TGFβ is active, and correlates with activators of TGFβ, following porcine coronary angioplasty

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Abstract

Objective: Restenosis following angioplasty involves processes that may be influenced by local production of cytokines. We investigated the expression of active and total transforming growth factor β (TGFβ) following porcine coronary angioplasty (PTCA), and have correlated this with the expression of potential in vivo activators of TGFβ: mannose-6-phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor and thrombospondin-1. Methods: Oversized porcine PTCA was performed and the arteries excised after selected intervals. Levels of in situ active and total (active plus latent) TGFβ were determined using a modified plasminogen activator–inhibitor/luciferase bioassay. Results: Levels of active TGFβ significantly increased 2 h to 7 days after angioplasty, compared to non-injured controls. Levels returned to baseline by 28 days. Active TGFβ in tissues adjacent to the injured artery did not change. Total TGFβ was significantly higher than controls 2–6 h after injury. M6P/IGF-II receptor mRNA was upregulated between 6 h and 3 days after injury, with protein detectable at 3–28 days. Thrombospondin-1 was detected between 1 h and 14 days. Conclusions: We conclude that balloon injury causes an early rapid increase in levels of active TGFβ, that correlates with the expression of TGFβ activators. Thus, TGFβ is a good potential target for anti-restenotic therapies. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Restenosis following percutaneous transluminal coronary angioplasty (PTCA) occurs in 30–40% of patients undergoing this procedure. The biological mechanisms are complex, involving recoil, extracellular matrix production, cellular proliferation and contraction of the vessel. A number of these processes are analogous to wound healing and scar formation elsewhere in the body. Transforming growth factor-β (TGFβ) has potent effects in the process of wound healing [1–3] and also appears to have a role in the arterial response to injury. The TGFβ gene is expressed in human restenotic lesions [4]. Synthesis of TGFβ is induced by interleukin-1β [5], which is released from infiltrating inflammatory cells at the site of injury [6]; and the expression of βig-h3, a TGFβ-inducible cell-adhesion molecule, is elevated in restenotic lesions [7]. We show here that TGFβ1 protein is present in vascular smooth muscle cells after PTCA in the porcine model, and others have shown that it may modulate the differentiation of adventitial fibroblasts to myofibroblasts [8]. In addition, direct gene transfer of a TGFβ1 expression plasmid into normal porcine arteries causes neointimal thickening and extracellular matrix production [9]. However, no study has thus far been able to distinguish whether the TGFβ detected is in its active or latent state in the artery wall.

Scar-free healing is a characteristic of fetal incisional wounds where, compared to adult scar-forming wounds, there is reduced inflammation and TGFβ expression [10,11]. Mimicking the TGFβ profile of a fetal wound, by
application of TGFβ neutralising antibodies to an adult wound at the time of injury, results in reduced scar formation [12,13]. This approach has also been successful in inhibiting intimal hyperplasia in a rat carotid injury model [14].

TGFβs are secreted associated with a latency associated peptide (LAP) as part of a biologically inactive complex (L-TGFβ) which is unable to interact with cell surface receptors. Activation of TGFβ is complex. Binding of L-TGFβ to the mannose-6-phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor, that is itself complexed to the urokinase plasminogen activator receptor, invokes a conformational change of the molecule, allowing proteolytic cleavage of the active TGFβ out of the latent complex [15,16]. Plasmin, under the control of tissue plasminogen activator, urokinase plasminogen activator [15,16], the plasminogen activator inhibitors (PAI), and transglutaminase [17] also appear to be required. Thrombospondin-1 (TSP-1) is also an important activator of TGFβ in vivo [18]. TSP-1 interacts with the N-terminal region of the LAP, forming a trimolecular complex within which a conformational change takes place, altering the immunoreactivity of TGFβ, and making it accessible to its receptor [19–21]. In activated macrophages, the TSP-1 receptor, CD36, is also involved [22], localising the TSP-1/L-TGFβ complex to the cell surface and making it accessible to cleavage by plasmin.

