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Plasminogen Activator Inhibitor 1 Expression is Regulated by the Inflammatory Mediators Interleukin-1α, Tumor Necrosis Factor-α, Transforming Growth Factor-β and Oncostatin M in Human Cardiac Myocytes

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K. Macfelda, T. W. Weiss, C. Kaun, J. M. Breuss, B. Kapeller, G. Zorn, U. Oberndorfer, M. Voegele-Kadletz, R. Huber-Beckmann, R. Ullrich, B. R. Binder, U. M. Losert, G. Maurer, R. Pacher, K. Huber and J. Wojta. Plasminogen Activator Inhibitor 1 Expression is Regulated by the Inflammatory Mediators Interleukin-1α, Tumor Necrosis Factor-α, Transforming Growth Factor-β and Oncostatin M in Human Cardiac Myocytes. *Journal of Molecular and Cellular Cardiology* (2002) **34**, 1681–1691. Accumulating evidence points towards a role for proteases and protease inhibitors in tissue remodelling and repair in a variety of organs. In particular—besides the matrix metalloprotease system—the plasminogen activator (PA)/plasmin system has been implicated in these processes in the heart. Urokinase type PA (u-PA) and PA inhibitor type 1 (PAI-1) seem to modulate cardiac rupture and infarct healing. In this study we aimed to investigate whether inflammatory mediators can regulate the expression of components of the PA/plasmin system in human adult cardiac myocytes (HACM). We could demonstrate that HACM, isolated from pieces of myocardial tissue by mechanical dispersion and characterized by positive immunostaining for the cardiac markers troponin I, tropomyosin, cardiotin and myocardial muscle-actin, *in vitro* express PAI-1 and tissue type PA (t-PA) whereas u-PA was not detectable in these cells. PAI-1 protein production was increased up to twofold by interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) and up to fivefold by transforming growth factor-β (TGF-β) and oncostatin M (OSM). Similar changes were observed in PAI-1 transcript levels after cytokine treatment. t-PA production in HACM was not affected by these agonists. No effect of these cytokines on PAI-1 production in fibroblasts isolated from human myocardial tissue was seen. In an *ex vivo* model we could show that incubation of pieces of human myocardial tissue with these cytokines also resulted in an increase in PAI-1 in cardiac myocytes as evidenced by immuno-histochemistry. Furthermore we found increased

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PAI-1 expression in myocardial tissue from a patient suffering from acute myocarditis. Thus for the first time we provide evidence that inflammatory mediators modulate PAI-1 expression in human adult cardiac myocytes *in vitro* and *ex vivo* and could demonstrate that PAI-1 expression is increased in the *in vivo* setting under inflammatory conditions. If the effect on PAI-1 expression brought about by IL-1α, TNF-α, TGF-β and OSM is not only operative under *in vitro* and *ex vivo* conditions but also in the *in vivo* setting one could speculate that these cytokines contribute to upregulation of PAI-1 in myocardial tissue and that PAI-1, when upregulated in myocardial tissue during inflammatory processes, could serve as a defence mechanism against excessive matrix degradation by proteases. Thus we propose a role for PAI-1 produced in the heart by cardiac myocytes in cardiac remodelling and repair processes.

KEY WORDS: PAI-1; Cardiac myocytes; Inflammatory mediators; Cardiac repair.

Introduction

Recently accumulated evidence points towards a major role for the plasminogen activator (PA)/plasmin system in wound healing and tissue repair in various organs.[1–4] Cardiac repair after myocardial infarction is a highly complex process involving inflammation, matrix remodelling and adaptive responses of cardiac myocytes. In patients who survive acute infarction, scar formation, wall-thinning of the infarcted region during the repair process and cardiac rupture are well recognized complications of this disease.[5,6] A recent publication has implicated the PA/plasmin system in cardiac repair mechanism pathways after acute myocardial infarction.[7] In this study it could be shown that mice deficient in urokinase-PA (u-PA) were completely protected against cardiac rupture after myocardial infarction.[7] However these mice showed impaired scar formation and infarct revascularization and died of cardiac failure, emphasizing the importance of proteolysis during later stages of the repair process.[7] The fact that temporary administration of the major physiological inhibitor of u-PA, namely PA inhibitor-1 (PAI-1) into wild type mice completely protected these animals against cardiac rupture but on the other hand did not impair infarct healing highlights the need for a refined balance between proteases and protease-inhibitors in such disease processes characterized by tissue injury and repair.[7]

With more than 1.5 million people suffering from acute myocardial infarction in the United States every year, insight into the regulation of cardiac repair mechanisms after myocardial infarction seems to be highly relevant. Studying modulation of components of the PA/plasmin system in the heart might contribute to our understanding of this complex process. PAI-1 expression has been demonstrated in the heart of mice and rats.[8,9] However, not much is known about the regulation of its expression in human myocardium. Inflammatory mediators like interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), oncostatin M (OSM) and transforming growth factor-β (TGF-β) have been shown to regulate PAI-1 expression in other cell types such as endothelial cells, smooth muscle cells and fibroblasts.[10–16] We therefore tested the hypothesis whether these cytokines are possible regulators of PAI-1 expression in human cardiac myocytes and thus might contribute to the process of cardiac repair after myocardial infarction by regulating the proteolytic balance between PAs and their inhibitor PAI-1.

