in vivo *24*: 647-652 (2010)

uPA, uPAR and TGFβ$_1$ Expression during Early and Late Post Myocardial Infarction Period in Rat Myocardium

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Abstract. *The expression patterns of transforming growth factor beta 1 (TGFβ_1), urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) were analysed after artery ligation-induced myocardial infarction (MI) in the rat myocardium. uPA and uPAR expressions were significantly increased both at transcriptional and protein level during early phase post MI period (uPA at 1 hour and uPAR at 24 hours post infarction). TGFβ1 mRNA expression profile revealed a significant increase of TGFβ1 expression from day 4 up to 8 weeks post infarction. These data suggest that the need for an increasing TGFβ_1 bioavailability during the post-infarction period in rat myocardium is achieved in the early post MI period by an increased expression of uPA/uPAR proteolytic system (indirect activation of latent TGFβ_1) and in the late post MI period by direct regulation of TGFβ_1 expression. It is therefore concluded that differential regulation of the TGFβ_1 bioavailability may be a crucial step of the repair mechanisms during the post MI infarction period in the rat myocardium.*

Myocardial infarction (MI) induces a process of cardiac muscle regeneration, which includes gross morphological, histological, and molecular changes of both the infracted and residual non-infracted myocardium (1-3). Wound healing is a complex process of invasion, transformation and apoptosis of various cell types which is highly regulated and may well be subject to regulatory influences, disturbances and imbalances (4).

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Key Words: TGFβ$_1$, uPA, uPAR, myocardial infarction.

Myocardial extracellular matrix (ECM) mainly consists of collagen, fibronectin and elastin, and forms a three-dimensional network which supports the cellular and structural integrity of the heart. The imbalanced production and degradation of the ECM is an important process for the development of myocardial dysfunction after MI (5). Notably, ECM participates in the regulation of the activity of various growth factors, such as insulin-like growth factors (IGFs) and transforming growth factor beta 1 (TGFβ$_1$), in physiological and pathophysiological processes (6-10). Urokinase-type plasminogen activator (uPA) and plasmin are implicated in several non-fibrinolytic processes, which lead to ECM degradation, either directly by proteolytic cleavage of extracellular matrix components, or indirectly through the activation of matrix metallo-proteinases (MMPs) in several tissues and pathologies (11, 12). However, uPA and MMPs regulate growth factor activities, such as the hydrolysis of the IGF-binding proteins (IGFBPs) and the activation of the latent-TGFβs and other growth substances as noted in various tissues (13-15).

TGFβs are a family of multifunctional growth factors, namely TGFβ$_1$, β2 and β3. A common characteristic of all TGFβs is their ability to bind to extracellular proteins and to be stored in the ECM until activation by a physiological process, such as wound healing (16). *In vitro*, TGFβs can affect stem cell proliferation and differentiation in a dose-dependent manner, however, it seems that TGFβs can inhibit, induce or have no effect on stem cell proliferation *in vivo*, depending on whether platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs) or IGFs are present (17). Moreover, it appears that TGFβs have an influence on ECM reorganization throughout the regeneration of myocardium; thus excessive TGFβ-induced deposition of ECM at the site of injury can lead to fibrosis (18).

This study assessed the pattern of the expression levels of uPA, uPAR and TGFβ$_1$, at both mRNA and protein levels, after coronary artery ligation-induced MI during the early and late phases of MI in rat myocardium.

Table I. *Primer sequences and PCR product sizes.*

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product
TGFβ1	GGACTACTACGCCAAAGAAG	GCCTGAGTGGCTGTCTTTTGA	294 bp
uPA	ACTGTGGCTGTCAGAACGG	GAACCCCGACAACCAGAG	298 bp
uPAR	AATGGTGGCCCAGTTCTG	TCCCTGCTCCTGACCCT	362 bp

Materials and Methods

Experimentally induced myocardial infarction in rats. Seventy-two male Wistar rats, weighting 280-330 g, were used in this study. The protocol was approved by the local Ethics Committee. MI was induced according to the procedure previously described (19) and the infarct size was confirmed by the tetrazolium method (19). Briefly, in the anaesthetised rat, the heart was excised and cut from apex to base transversely in 2 mm-thick sections. The sections were then incubated in a 1% solution of triphenyltetrazolium chloride (TTC) for 10 to 15 min until viable myocardium was stained brick red. Infarct-related myocardium fails to be stained with TTC. The tissue sections were then fixed in a 10% formalin solution and weighed. Colour digital images of both sides of each transverse slice were obtained. Based on the observed size of the infarct and on the indicative tissue sections stained with TTC, the infarct zone was measured to be 20-40% as previously published (19). The non-infarct area was collected for use in this study.