Notwithstanding these data, there is considerable debate regarding the precise role of TGFβ in the arterial response to injury. This arises because of the very considerable uncertainties regarding the measurements of active and total TGFβ, the (until recently) poorly understood mechanism of TGFβ activation, as well as the non-viability of TGFβ knockout mice. We report here the results of a study of porcine coronary artery injury where a novel bioassay has been used that accurately assesses active TGFβ levels following angioplasty, for the first time. This has been correlated with TGFβ levels and expression of the M6P/IGF-II receptor complex.

2. Methods

2.1. Porcine coronary angioplasty model

Coronary artery injury was induced by oversized PTCA of the left anterior descending (LAD) and the right coronary artery (RCA) in 20 Yorkshire White pigs, 25–30 kg in weight, as previously described [23]. The investigation conforms with UK Home Office regulations. The site of balloon inflation was selected such that the balloon:artery ratio was 1.25:1, as assessed by quantitative angiography. At selected intervals (1, 2, 6, 18 h and 3, 7, 14, 28 days) post-PTCA, the animals were sacrificed and the heart excised. The angioplastied vessels were identified and removed, preserving the adventitia. The circumflex coronary artery was also explanted and used as untreated controls. All arteries were serially cross-sectioned, without further dissection, into 2–3 mm thick blocks (12–14 per artery) and sequential blocks were preserved in liquid nitrogen, formalin-fixed and embedded into paraffin wax, or embedded into 8% methylcellulose and frozen slowly in liquid nitrogen, prior to subsequent analysis. Samples of myocardium and fat immediately surrounding the excised artery were also taken for analysis.

2.2. Histology–quantitative morphology

Transverse coronary artery sections were cut from each paraffin-embedded block and stained with H&E to determine the location and extent of injury. Arterial blocks with definite evidence of balloon injury (disruption in the internal elastic lamina, IEL) were identified and, together with sections from undilated arteries (controls), were used for analysis. Sections from apparently non-injured areas of angioplastied artery were not used for immunohistochemical or Western blot analysis because of the possibility of unseen damage caused by the angioplasty catheter affecting the interpretation of data. However, non-injured areas of angioplastied arteries (embedded into methylcellulose) were analysed for TGFβ levels via the PAI/L assay, and treated as a separate group of data.

2.3. Determination of active and latent TGFβ

The amount of active and latent TGFβ present at each timepoint was detected using a modified plasminogen activator-inhibitor/luciferase (PAI/L) assay [24]. Briefly, mink lung epithelial cells (MLECs) stably transfected with a truncated TGFβ-inducible PAI-1 promoter fused to a firefly luciferase reporter gene (generating a highly sensitive TGFβ-responsive cell line [25]), were plated into 24-well plates in complete DMEM and allowed to attach. The serum-containing medium was then replaced with serum-free DMEM containing 0.1% (w/v) pyrogen-poor BSA (DMEM–BSA medium), and sterile coverslips carrying 20 μm cryosections placed, upside down, onto the MLECs. Each coverslip contained a single arterial tissue section, which had been trimmed of extra-arterial myocardial tissue prior to embedding into methylcellulose. A standard curve of TGFβ activity was generated using serial dilutions of recombinant human TGFβ1 (R&D Systems). Triplicates of each sample and TGFβ standard were incubated with the MLECs overnight at 37°C. The MLECs were then washed with PBS, lysed, and luciferase activity in the lysates determined using a luciferase assay kit (Labtech), measured with a luminometer.

Total (active plus latent) TGFβ in the arteries was determined by submerging the coverslips carrying the cryosections in DMEM–BSA medium and incubating at 80°C in order to heat activate latent TGFβ. The coverslips were then transferred, together with the medium, onto MLECs for TGFβ quantification in the PAI/L assay.