Materials and Methods

Materials

Recombinant human (rh) IL-1α, rh TNF-α, rh TGF-βr, rh OSM, rh HGF, rh LIF, and rh EGF were purchased from R&D Systems (Minneapolis, MN, USA). Human α-thrombin was obtained from American Diagnostica (Greenwich, CT, USA). Morpholinopropane sulfonic acid (Serva, Germany), piperazine-N,N′-bis[2-ethane sulfonic acid] (PIPES; Sigma, St Louis, MO, USA), Seakem LE Agarose (FMC Bioproducts, Rockland, ME, USA), dCTP [Aloha-32P] (ICN Pharmaceuticals, Costa Mesa, CA, USA), were obtained from the sources indicated.

Isolation and cultivation of human adult cardiac myocytes (HACM)

Primary cultures of human adult cardiac myocytes (HACM) were prepared from ventricular tissue obtained from donor hearts from patients undergoing heart transplantation. Ventricular tissue was minced into fine fragments with scissors in phosphate buffered saline, pH 7.4 (PBS) without enzymatic digestion. The fragments were rinsed twice with PBS to remove red blood cells and passed through a cell strainer (40 μm, Gibco-Life Technologies, Paisley, UK) to remove debris and large aggregates. The cellular filtrate was centrifuged at 1200 rpm for 10 min and the resulting pellet was washed twice with PBS. Cells were resuspended in

Dulbecco's Minimal Essential Medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (all obtained from Gibco), placed in a Petri-dish and incubated for 60 min at 37°C in a humidified atmosphere of 5% CO_2: 95% air to separate myocytes from fibroblasts by preplating. After incubation the non-attached cells were decanted, centrifuged and rinsed twice with PBS. Thereafter, the cell pellet was resuspended in DMEM containing 10% FCS as well as 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml transferrin (Sigma) and 10 μg/ml insulin (Sigma). This procedure resulted in cell populations of >95% rod-shaped cells with a viability of >90% [Fig. 1(A)]. HACM were seeded at a cell density of approximately 1×10^4 cells/cm^2 into culture flasks that had been coated with fibronectin (Roche, Basel, Switzerland). Fibronectin coating was performed by adding 5 μg/cm^2 fibronectin in PBS, pH 7.4 to each culture flask followed by incubation at room temperature for 1 h prior to the addition of cardiac myocytes. The unbound fibronectin was decanted before the addition of myocytes. Thereafter, cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2:95% air. Confluent cultures of HACM that had altered their shape from rod to oval and round were obtained after approximately 10 days under these conditions. The attached cardiomyocytes flattened out transversely and extended cytoplasmatic processes of various shapes and sizes [Fig. 1(B)]. These cells were either transferred into 24 well plates treated with fibronectin as described above for cytokine-experiments or were seeded onto chamber slides (Falcon, Heidelberg, Germany) for immuno-histochemical characterization (see below).

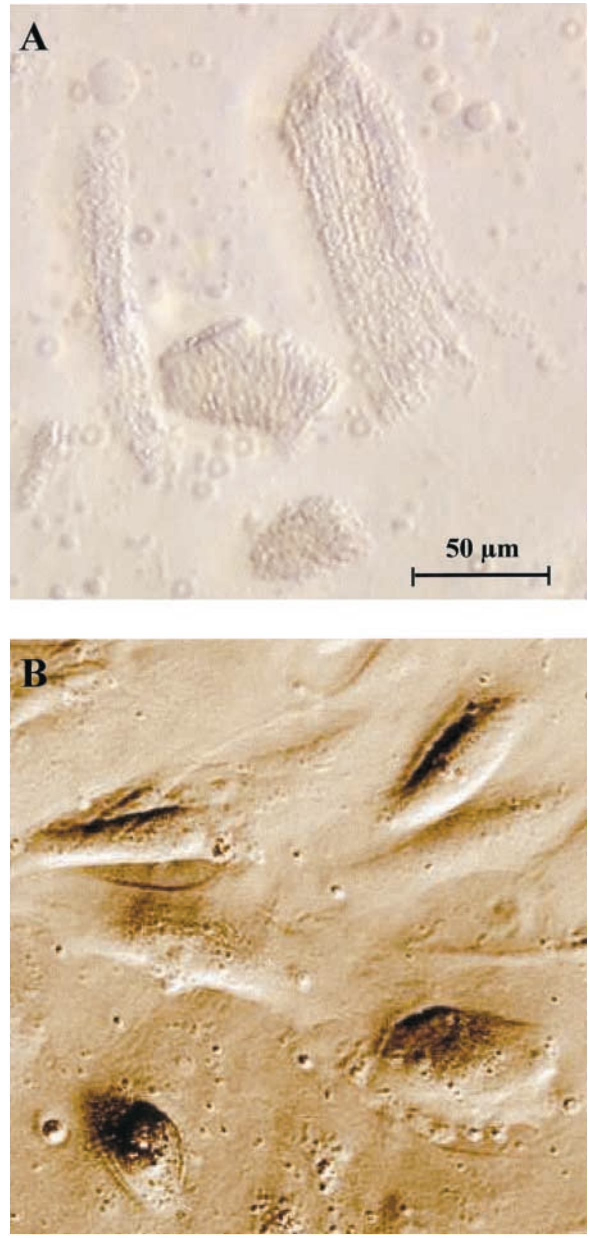

Figure 1 Phase contrast micrographs of human adult cardiac myocytes (HACM). HACM immediately after isolation at day 0 (panel A), and after 10 days in culture (panel B).

