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TGF-β1 overexpression in the transversalis fascia of patients with direct inguinal hernia

G. Pascual, C. Corrales, V. Gómez-Gil, J. Buján and J. M. Bellón

Faculty of Medicine, University of Alcala, Alcalá de Henares, Madrid, Spain

Abstract

Background The aetiology of inguinal hernia includes changes in collagen turnover and metalloproteinase (MMP) expression, and direct hernia has been linked to increased MMP-2 expression. Since transforming growth factor β1 (TGFβ1) plays a role in tissue remodelling, this growth factor could directly affect metalloproteinase secretion and thus the proteolytic activity of these enzymes. We hypothesized that TGFβ1 expression could also be altered in direct inguinal hernias.

Materials and methods Tissue specimens were obtained from the transversalis fascia (TF) of organ donors (controls; $n = 10$) and patients with inguinal hernia (indirect; $n = 20$/direct; $n = 20$), who were also divided into two groups according to age (20–40/41–60 years). Tissue sections were immunohistochemically labelled using anti-LAP TGFβ1 (latent form) and anti-TGFβ1 (active form) antibodies, and fragments of tissue were subjected to Western blot analysis.

Results No significant differences in LAP-TGFβ1 expression were detected between specimens from control and hernia patients. However, significantly higher levels of active TGFβ1 were detected in the TF of patients with direct hernia ($P < 0{\cdot}05$). Age affected the expression of the growth factor in its active form, and significant differences emerged between direct hernias and controls/indirect hernias only in the younger age groups.

Conclusions Our findings indicate overexpression of the active form of TGFβ1 in the TF of young patients with direct hernia. This overexpression reflects an attempt to counterbalance the enhanced matrix degradation process observed in these patients, identifying a subset of patients requiring the use of a prosthetic material for primary hernia repair.

Keywords Extracellular matrix, growth factors, hernia repair, inguinal hernia, metalloproteinases, TGFβ, transversalis fascia

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Introduction

The surgical treatment of inguinal hernia continues to be among the most common general surgery procedures. It has been estimated that more than 20 million patients worldwide undergo surgery for hernia each year [1] and, in the USA, some 700 000 hernias are repaired per year [2].

Despite several technical advances in the surgical treatment of hernias – essentially the use of prosthetic materials prompted by Lichtenstein *et al.* [3] – hernia recurrence continues to be a major problem. Recurrence is related to the type of hernia, with direct hernias being the most likely to recur. The causes and aetiology of the inguinal hernia remain unclear. Congenital factors have been linked to the aetiology of indirect hernia although regardless of hernia type, the integrity of the posterior wall of the inguinal canal depends on the orientation of the canal itself and especially of the transversalis fascia (TF). According to Pans *et al.* [4], it is the eventual disruption of the TF that gives rise to a hernia.

The question that now needs to be addressed is why does the TF fail in some people and not in others? There is evidently an underlying biological cause to explain the behaviour of the TF.

The biological factors proposed in the studies performed by Read [5–7] have gradually gained acceptance over the years. In a clarifying scheme, Jansen *et al.* [8] placed inguinal hernias within the context of conditions generated

Departments of Surgery (C. Corrales, J. M. Bellón) and Medical Specialties, Faculty of Medicine, University of Alcala, Alcalá de Henares, Madrid, Spain (G. Pascual, V. Gómez-Gil, J. Buján).

Correspondence to: Prof Juan M Bellón, Department of Surgery, Faculty of Medicine, University of Alcalá, Ctra. Madrid-Barcelona Km 33,600, 28871 Alcalá de Henares, Madrid, Spain.
Tel.: 91-885-45–40; fax: 91-885-48–85; e-mail: juanm.bellon@uah.es

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by an abnormality in the components of the extracellular matrix (ECM). Among these components, we find collagens, proteoglycans, and several types of glycoprotein.

Cytokines such as transforming growth factor beta 1 (TGFβ1) are known to be involved in tissue remodelling. TGFβ1 is a polypeptide that regulates different cell functions such as proliferation, migration and differentiation, and also plays an important role in extracellular matrix production. It is released as an inactive high-molecular weight mass complex, comprised of the mature form of TGFβ1, latency associated peptide (LAP) and latent TGFβ1 binding protein (LTBP). The latter participates in storing the latent form of TGFβ1 in the extracellular matrix. The biologically active form of TGFβ1 is then released by proteinases and glycosidases [9,10] that may be secreted by mast cells and leukocytes.

