseases characterized by local capillary clotting.

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