Characterization of human adult cardiac myocytes (HACM)

In order to characterize cultured HACM the cells were immuno-histochemically stained for troponin I, tropomyosin, cardiotin and myocardial muscle-actin. Cells were also stained for desmin, vWF and fibroblast specific antigens to rule out contamination of the cultures with smooth muscle cells, endothelial cells and fibroblasts, respectively.

Briefly, the cells were cultured on chamber slides, washed 3 times with PBS and fixed with cold acetone at 4°C for 10 min. After washing three times with PBS containing 0.02% Tween 20, the cells were exposed to antibodies against troponin I (dilution 1:100; Santa Cruz, Santa Cruz, CA, USA), tropomyosin (dilution 1:50; Sigma), cardiotin (dilution 1:100; Chemicon, Temecula, CA, USA), myocardial muscle-actin (dilution 1:50; Dako; Glostrup, Denmark), smooth muscle-actin (dilution 1:100, Dako), vWF (dilution 1:50; Dako) and fibroblast specific antigens (dilution 1:100; antibody 1B10, Dako; antibody 5B5, Dako) for 1 h at room temperature. The control group cells were incubated with PBS under the same conditions. The cells were washed three times with PBS containing 0.02%

Tween 20. For immunofluorescent staining the second antibody, a goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC; Dako) diluted 1:10 in PBS containing 1% BSA, was added. The cells were incubated with the second antibody under dark and humid conditions for 1 h at room temperature and the slides were analysed by using an immunofluorescence microscope and Image Analysis™ software (SYS, Muenster, Germany). Replicate slides treated as described above were used for phosphatase-based immuno-histochemical analysis employing an LSAB Staining Kit (Dako). The cells were incubated with the second antibody for 30 min at room temperature, then streptavidin alkaline phosphatase was added followed by incubation for 15 min at room temperature. Finally the chromogen substrate solution (Fuchsine) was added for 10 min at room temperature. For counter-staining Papanicolaou 1B solution (Merck, Darmstadt, Germany) was used. LSAB stained cells were analyzed by phase contrast microscopy using the same software as described above.

Only cultures in which >95% of the cells stained positive for cardiac myocyte markers (troponin I, tropomyosin, cardiotin and myocardial muscle actin) were used in this study. In these cultures contamination with smooth muscle cells, endothelial cells and fibroblasts as judged by staining for smooth muscle actin, vWF and fibroblast specific antigens was <2%.

Isolation and characterization of human adult cardiac fibroblasts (HACFB)

Human adult cardiac fibroblasts (HACFB) were isolated by the explant technique and characterized as described recently.[17] Briefly pieces of myocardial tissue were placed in a Petri dish and covered with a drop of Medium 199 (M199, Sigma) containing 10% FCS as well as 100 U/ml penicillin and 100 μg/ml streptomycin. After 3 to 5 days the explants became adherent and the Petri dish was filled with M199 containing FBS and antibiotics as described above. HACFB growing out from the explants were grown to confluence and cultured at 37°C in a humidified atmosphere of 5% CO_2:95% air. >95% of these cells stained positive for fibroblast specific antigen. HACFB did neither stain for the cardiac myocyte markers troponin I, tropomyosin, cardiotin and myocardial muscle actin, nor for the endothelial marker vWF nor for smooth muscle actin ruling out contamination with myocytes, smooth muscle cells and endothelial cells, respectively.

Treatment of human adult cardiac myocytes (HACM) or human adult cardiac fibroblasts (HACFB) with cytokines

Human adult cardiac myocytes (HACM) or human adult cardiac fibroblasts (HACFB) were incubated in DMEM containing 0.1% bovine serum albumin (BSA; Sigma) for 24 h prior to treatment with the respective cytokine. Thereafter the medium was replaced with fresh M199 containing 0.1% BSA, and IL-1α, TNF-α, TGF-β or OSM, respectively, were added at the concentrations indicated. After incubation for 24 h, the culture supernatants were collected following removal of cell debris by centrifugation and stored at −70°C until used. The total cell number of the respective cultures after trypsinisation was counted with a haemocytometer. In selected experiments HACM incubated as described were transferred to chamber slides for immunohistochemical analysis. No difference in staining characteristics to HACM stained before cytokine treatment was seen.

Plasminogen activator inhibitor-1 (PAI-1), tissue type-plasminogen activator (t-PA) antigen and urokinase type plasminogen activator (u-PA) antigen assays

PAI-1, t-PA and u-PA antigen in conditioned media were determined by specific enzyme-linked immunosorbent assays (ELISAs) using monoclonal antibodies (Technoclone, Vienna, Austria). The PAI-1 ELISA measures active, complexed and latent PAI-1. The t-PA ELISA measures free and complexed t-PA. The u-PA ELISA measures single chain and two chain u-PA.

Northern blots

Total cellular RNA was prepared by the guanidinium thiocyanate-phenol-chloroform extraction from confluent cells treated as indicated. RNA samples were electrophoresed in 1.2% agarose gels, transferred to a Duralon-UV™ membrane (Stratagene, La Jolla, CA, USA). Hybridisations were performed over night in 50 mM PIPES, 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, 5% SDS, containing 10^6 cpm/ml of the ^{32}P labelled cDNA probes for human PAI-1 or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After hybridisation the membranes were washed in 5% SDS, 1 × SSC at room temperature for 10 min, then washed in the same buffer at 57°C for 20 min three times. Autoradiography was performed with XAR-5 X-ray films (Eastman Kodak, Rochester, NY, USA) at −70°C.