TGFβ1 has been identified as an important modulator of metalloproteinases (MMPs) the enzymes regulating the synthesis and degradation of collagen and many other ECM components [11]. Some authors [12–14] have reported that this cytokine regulates the expression of MMP-2 in several cell types including fibroblasts and endothelial cells, as well as in different animal models [15].

The aim of this study was to investigate TGFβ1 expression in direct and indirect hernia, by examining the latent (LAP-TGFβ1) and active forms of the growth factor. Effects of patient age were also examined.

Materials and methods

Patients

Control TF specimens were obtained from donor patients who were free from any disease at the time of organ procurement for transplant. Pathological specimens were obtained from patients whose only condition was a primary inguinal hernia during elective surgery for hernia repair. Specimens of the TF were taken from the same region, the central area of the posterior wall of the inguinal canal. The study was approved by the local ethics committee and informed consent to participate was obtained from each of the subjects.

The following study groups were established:
Group I: Control group (n = 10); TF specimens obtained from organ donors aged 20–40 years (n = 5) (mean age ± SE; 28·20 ± 3·27) and 41–60 years (n = 5) (52·80 ± 3·24).
Group II (n = 20); TF specimens obtained from patients with direct hernia aged 20–40 years (n = 10) (mean age ± SE; 34·50 ± 2·10) and 41–60 years (n = 10) (50·30 ± 1·59).
Group III (n = 20); TF specimens obtained from patients with indirect hernia aged 20–40 years (n = 10) (mean age ± SE; 33·50 ± 1·32) and 41–60 years (n = 10) (54·17 ± 3·11).

Immediately after procurement, the TF specimens were placed in sterile culture medium (MEM) and stored at 4 °C for their transfer to the laboratory, where they were divided into two fragments: one fragment was processed for light microscopy (immunohistochemistry) and the other fragment was frozen at –80 °C until the time of Western blot analysis.

Immunohistochemistry

For the immunohistochemical studies, specimens were fixed, embedded in paraffin and cut into 5 μm slices using a microtome (Microm, Barcelona, Spain). These sections were then deparaffinated, hydrated and equilibrated in phosphate-buffered saline (PBS) (pH 7·4). We used a goat monoclonal antihuman LAP-TGFβ1 antibody (dilution 1 : 20) (R & D Systems, Minneapolis, MN, USA) and a mouse monoclonal antihuman TGFβ1 antibody (dilution1 : 500) (Chemicon, Temecula, CA, USA). The antigen-antibody reaction was detected by alkaline phosphatase or peroxidase-labelled avidin-biotin procedures. The chromogenic substrate contained alpha-napthol and fast-red or diaminobenzidine (DAB). Nuclei were counterstained with Carazzi hematoxylin. After immunostaining, the tissue sections were examined under a light microscope (Zeiss, Jena, Germany).

Protein extraction and immunoblotting

For Western blot analysis, the total protein contents of the specimens were extracted. Tissue samples were cut into small pieces immersed in the extraction buffer [50 mM TrisHCl pH 7·6, 1%Triton X-100, 200 mM NaCl, 100 mM $CaCl_2$ $2H_2O$ and 4% protease inhibitor cocktail (Roche, Penzberg, Germany)] and milled using a grinder (Universal Mühle M20, Ika Industries, Staufen, Germany). Once milled, the resultant solutions were centrifuged at 12 000 g for 20 min and the supernatants recovered in clean Eppendorf tubes. The proteins were then separated by electrophoresis on a 14% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) under reducing conditions and in the presence of 10% 2-beta-mercaptoethanol according to the modified method of Laemmli [16].

Equal aliquots (20 μL) containing approximately 10 μg of protein were diluted in sample buffer (50 mM Tris-base, pH 6·8, containing 50% glycerol, 0·125% bromophenol blue, 15% SDS in the presence of 10% 2-beta-mercaptoethanol) and heated at 100 °C for 5 min before loading. A broad-range prestained SDS-PAGE standard (Bio-Rad Laboratories, Hercules, CA, USA) served as a molecular weight marker. After 2 h of electrophoresis at 100 V, the separated proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) and subjected to 210 mA for two hours at room temperature. The membranes were blocked overnight at 4 °C with 5% dry milk in PBS using 0·05% Tween 20. The following primary antibodies were applied to the blotted membranes for 2 h at room temperature: anti-LAP TGFβ1 (1 : 500) and anti-TGFβ1 (1 : 5000). The secondary antibodies, antimouse/goat IgG-horseradish peroxidase (Sigma, St. Louis, MO, USA) (1 : 10 000), were incubated with the membranes for 1·5 h at room temperature. The blots were developed using the SuperSignal Westpico Chemiluminescent