The amount of TGFβ present in each section was
normalised to tissue volume. The volume of the tissue sections was measured from serial cryosections from the same specimen, stained with H&E. Colour images of each section were captured using a digital image analysis system and the total coronary artery cross-sectional area of the section measured. The volume of each section was then calculated from the area and thickness of the section. The average volume of one artery section was 0.08–0.2 mm³.

2.4. Western blotting

Levels of TSP-1 were determined by Western blotting. Protein from each timepoint was extracted into RIPA lysis buffer (1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS) and 50 µg of each sample separated by SDS–PAGE, followed by semi-dry transfer onto nitrocellulose membrane. The membrane was blocked with 5% (w/v) dried milk–TBS prior to incubation with monoclonal anti-TSP-1 antibody (NeoMarkers). The membrane was then washed with 0.1% Tween-20/TBS before incubation with HRP-labelled secondary antibody. Protein bands were visualised before blocking non-speciﬁc binding with 5% (w/v) dried milk/PBS as diluent. Sections were washed with PBS prior to incubation with secondary antibodies, followed by peroxidase-anti-peroxidase (PAP) complex (TSP-1) or avidin–biotin complex (ABC) (TGFβ, mac-387). Finally, antibody binding was visualized with 3,3′diaminobenzidine tetrahydrochloride (DAB) and counterstained with Carazzi’s haematoxylin.

M6P/IGF-II receptor was localised within cryosections cut from the same arterial segments used in the PAI-1/L assay and acetone ﬁxed prior to storage, following the protocol described in [26]. Briefly, sections were thawed followed by blocking non-specific binding with 5% (w/v) dried milk/PBS. Primary antibody (1:1500 in 5% (w/v) dried milk/0.5% (v/v) Triton X-100/PBS; gift to MWJF) was applied overnight at 4°C before detection via a biotinylated secondary antibody and ABC. Antibody binding was visualised with DAB and counterstained with Carrazzi’s haematoxylin.

For all immunohistochemistry experiments, negative controls were performed. Sections were incubated with the omission of primary antibody (substituting antibody diluent or the appropriate non-immune IgG in each case). Only negative control sections stained with non-immune IgG gave some slight non-specific staining. Antibody speciﬁcity was confirmed using appropriate positive control tissue.

2.7. Statistics

Comparisons between control arteries and active or total TGFβ expression at each timepoint after PTCA were made using the Kruskal–Wallis test (nonparametric ANOVA) followed by Dunn’s multiple comparison test. The Tukey–Kramer multiple comparisons test was used to compare differences between active TGFβ levels in injured angiop-
Fig. 1. Levels of active (a) and total (b) TGFβ after porcine PTCA compared to non-injured arteries (baseline) and non-injured tissues surrounding the injured area, detected using a modified PAI-1/L assay. ***, P<0.001; **, P<0.01; *, P<0.05 compared to control; §, P<0.05 compared to 2 h, 18 h, 28 days; ‡, P<0.001 compared to 3 days; ♂, P<0.01 compared to 3 days). (c) Levels of active TGFβ in sections of injured angioplastied arteries 6 h post-PTCA compared to sections of angioplastied arteries without evidence of injury. *, P<0.01 compared to non-injured PTCA; §, P<0.001 compared to control. Error bars denote standard error of the mean.
lasted arteries, non-injured angioplastied arteries and non-angioplastied arteries. Values of $P<0.05$ were considered significant.

3. Results

The 28 coronary arteries which had undergone PTCA yielded 80 sections (5–19 per timepoint) with evidence of injury (Table 1). Twenty-four sections were taken from the control arteries (non-angioplastied vessels). Power calculations using a significance level of 0.05 and a power of 90% confirmed that the number of arteries and sections analysed per timepoint were sufficient for a valid study.