Ex-vivo immuno-histochemical determination of plasminogen activator inhibitor-1 (PAI-1) in human myocardial tissue

Small pieces (approximately 0.2 cm^3) of fresh myocardial tissue were incubated in DMEM containing 0.1% BSA in the presence or absence of 200 U/ml IL-1α, 2000 U/ml TNF-α, 0.5 ng/ml TGF-β or 100 ng/ml OSM for 24 h at 37°C. Thereafter the tissue was rinsed with PBS and frozen sections were prepared in Tissue Tek™ OCT (Sakura Finetechnical, Tokyo, Japan) in liquid nitrogen. Four μm sections were cut and fixed with cold acetone (4°C). To inhibit endogenous peroxidase (POX), POX-block was performed with periodic acid. After incubation with normal goat serum (dilution 1:100; Dako) a monoclonal anti PAI-1 antibody (5PAI-12, Technoclone) at a concentration of 10 μg/ml was added and the sections were incubated for 1 h at room temperature. Thereafter a biotinylated antibody (dilution 1:100; Dako) was added for 1 h. After rinsing with PBS the sections were incubated with a streptavidin-POX complex (dilution 1:100; Dako) 30 min at room temperature. PAI-1 was visualized by addition of DAB (Diaminobenzidine; Dako) for 20 min. Counterstaining was performed with Hematoxyilin (Merck).

Immuno-histochemical determination of plasminogen activator inhibitor-1 (PAI-1) in healthy human myocardial tissue and in human myocardial tissue with acute inflammation

Paraffin embedded pieces of healthy human myocardium obtained from a donor heart that was unsuitable for transplantation and paraffin embedded pieces of myocardium obtained by biopsy from a patient suffering from acute myocarditis were subjected to immuno-histochemical staining for PAI-1 as described above.

Statistical analysis

Data were compared statistically by Student's *t*-test for paired observations. Values of $P \leq 0.05$ were considered significant.

Results

As can be seen from Figure 1(A) freshly isolated human adult cardiac myocyte exhibited the characteristic rod-shaped phenotype. After approximately 5–10 days in culture the cells had flattened and acquired a round to oval shape with various cytoplasmatic processes [Fig. 1(B)]. When such confluent cells after 5–10 days in culture were subjected to immunostaining as described in the Materials and Methods section, only cultures in which >95% of the cells stained positive for cardiac myocyte markers (troponin I, tropomyosin, cardiotin and myocardial muscle actin) were further used in this study. In these cultures contamination with smooth muscle cells, endothelial cells and fibroblasts as judged by staining for smooth muscle actin, vWF and fibroblast specific antigens was <2%.

Table 1 summarizes the results obtained when HACM isolated from five different hearts were treated with IL-1α (200.0 U/ml), TNF-α (2000.0 U/ml), OSM (100.0 ng/ml) or TGF-β (0.5 ng/ml), respectively. As can be seen in all five preparations of HACM the cytokines increased the production of PAI-1. No effect of these cytokines on tissue type-PA (t-PA) production by HACM was observed (Table 2). u-PA was not detected in conditioned media obtained from HACM (data not shown).

Table 1 Influence of IL-1, TNF-α, TGF-β or OSM on PAI-1 production in human adult cardiac myocytes (HACM)

	Donor #2	Donor #3	Donor #4	Donor #5	Donor #6
Control	103.8 ± 12.1	105.2 ± 12.7	93.3 ± 11.0	339.9 ± 26.1	91.2 ± 10.8
IL-1 (200 U/ml)	n.d.	197.3 ± 5.2	224.5 ± 26.3	777.7 ± 58.9	204.5 ± 17.8
TNF-α (2000 U/ml)	233.0 ± 19.5	215.5 ± 3.6	244.4 ± 8.6	815.6 ± 64.3	262.7 ± 23.9
TGF-β (0.5 ng/ml)	393.6 ± 31.3	367.3 ± 7.2	436.0 ± 46.9	1280.3 ± 98.2	543.4 ± 45.3
OSM (100 ng/ml)	496.0 ± 36.2	486.0 ± 13.2	n.d.	1417.6 ± 97.5	n.d.

Confluent monolayers of HACM were incubated for 24 h in the absence or presence of the respective cytokine at concentrations indicated. Conditioned media of such treated cells were collected and PAI-1 antigen was determined as described in the Materials and Methods section. Values are given in ng/10^4 cells/24 h and represent mean values ± SD of three independent determinations. n.d.: not determined.

Table 2 Influence of IL-1, TNF-α, TGF-β or OSM on t-PA production in human adult cardiac myocytes (HACM)

	Donor #2	Donor #3
Control	1.9 ± 0.2	3.0 ± 0.4
IL-1 (200 U/ml)	2.0 ± 0.2	3.2 ± 0.2
TNF-α (2000 U/ml)	1.6 ± 0.1	2.7 ± 0.5
TGF-β (50 ng/ml)	2.1 ± 0.1	3.2 ± 0.4
OSM (100 ng/ml)	2.2 ± 0.2	3.5 ± 0.4

Confluent monolayers of HACM were incubated for 24 h in the absence or presence of the respective cytokine at concentrations indicated. Conditioned media of such treated cells were collected and t-PA antigen was determined as described in the Materials and Methods section. Values are given in ng/10^4 cells/24 h and represent mean values $\pm$ SD of three independent determinations.