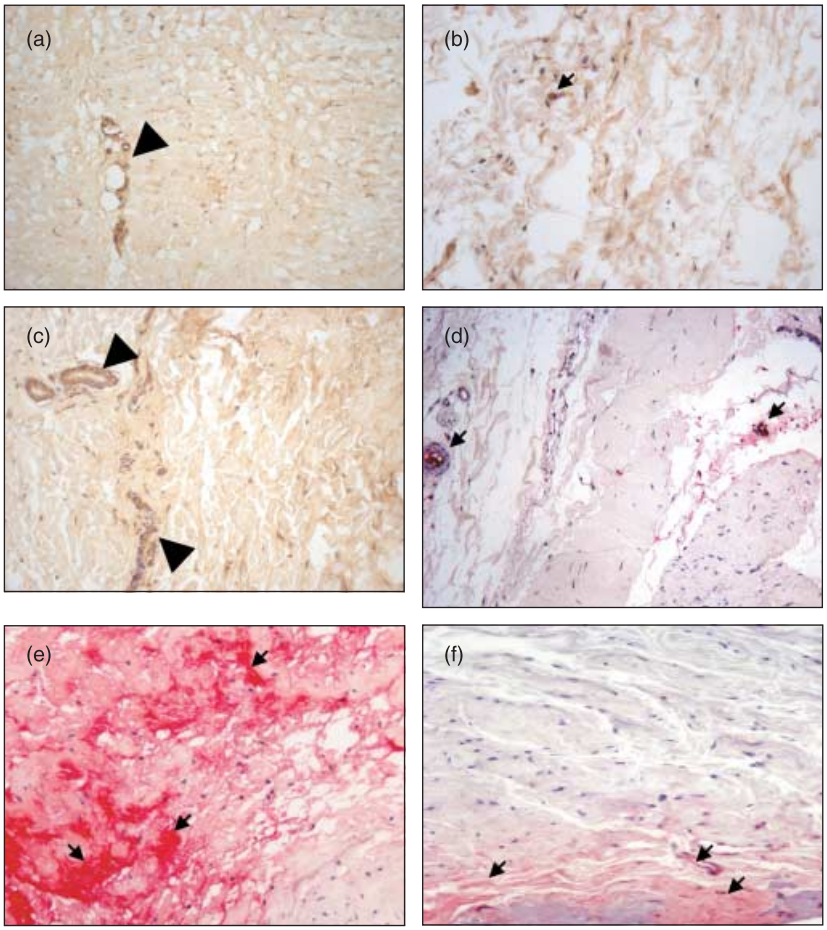

Figure 1 LAP-TGFβ1 immunostaining in tissue sections. Transversalis fascia from control donor patients (a; X200), patients with direct inguinal hernia (b; X500) and patients with indirect inguinal hernia (c; X200). The different groups showed no significant differences in staining with the anti-LAP TGFβ1 antibody. (▶: vascular areas; →: cells). Active TGFβ1 immunostaining in tissue sections. Transversalis fascia from control donor patients (d; X200), patients with direct inguinal hernia (e; X400) and patients with indirect inguinal hernia (f; X400). Staining was significantly greater in the group of patients with direct hernia. (→: staining).

kit (Pierce, Rockford, IL, USA). Positive bands were visualized on X-ray film.

Mouse antiβ actin antibody (Calbiochem, Inc. La Jolla, CA, USA) was used as a loading control. Western blot bands were quantified using the Scion Image program. The ratio between LAP TGFβ1 or TGFβ1 and β-actin was obtained to standardize the results. Results were expressed as the mean ± SE of 10 representative experiments for the control group, and of 10 experiments each for the indirect and direct hernias. Statistical analysis was performed with the Graph Pad Prism program using the Mann-Whitney test to compare two independent groups of sampled data. The level of significance was set at $P < 0{\cdot}05$.

Results

LAP-TGFβ1

Immunohistochemistry

In TF specimens of the control group, the extracellular matrix was homogeneously yet only discretely stained with the anti-LAP TGFβ1 antibody in both age groups. Zones with blood vessels were more intensely stained (Fig. 1a). In TF specimens obtained from patients with direct (Fig. 1b) or indirect (Fig. 1c) hernias, the expression pattern of the latent form of TGFβ1 was similar to that observed in the control group.

Western blotting

LAP TGFβ1 expression analysed by Western blotting was not different among control subjects and patients with direct or indirect inguinal hernias, excluding (Fig. 2a) or including (Fig. 2b) the age factor.