Active TGFβ was significantly increased 2 h to 7 days post-PTCA compared to control arteries (Fig. 1a), returning to baseline at 28 days. However, there was no significant difference in active TGFβ expression over time, except for at day 3 (Fig. 1a). The level of active TGFβ in tissue immediately surrounding the injured artery (epicardium/myocardium/fat) were similar to baseline levels found in non-injured control arteries. Similarly, sections of angioplastied arteries without evidence of injury displayed a lower level of active TGFβ compared to injured sections from the same artery ($P<0.01$) (Fig. 1c). Total (active plus latent) TGFβ was also significantly raised between 2 and 6 h post-PTCA compared to controls ($P<0.001$) and over time ($P<0.001$ and $P<0.01$ compared to 3 days, respectively), with levels returning to baseline by 3 days. Total TGFβ then increased again, compared to controls, at 7 days, before returning to baseline by 28 days, although this increase was not statistically significant (Fig. 1b). From 18 h post-PTCA, there was no significant change in total TGFβ levels over time.

Immunohistochemistry detected total (active plus latent) TGFβ in the endothelium and media of control arteries, with the remnants of endothelium in all the injured sections staining positively at up to 18 h. After injury, the staining pattern of TGFβ altered, appearing more diffuse. TGFβ was found in neointima from 7 to 28 days; in the media throughout, but maximally at 3 days; and in the adventitia between 3 and 14 days, returning to baseline by 28 days (Fig. 2). TGFβ was also found in the inflammatory cell infiltrate (defined by mac-387 staining, not shown) at 1–18 h (Fig. 2). Negative control sections did not stain for TGFβ1.

Western blotting showed TSP-1 to be expressed between 1 h and 14 days post-PTCA (Fig. 3). Immunohistochemical staining showed TSP-1 to be absent within non-injured vessels, although residual platelets on the luminal surface were positive (Fig. 4a). As expected, after injury TSP-1 was expressed in high levels in the thrombus (Fig. 4b), located in the platelets and in the fibrous strands. From 1 to 6 h after injury TSP-1 expression was detected in some medial cells, although the majority remained negative, however, by 18 h, the media stained strongly for TSP-1 (not shown). At 6 and 18 h, TSP-1 expression was also detectable in the inflammatory infiltrate present in the connective tissues surrounding the artery (Fig. 4b). The adventitia stained negatively for TSP-1 until 3 days post-PTCA, when weak staining was seen in the area of adventitia around the site of breach in the external elastic lamina (EEL). At 7–14 days, the neointima stained strongly for TSP-1 with weaker staining in the neoadventitia (Fig. 4c). At 28 days post-PTCA, levels of TSP-1 were almost undetectable by immunohistochemical staining, with only the neointima giving a very weak positive stain.

M6P/IGF-II receptor RNA was detectable by RT-PCR in control vessels and in injured arteries between 2 h and

![Fig. 1. (continued)](image-url)
Fig. 2. Immunohistochemical staining for total (active plus latent) TGFβ showing it to be present in the endothelium and media in non-injured arteries (a), and in the neointima, media and adventitia at 14 days post-PTCA (b). TGFβ is also detected in the inflammatory infiltrate at 18 h (c). n, neointima; m, media; adv, adventitia. Arrow depicts IEL. Original magnification 20× (a, b); 100× (c).
14 days post-PTCA, with increased levels at 2 h to 3 days (Fig. 5). Immunohistochemical staining showed the M6P/IGF-II receptor to be absent in non-injured vessels (Fig. 6a). The inflammatory infiltrate also stained negatively at all timepoints after injury (Fig. 6b). At 3 days, weak positive staining was seen in the adventitial cells around the site of breach in EEL (Fig. 6c and d). The neointima stained strongly for the M6P/IGF-II receptor at 7–14 days, with sparser staining at 28 days (Fig. 6e and f).

4. Discussion

This is the first study to describe the active and latent state of TGFβ in the artery after PTCA, and to correlate the presence of active TGFβ with that of its activators. The study shows that TGFβ is present in its active state following PTCA and that the M6P/IGF-II receptor and TSP-1, both involved in the activation of TGFβ in vivo, are upregulated with a temporal relationship to TGFβ that suggests latent TGFβ may be activated at the site of injury by these mechanisms.