Table 3 Influence of IL-1, TNF-α, TGF-β or OSM on PAI-1 production in human adult cardiac fibroblasts (HACFB)

	Donor #1	Donor #2
Control	252.6 ± 47.3	341.9 ± 40.0
IL-1 (200 U/ml)	260.7 ± 36.8	352.7 ± 35.9
TNF-α (2000 U/ml)	236.1 ± 29.7	n.d.
TGF-β (50 ng/ml)	247.0 ± 15.9	n.d.
OSM (100 ng/ml)	273.0 ± 33.9	333.6 ± 23.8

Confluent monolayers of HACFB were incubated for 24 h in the absence or presence of the respective cytokine at concentrations indicated. Conditioned media of such treated cells were collected and PAI-1 antigen was determined as described in the Materials and Methods section. Values are given in ng/10^4 cells/24 h and represent mean values $\pm$ SD of three independent determinations. n.d.: not determined.

As can be seen from Table 3 these cytokines did not affect PAI-1 production in HACFB.

Table 4 shows that other known modulators of the fibrinolytic system such leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and thrombin did not affect PAI-1 production in HACM.

HACM from three different donors were treated with increasing concentrations of IL-1α (0.002–200 U/ml), TNF-α (0.02–2000 U/ml), TGF-β (0.0005–50 ng/ml) or OSM (0.001–100 ng/ml), respectively. A similar dose dependent increase in PAI-1 production by all three preparations of HACM in response to the respective cytokine was observed. A representative experiment using cells from one donor is depicted in Figure 2. Maximum effects were obtained with 200 U/ml of IL-1α, 2000 U/ml of TNF-α, 0.5 ng/ml of TGF-β or 100 ng/ml of OSM (control: 209.0 ± 14.5 ng/10^5 cells/24 h; IL-1α: 389.8 ± 15.2 ng/10^5 cells/24 h; TNF-1α: 464.0 ± 21.9 ng/10^5 cells/24 h; TGF-β: 538.9 ± 34.8 ng/10^5 cells/24 h; OSM: 1189.1 ± 29.7 ng/10^5 cells/24 h).

As shown in Figure 3 the effect of the respective cytokines was also evident on the level of

Table 4 Influence of LIF, HGF, EGF or thrombin on PAI-1 production in human adult cardiac myocytes (HACM)

	Donor #2	Donor #3
Control	103.8 ± 12.1	105.2 ± 12.7
LIF (100 ng/ml)	95.3 ± 10.7	95.0 ± 6.5
HGF (100 ng/ml)	99.3 ± 12.0	115.3 ± 12.1
EGF (500 ng/ml)	110.0 ± 6.0	97.7 ± 9.4
Thrombin (10 U/ml)	120.4 ± 19.6	123.0 ± 11.3

Confluent monolayers of HACM were incubated for 24 h in the absence or presence of the respective compounds at concentrations indicated. Conditioned media of such treated cells were collected and PAI-1 antigen was determined as described in the Materials and Methods section. Values are given in ng/10^4 cells/24 h and represent mean values $\pm$ SD of three independent determinations.

specific mRNA expression. IL-1α (200.0 U/ml), TNF-α (2000.0 U/ml), OSM (100.0 ng/ml) or TGF-β (50 ng/ml) increased PAI-1 mRNA levels in HACM.

When pieces of fresh myocardial tissue were incubated with IL-1α, TNF-α, TGF-β or OSM immuno-histochemistry with anti PAI-1 antibodies revealed an increase in PAI-1 in cardiac myocytes as evidenced by the more intense and extensive staining (Fig. 4).

When healthy myocardial tissue and myocardial tissue from a patient suffering from acute myocarditis was subjected to immuno-histochemistry-analysis for PAI-1 a more intense staining for this protein was evident in the diseased heart (Fig. 5).

Discussion

More than 1.5 million people suffer from acute myocardial infarction in the United States every year. A highly complex process involving inflammation, matrix remodelling and adaptive responses of cardiac myocytes regulates cardiac repair after myocardial infarction. There is accumulating evidence that activation of proteases and their regulated inhibition are key events in tissue repair processes. In line with this view matrix metalloproteases (MMPs) have been implicated in ventricular remodelling and dilation after myocardial infarction. It was shown that inhibition of matrix metalloproteases attenuates left ventricular enlargement after experimental myocardial infarction in mice.[18] Furthermore in samples of human ventricular tissue obtained from patients with heart failure a downregulation of tissue inhibitors of metalloproteases (TIMPs) and an upregulation of MMP 9 has been observed.[19] In an *in vitro* study it has been shown that the proinflammatory cytokines TNF-α and IL-1β modulate the expression of TIMPs and metalloproteases in cardiac cells.[20] Recently also