RNA extraction and RT- PCR conditions. The rat cardiac tissues were homogenised (Ultra-Turrax T25; Thermo Fisher Scientific, Cheshire, UK) at 15-20,000 rpm and total RNA was extracted according to the Trizol Reagent protocol (Invitrogen Corp, Carlsbad, CA, USA). Diethylpyrocarbonate (DEPC)-treated water was used for the dilution of the RNA pellet. RNA concentration was estimated spectrophotometrically (Genova; Jenway, Essex, UK) by absorbance at 260 nm, and the purity by the ratio of the absorbance at 260/280 nm. RNA integrity was assessed by visualisation of the 18S and 28S ribosomal RNA patterns in a denaturing agarose gel. Two micrograms of total RNA of each sample were reverse-transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen Corp) as described previously (19). In order to study the relative mRNA expression of the TGFβ_1, uPA and uPAR in each PCR reaction, each target cDNA was co-amplified with 18S internal standard (Ambion, Austin, TX, USA) and its expression is given as a ratio of the target cDNA/18S. This procedure compensates for differences in the initial amounts of total RNA and in RT efficiency. In order to decrease the 18S signal so as to attain a linear amplification of each target mRNA and 18S to the same range, 18S primers and competimers were mixed at ratios which ranged from 1:2 to 1:4 depending on the abundance of the target mRNA. For both controls and pathological samples of every group, the same premixed reagents were used in order to minimise the differences in PCR efficiency. The relative quantitative RT-PCR method used in the present study has been extensively validated as previously published (20, 21).

Primer sequences for rat TGFβ_1 (Accession number: NM0215 78.2), uPA (accession number: NM01085.3) and uPAR (accession number: BC127499.1) were specifically designed according to the sequences published at NIH (NCBI) and their exact sequence is presented in Table I. Primers were designed using the Primer Select computer program (DNAStar, Madison, WI, USA) and synthesized by Invitrogen Corp. The PCR mix for the amplification was constructed according to the Taq Master Mix Kit protocol (Qiagen, Valencia, CA, USA) and the reactions were carried out in a PTC-200 Peltier Thermal Cycler (MJ Research Inc, Waltham, MA, USA). Annealing temperatures were at 54°C for TGFβ_1, at 56°C for uPA and at 57°C for uPAR. Extension temperatures were at 72°C for all the genes. The reaction was accomplished after 35 cycles according to the results of linearity tests for each target mRNA and 18S. The expected sizes of the specific PCR products were initially verified by electrophoretic separation on an 2% agarose gel and all target sequences were identified by sequencing analysis to ensure specificity of the primers and to further verify each target mRNA. In preliminary experiments, each primer set was tested for its compatibility with the 18S primers.

Protein extracts and Western blot analysis. Total proteins were obtained from rat myocardium by lysis of 10 mg of the tissue in 1 ml RIPA Buffer (50 mM Tris-HCl; 150 mM NaCl) containing 0.55 ml Nonidet P-40, protease 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin and phosphatase inhibitors (1 mM sodium ortovanadate, 1 mM NaF); all obtained from Sigma, St. Louis, MO, USA. The samples were homogenised (homogeniser Ultra-Turrax T25; Thermo Fisher Scientific) in the RIPA buffer and then incubated on ice for 20 min. The homogenates were cleared by centrifugation (12.000 rpm, 4°C, 30 min). The extract was stored at –80°C. Protein concentrations were determined by Bio-Rad protein assay (BIO-RAD Laboratories, Hercules, CA, USA).

Equal amounts (50 μg) of protein extracts from the myocardial tissues were used for Western blot analysis under reducing conditions, following the procedure described previously (19). A goat polyclonal antibody (H-140, sc-6831) was used at dilution 1:200 for the detection of uPA and a rabbit polyclonal antibody (FL-290, sc-10815) was at the same dilution for uPAR; all obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test. The level of statistical significance was set at $p<0.05$. All data are presented as mean±standard error of the mean (SEM).

Results

Evaluation of the infarction. In all experimental animals, ligation of the left anterior descending artery provoked an infarct extending from 20% up to 40% of the rat

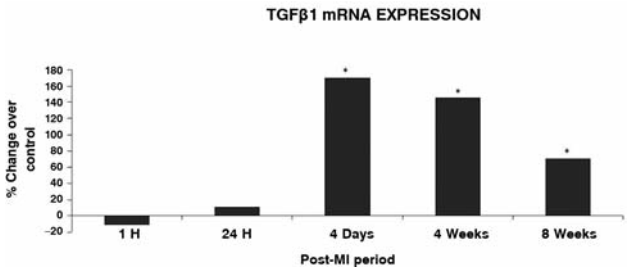

Figure 1. *mRNA expression of TGFβ_1 at different time points after infarction. Bars represent mean values (mean±SEM of 6 measurements) of TGFβ_1 mRNA expression, which was normalised to each corresponding ribosomal 18s and expressed as % change over mRNA levels of sham-operated rats (controls).* Statistically significant values: p<0.05.*