Active TGFβ levels are significantly increased between 2 h and 7 days after PTCA. This activation pattern is similar to that seen during the healing of rodent dermal wounds [24]. Immediately after wounding, platelets accumulate at the site of the wound and are thought to be an important early source of TGFβ after wounding. Since platelets accumulate at the site of damage following PTCA and release their stores of TGFβ this may be the source of the initial rise in total TGFβ levels seen within hours of injury. In the porcine PTCA model, there is also an influx of inflammatory cells, including macrophages, within hours of injury [27]. The timing of this inflammatory influx coincides with that of the initial rise in TGFβ levels and thus, may also contribute to active TGFβ levels within the arterial wall. Three days after PTCA, polymorphonuclear leukocytes and VSMCs, both sources of TGFβ, are present in thrombus-filled breaches in the media. Adventitial expansion is also first observed at this timepoint, with α-smooth muscle actin-positive cells present for the first time in the neoadventitia. Adventitial cells, 3 days post-PTCA, also express high levels of IL-1β [6], which is known to induce expression of TGFβ. Alternatively, or additionally, the level of TGFβ activity seen at 3 days may be due to the release of active TGFβ from pools of soluble or extracellular matrix TGFβ-binding proteins [28–36], or from the release of the small L-TGFβ complex from the fibrin clot following the degeneration of the clot by plasmin [37], or by endothelial cells and fibroblasts which migrate into the injured area, and which may produce and activate TGFβs [14,38].

It is likely that all of these potential sources of TGFβ contribute to the presence of TGFβ following PTCA demonstrated here. This indicates that TGFβ production and activation is a central event in the biology of the
Fig. 4. Immunohistochemical staining for TSP-1. TSP-1 is only found in the residual platelets (arrowed) of control arteries (a). After PTCA, TSP-1 is found in the thrombus and inflammatory cell infiltrate (arrowed) at 18 h (b), and the neointima, neoadventitia, and remains of underlying thrombus at 14 days (c). Arrowheads depict breach in the internal elastic lamina. Original magnification 40×(a, b); 4× (c).
arterial response to injury. This is reinforced by the observation that areas adjacent to the site of injury do not show any elevation in active or total TGFβ as well as the finding of significantly attenuated levels in arteries that underwent PTCA but did not demonstrate an injury.

Levels of active TGFβ in our model correlate with the temporal expression of the L-TGFβ activators TSP-1 and M6P/IGF-II receptor. TSP-1 is secreted by platelets, endothelial cells, VSMCs, fibroblasts, macrophages and monocytes and therefore, has the potential to play a significant role in the response to injury. TSP-1 is transcriptionally controlled by a serum-response element that up-regulates TSP-1 synthesis rapidly after injury and then down-regulates it during later stages of healing or remodeling when growth factors are low. In our model, Western blotting first detected TSP-1 1 h after injury, with levels remaining detectable throughout the timecourse of injury, suggesting that TSP-1 could be regulating latent-TGFβ activation after PTCA. Indeed, increased TSP-1 expression is frequently associated with increased TGFβ activity in vivo [39–41]. We found that TSP-1 after injury was localised mainly in platelets and thrombus, with a weak presence in the media. The inflammatory cell infiltrate also contained TSP-1 6 h after PTCA. By 18 h, TSP-1 staining was strong in the media, and remained high until 7 days. At 7 and 14 days post-PTCA, TSP-1 staining was strongest in the neointima. This indicates that the major sources of TSP-1 in the first few hours after PTCA are platelets and inflammatory cells, and VSMCs from around 18 h. This agrees with other studies using a rat carotid model of injury [42]. Both the media and neointima showed an extracellular staining pattern rather than the nuclear stain seen with inflammatory cells. This is expected because TSP-1 is secreted by VSMCs, fibroblasts, and endothelial cells and so would not be totally nuclear in location. The pattern of TSP-1 localisation also closely correlates with that of TGFβ, which is present in the inflammatory infiltrate early after injury, in increased levels in the VSMCs of the media from 6 h and in the adventitia from 3 days, and is also highly expressed in the neointima and neoadventitia 7–28 days after injury. In addition, TGFβ is detected in all layers of non-injured arteries. This is to be expected as TGFβ is ubiquitously expressed, although our data from the PAI/L assay suggests it is unlikely to be active. The TGFβ antibody used in this study, cannot distinguish between active and latent forms. Thus, we cannot be certain of the spatial distribution of the active TGFβ detected using the PAI/L assay. However, the correlation of the temporal pattern of TSP-1 staining with that of active TGFβ (detected by the PAI/L assay) following injury, together with the observation that TGFβ and TSP-1 are present in the same cell types and layers within the artery following injury, makes TSP-1 activation of L-TGFβ feasible. This hypothesis is reinforced by studies indicating TSP-1 to have both an autocrine and a paracrine pattern of TGFβ activation [18].