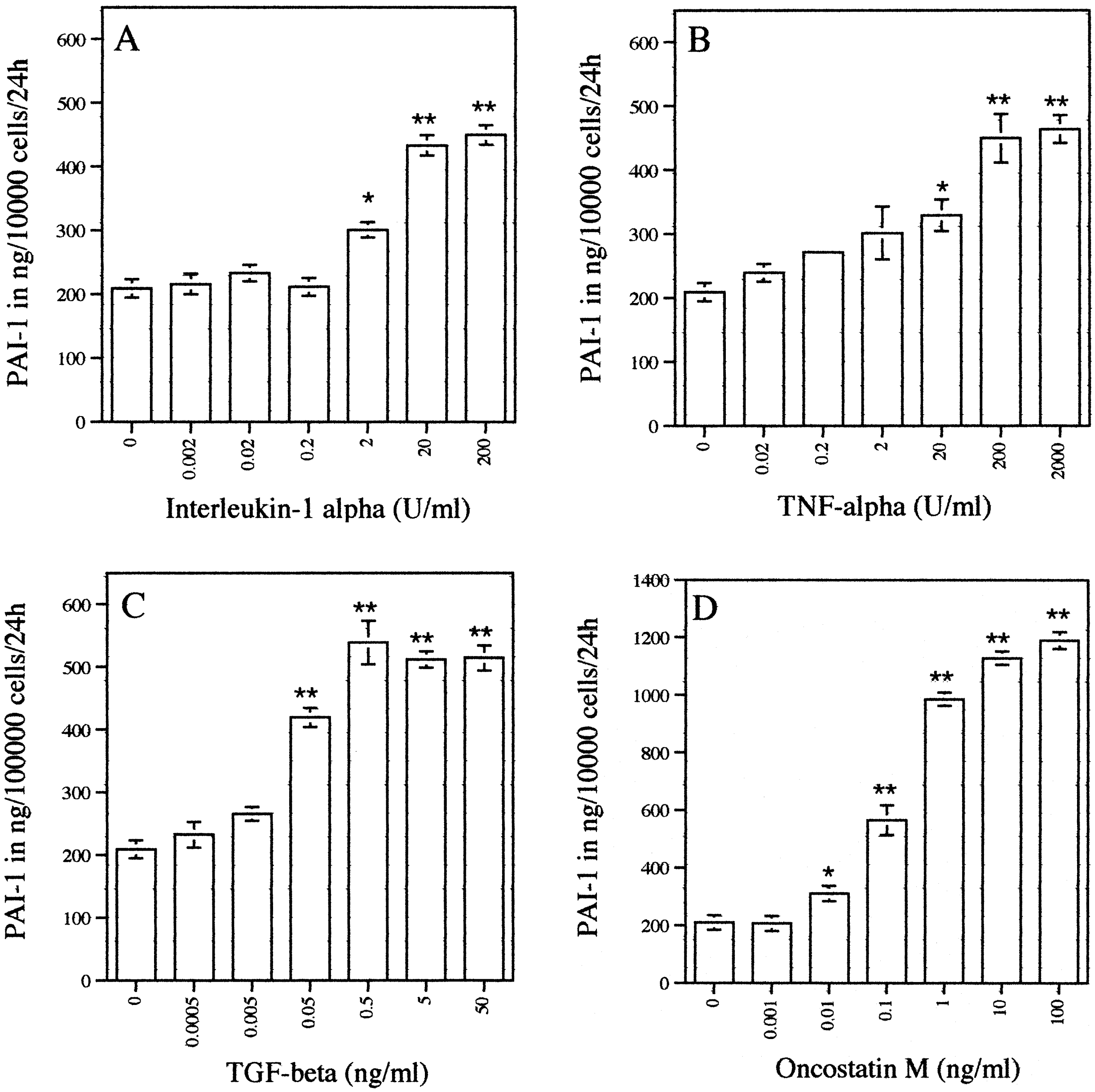

Figure 2 Effect of IL-1α, TNF-α, TGF-β and OSM on PAI-1 production in human adult cardiac myocytes (HACM). Confluent monolayers of HACM were incubated for 24 h in the absence or presence of rh IL-1α (panel A), rh TNF-α (panel B), rh TGF-β (panel C) or rh OSM (panel D) at the indicated concentrations. Conditioned media of such treated cells were collected and PAI-1 was determined as described in the Materials and Methods section. Values are given in ng/10^4 cells/24 h and represent mean values $\pm$ SD of three independent determinations. Experiments were performed three times with a representative experiment shown. $^{**}P<0.01$, $^{*}P<0.05$.

the plasminogen activator/plasmin system has been implicated in tissue remodelling and repair processes in the heart. It could be shown that u-PA and PAI-1 were upregulated in swine in ventricular hypertrophy and during coronary occlusion.[21,22] Furthermore a role for u-PA and PAI-1 has been established in scar formation and cardiac rupture after myocardial infarction.[7] Cardiac rupture is a well recognized complication in patients who survive acute infarction and is responsible for 4–24% of all in-hospital deaths related to myocardial infarction.[5,6] In the abovementioned study it was shown that mice deficient in u-PA were completely protected against cardiac rupture but showed impaired scar formation and revascularization of the infarcted area and died of heart failure. Interestingly, adenoviral-assisted administration of PAI-1 protected wild type mice from cardiac rupture but did not impair infarct healing.[7] This study points towards an important role for PAI-1 in a setting in