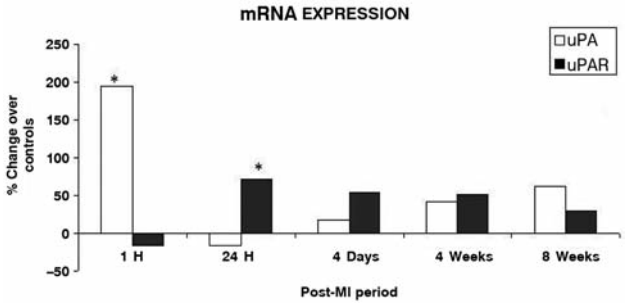

Figure 2. *mRNA expression of uPA and uPA.R at different time points after infarction. Bars represent mean values (mean±SEM of 6 measurements) of respective mRNA expression, which was normalised to each corresponding ribosomal 18s and expressed as % change over mRNA levels of sham-operated rats (controls).* Statistically significant values: p<0.05.*

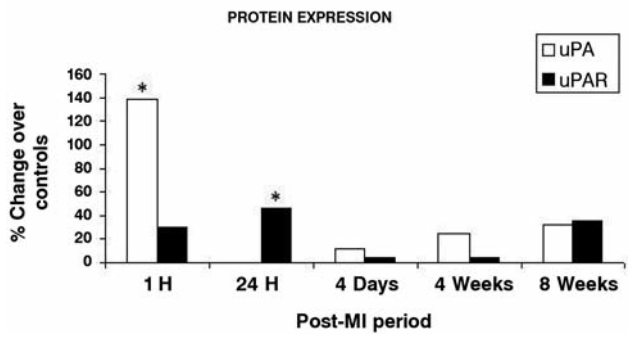

Figure 3. *Expression of uPA and uPA.R at the protein level. Immunoblotting for GAPDH served as a control for protein loading. Expression values (means±SEM of 6 measurments) were normalised to those of corresponding GAPDH in the same immunoblot and expressed as fold of change of the sham-operated rats (controls).* Statistically significant values: p<0.05.*

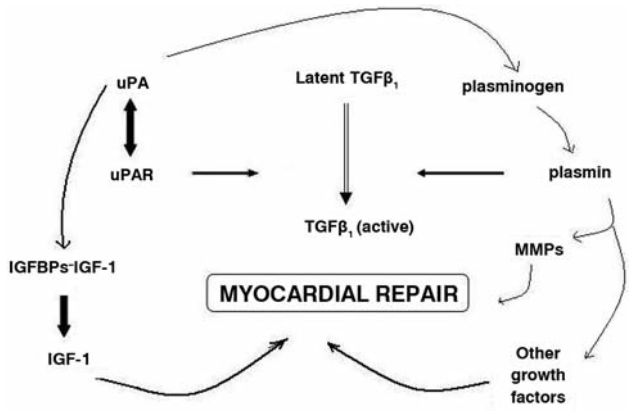

Figure 4. *uPA/TGFβ1/IGFs/IGFBPs bioregulation system in extracellular matrix remodelling during the myocardial repair processes. ECM: Extracellular matrix; IGFs: Insulin-like growth factors; IGFBPs: Insulin-like growth factor binding proteins; MMPs: Matrix metalloproteinases; TGFβs: Transforming growth factors beta; uPA: Urokinase-type plasminogen activator; uPAR: Urokinase-type plasminogen activator receptor.*

myocardium. Viable myocardium was stained brick red while infarcted myocardium failed to be stained with TTC. In each animal, the infarction was confirmed initially by the cyanotic colour of the injured myocardium and by the deviation of the electrical axis in the electrocardiogram (ECG) as previously published (19).

Pattern of TGFβ_1 expression in the post MI period in rat myocardium. The mRNA expression profile of TGFβ_1 during the post-infarction period revealed that only after 4 days there was an increase of TGFβ_1 expression which lasted up to 8 weeks (Figure 1).

Pattern of uPA/uPAR expression in the post MI period in rat myocardium. The expression pattern of uPA and uPAR during the post-infarction period showed significant increase during the early post MI period (at 1 hour for uPA) and (after 24 hours for uPAR) at both transcriptional and protein levels (Figures 2 and 3).

Discussion

MI is one of the most frequent cardiovascular events in the Western world. Over the last two decades, early mortality after MI has been significantly reduced as a consequence of thrombolytic and antiarhythmic therapies. Consequently, more patients survive the acute phase after MI and enter the wound-healing process that occurs in the days and weeks after MI, which will gradually lead to hypertrophy and heart failure (22-25).