M6P/IGF-II receptor expression is detectable by RT-PCR in both control and injured vessels, but is upregulated between 2 h and 3 days after injury. This temporal pattern of expression almost exactly correlates with that of active TGFβ, suggesting that this receptor may play a role in the activation of L-TGFβ after PTCA. The spatial expression of the M6P/IGF-II receptor, detected via immunohistoch- emical staining, showed the M6P/IGF-II receptor to be present in the neointima at 7–28 days post-injury, directly correlating with the spatial expression of TGFβ in this arterial layer. At 3 days, the M6P/IGF-II receptor was also detected in adventitial cells located at the site of breach of the EEL, in an identical staining pattern to that of TGFβ. However, the M6P/IGF-II receptor was not detected in the media or the inflammatory infiltrate after injury. This staining pattern suggests that the M6P/IGF-II receptor is not involved in L-TGFβ activation early (within hours) after injury but has a potential role to play in TGFβ activation within the neointima, whereas TSP-1 has the potential to be involved in TGFβ activation throughout all the arterial layers and at all timepoints.

Our data suggests that at least two mechanisms of TGFβ activation could be employed by the artery following balloon injury, the TSP-1 mechanism perhaps being more central. Other mechanisms of TGFβ activation, involving the integrin αvβ6, have also been reported [43]. However, as αvβ6 is principally expressed on epithelial cells, this mechanism is assumed to play an insignificant role in our model.

Our data support strategies that inhibit TGFβ activation to limit neointima formation after PTCA. Neutralising TSP-1 has been shown to reduce neointima formation in balloon-injured rat carotid arteries when delivered intraarterially by use of the dwell technique [44] and it is possible that this reduction in neointima could, in part, be due to reduced levels of active TGFβ. Inhibiting TGFβ

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Fig. 5. RT-PCR of M6P/IGF-II receptor expression in porcine coronary arteries after PTCA.
Fig. 6. Immunohistochemical staining for the M6P/IGF-II receptor. Absent in non-injured vessels (a) and in the inflammatory infiltrate after injury (b), M6P/IGF-II receptor is found in the adventitial cells around the site of breach in EEL at 3 days (c, d) and in the neointima at 14 days (e), and 28 days (f). (d) Higher power of boxed area marked in (c). Arrowheads depict IEL. Original magnification 20× (a, e); 10× (c); 100× (b, d, f).
activation via local delivery of mannosene-6-phosphate (which inhibits the formation of the L-TGFβ/M6P/IGF-II receptor complex) results in the reduction of scar formation in rodent, porcine and human dermal incisional wounds [MWJF, unpublished data]. This may be a possible future therapeutic intervention post-PTCA.

In summary, we have shown active TGFβ levels rise significantly early after PTCA, returning to baseline by 28 days when, in the porcine model, remodelling of the restenotic lesion is essentially complete. Levels of TGFβ activity correlate with the expression of the M6P/IGF-II receptor and TSP-1 which are involved in distinct mechanisms of TGFβ activation in vivo. It is highly likely, therefore, that TGFβ activation is a good target for anti-restenotic therapies.

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