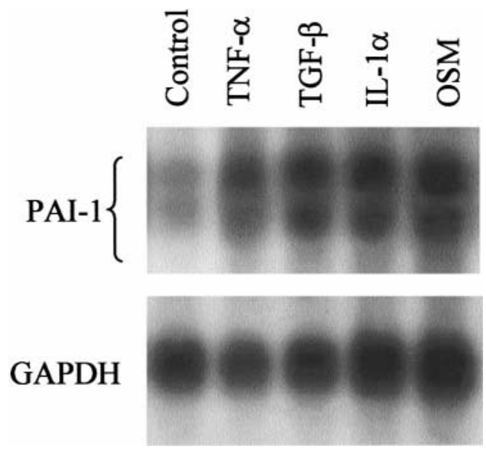

Figure 3 Effect of IL-1α, TNF-α, TGF-β and OSM on PAI-1 mRNA expression in human adult cardiac myocytes (HACM). Confluent monolayers of HACM were incubated for 8 h in the absence (lane 1) or presence of rh TNF-α (2000 U/ml; lane 2), rh TGF-β (0.5 ng/ml; lane 3), rh IL-1α (200 U/ml; lane 4), or rh OSM (100 ng/ml; lane 5). mRNA of such treated cells was prepared and PAI-1 and GAPDH mRNA was visualized by Northern Blotting as described in the Materials and Methods section. Experiments were not statistically analyzed but performed two times with a representative experiment shown.

which highly regulated spatio-temporal expression of components of the PA/plasmin system seems to be critical. It should also be noted that a recent study has implicated the PA/plasmin system also in the modulation of repair processes in skeletal muscle.[23]

PAI-1 expression has been shown to be upregulated by endotoxin and inflammatory mediators in the hearts of mice and rats.[8,9] However, not much information is available on the regulation of PAI-1 in human cardiac cells. In this paper we provide evidence for the first time that human adult cardiac myocytes *in vitro* express PAI-1 and that this expression is significantly upregulated by the inflammatory mediators IL-1α, TNF-α, TGF-β and OSM in a dose dependent way. PAI-1 production was increased approximately twofold by IL-1α and TNF-α, whereas an up to fivefold increase in PAI-1 was seen when the cell were treated with either TGF-β or OSM. The possibility that the increase in PAI-1 production in response to the agonists was caused by contaminating cells in our cultures of human adult cardiac myocytes can be excluded by the fact that >95% of the cells in these cultures stained positive for cardiac myocyte markers whereas only <2% stained positive for smooth muscle cell, endothelial cell or fibroblasts markers. It should also be emphasized that we could demonstrate that in cultured human adult cardiac fibroblasts PAI-1

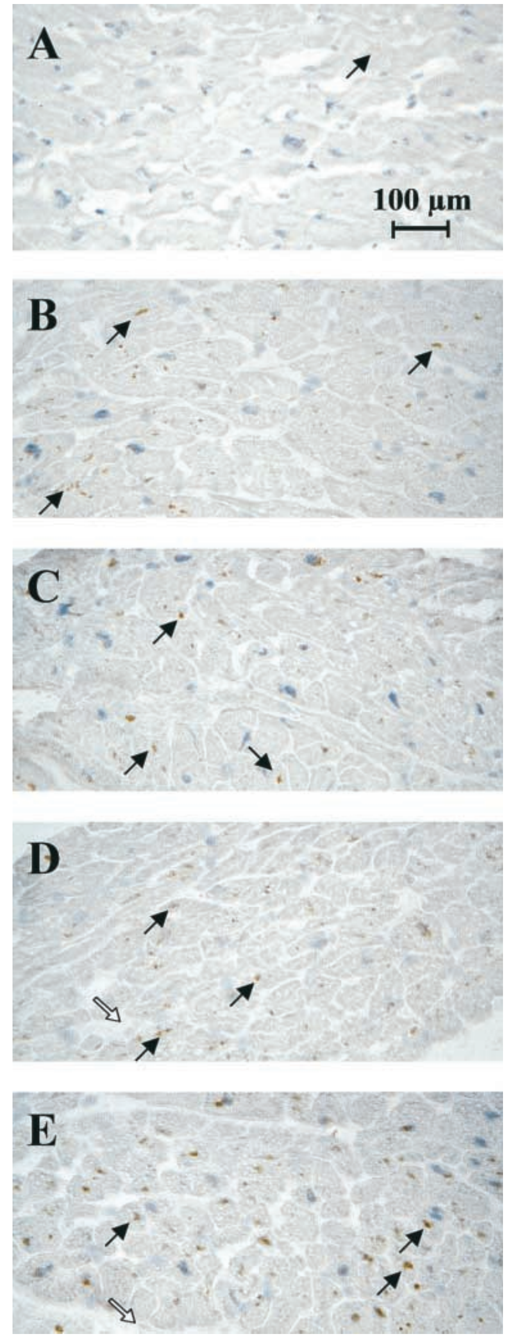

Figure 4 Effect of IL-1α, TNF-α, TGF-β and OSM on PAI-1 expression in human myocardial tissue. Pieces of fresh myocardial tissue were incubated in the absence (panel A) or presence of 200 U/ml IL-1α (panel B), 2000 U/ml TNF-α (panel C), 0.5 ng/ml TGF-β (panel D) or 100 ng/ml OSM (panel E) for 24 h at 37°C and PAI-1 was visualized as described in the Materials and Methods section. Note that the brown staining for PAI-1 is predominantly located within cardiac myocytes after cytokine treatment and is hardly visible in untreated tissue. Examples of PAI-1-positive cardiac myocytes are highlighted by black arrows whereas examples of PAI-1-negative non-myocytes are indicated by white arrows. Experiments were performed three times with a representative experiment shown.