A major mediator in cardiac repair processes is the activation of local growth substances in response to myocardial overload and injury (26). Several studies have

demonstrated TGFβ$_1$ as a significant regulator of remodeling processes, because of its anti-inflammatory effects and its involvement in the synthesis of ECM proteins (27-30). The activation of TGFβ is beneficial during the early phase after MI, acting cardioprotectively against ischaemic myocardial damage (31, 32). However, during the late phase of wound-healing processes, its uncontrolled expression leads to heart failure (33). The role of TGFβs in MI is also related to the co-expression and activation of other growth substances, such as IGFs, PDGF and bFGF (17).

Myocardium predominantly consists of myocardial cells and fibroblasts whose main purpose is to synthesise and to regulate the composition of ECM. The predominant presence of fibroblasts in myocardium indicates its dynamic regulation and reveals their significant physiological effects on the overall function of the heart. TGFβ plays a determinant role in the fibroblast proliferation and ECM production, especially of collagen and fibronectin, while reducing degradation of these components (18). Collagen deposition has a dual effect on cardiac structure and function. Increased collagen deposition is an indispensable prerequisite to prevent dilation of the infracted area, although excessive accumulation of collagen in the infracted and non-infarcted myocardium leads to tissue stiffness which results in myocardial dysfunction and ultimately contributes to heart failure (18). Consequently, degradation and synthesis of the ECM should be in balance in order to maintain physiological myocardial function.

The process of ECM degradation during cardiac wound healing is regulated by proteinases, including PAs (34) and MMPs (5). The main mammalian PAs are tissue-type plasminogen activator and uPA. uPA is an extracellular serine protease that binds to its receptor, uPAR, and generates plasmin from plasminogen by cleaving a specific Arg-Val peptide bond located within the protease domain. Plasmin, the active enzyme of the plasminogen/plasmin system, participates in the proteolytic process by degrading a variety of ECM components, thus activating several MMPs and growth substances (35-37). The generation of ECM degradation is tightly controlled by the balance between plasminogen activators and MMP inhibitors (6).

TGFβ$_1$ suppresses the activity of proteases by inducing synthesis of protease inhibitors, such as uPA inhibitor 1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs) (6). In addition, the cellular receptor M-6-P/IGF receptor CD222, interacts with uPAR and latent TGFβ$_1$ complex, and active TGFβ$_1$ is released promoting PAI-1 up-regulation which will act as a negative feedback loop to terminate TGFβ$_1$ activation (Figure 4).

Impaired inhibition of wound healing after myocardial infarction and completed protection against cardiac failure was demonstrated in uPA-deficient (10) and plasmin-deficient mice (38); facts that imply a significant role of the uPA-mediated plasminogen-plasmin system in myocardial wound healing process after myocardial infarction. Thus, it is intriguing to elucidate the expression of uPA/uPAR and TGFβ$_1$ system at the transcriptional and translational levels after MI.

The data of the present study supported such a role for TGFβ$_1$ activity during the early post-infarction period of rat myocardium. However, this increase of TGFβ$_1$ activity was not mediated by the induction of TGFβ$_1$ transcription. Apparently, the post-injury tissue needs the increased TGFβ$_1$ bioavailability both in the early and late post MI periods. In the early post MI period (1hour up to 4 days), this task is served mainly by an increased uPA/uPAR expression system, which in its turn, activates latent TGFβ$_1$ already present in ECM of myocardium. Interestingly, based on previous data (19) during this early post MI period, there is also an increase of the transcriptional activity of IGF-1, mainly its MGF isoform, which produces a selective MGF-E peptide. This is important because MGF is known to regulate the recruitment of stem cells in injured muscles (39-41). Indeed, TGFβs can inhibit, induce or have no effect on stem cell proliferation *in vivo*, depending on whether PDGF, bFGF or IGFs are present (39). In contrast, in the late post MI infarction period there was direct up-regulation of TGFβ$_1$ expression at the transcription level 4 and 8 weeks post MI which corresponds with the time of IGF-1 maximal activity (isoforms IGF-1Ea and MGF) in this model (19). Conceivably, the possible need of synergistic actions between TGFβ$_1$ and IGF-1 or MGF in the early and late stage post MI periods is differentially achieved by an increase of the transcription of IGF-1/MGF and TGFβ$_1$ genes and/or by the activation of stocks of inactive IGF-1 and TGFβ$_1$ in the ECM (latent TGFβ$_1$ and IGFBP-bound IGF-1). Therefore, this differential mode of activation of IGF-1 and TGFβ$_1$ biavailability suggests that different molecular events are taking place during the early and late post MI periods in the rat myocardium.

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Received 6 May 2010
Revised 10 May 2010
Accepted 21 May 2010