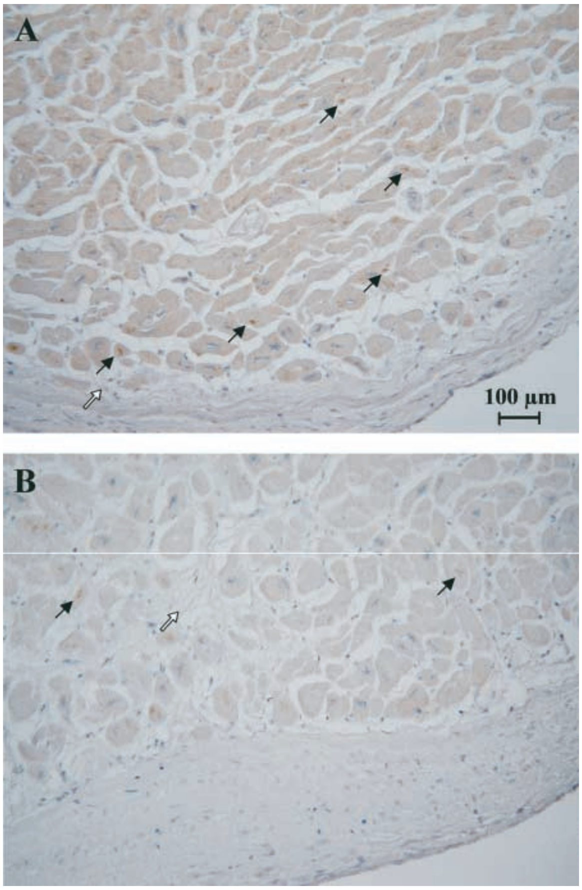

Figure 5 PAI-1 expression in healthy human myocardial tissue and in human myocardial tissue obtained from a patient suffering from acute myocarditis. PAI-1 was visualized as described in the Materials and Methods section. More intense and extensive PAI-1 staining in cardiac myocytes is visible throughout the tissue obtained from the patient suffering from acute myocarditis (panel A) as compared to the healthy myocardium (panel B). Examples of PAI-1 positive cardiac myocytes are highlighted by black arrows whereas examples of PAI-1 negative non-myocytes are indicated by white arrows.

production was not affected by these cytokines. The data obtained on the level of protein production were also confirmed on the level of specific mRNA expression. IL-1α, TNF-α, TGF-β and OSM induced an increase in PAI-1 specific mRNA in cardiac myocytes consistent with the differences determined by ELISA. Other modulators of PAI-1 expression in other cell types such as LIF, HGF, EGF, or thrombin did not affect PAI-1 production in human cardiac myocytes.[24–29] Furthermore we could demonstrate that, in accordance with previous studies in rats, human adult cardiac myocytes produce t-PA.[8,30] In contrast to PAI-1, however, neither of the mediators tested did affect t-PA production in these cells. u-PA was not detected in our cultures of cardiac myocytes on the protein level.

In addition to these *in vitro* results we could also show that IL-1α, TNF-α, TGF-β and OSM induce PAI-1 production in an *ex vivo* setting. When pieces of myocardial tissue were treated with these agonists a significant increase in PAI-1 was visualized in cardiac myocytes. In agreement with previous studies done by others a strong induction of PAI-1 was also seen in microvascular endothelium under these conditions (Data not shown).[10,14–16] Furthermore we provide evidence that PAI-1 expression is increased in the myocardium in the *in vivo* setting under inflammatory conditions, namely in myocardial tissue obtained from a patient suffering from acute myocarditis. It should be mentioned, however, that no difference in OSM expressing cells between healthy and inflamed myocardium was observed. This could be possibly due to the kinetics of the progression of the disease with OSM expressing cells not longer being present in significant numbers at the time when biopsy was performed (data not shown).

In conclusion, we provide evidence for the first time that inflammatory mediators modulate PAI-1 expression in human adult cardiac myocytes *in vitro* and *ex vivo* and we were able to show an increase in PAI-1 expression under inflammatory conditions *in vivo*. It should be noted that there is ample evidence for a role for IL-1α, TNF-α and TGF-β in the development and progression of heart disease whereas such a role for OSM has yet to be established.[31–34] Furthermore numerous studies have shown the presence of such mediators directly in the heart under inflammatory conditions.[35–38] If the effect on PAI-1 expression brought about by IL-1α, TNF-α, TGF-β and OSM is not only operative under *in vitro* and *ex vivo* conditions but also in the *in vivo* setting one could speculate that these cytokines contribute to upregulation of PAI-1 in myocardial tissue in inflammatory processes. Such upregulation of PAI-1 could serve as a defence mechanism against excessive matrix degradation by proteases. In such a setting PAI-1 would inhibit u-PA thereby preventing direct extracellular proteolysis by this PA on the one hand and activation of latent metalloproteases by u-PA which in turn would also degrade extracellular matrix on the other hand. Thus we propose a role for PAI-1 produced in the heart by cardiac myocytes in cardiac remodelling and repair processes.

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