Active TGFβ1

Immunohistochemistry

In TF specimens of the control group, staining for active TGFβ1 revealed the microvasculature (Fig. 1d). In TF specimens obtained from patients with direct hernias, expression was significantly higher compared to control levels (Fig. 1e) in both age groups although expression was greater in the younger age group. Staining was patchy in

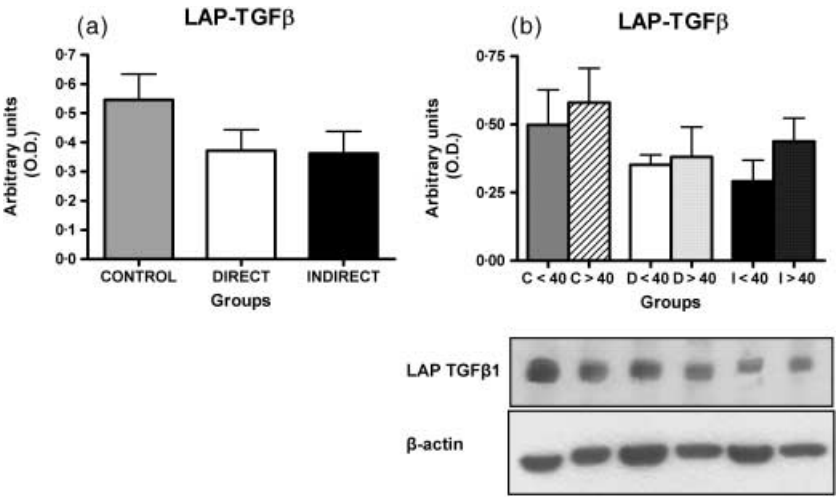

Figure 2 Western blotting for expression of the latent (LAP-TGFβ1) form of TGFβ1 in healthy and pathological specimens: (a) excluding the age factor, and (b) including the age factor. No significant differences were found between the study groups. A mouse antiβ actin antibody was used as a loading control. The intensities of the TGFβ1 bands were normalized to those of the β-actin bands. Data in each bar are the mean ± SE. (C < 40 (*n* = *5*): control under 40 years; C > 40 (*n* = *5*): control over 40; D < 40 (*n* = *10*): direct hernia under 40; D > 40 (*n* = *10*): direct hernia over 40; I < 40 (*n* = *10*): indirect hernia under 40; I > 40 (*n* = *10*): indirect hernia over (40).

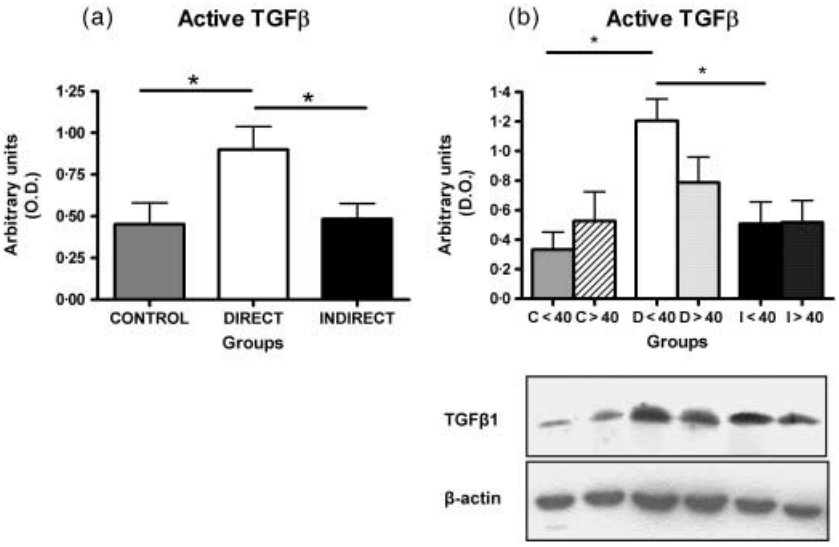

Figure 3 Western blotting for expression of the active form of TGFβ1 in healthy and diseased specimens: (a) excluding the age factor, and (b) including the age factor. Significantly greater active TGFβ1 expression was found in direct hernia TF specimens compared to the rest of the groups (**P* < 0·05). When age was considered, these differences were confined to the younger groups (**P* < 0·05). Mouse antiβ actin antibody was used as a loading control. The intensities of the TGFβ1 bands were normalized to those of the β-actin bands. Data in each bar are the mean ± SE (C < 40 (*n* = *5*): control under 40 years; C > 40 (*n* = *5*): control over 40; D < 40 (*n* = *10*): direct hernia under 40; D > 40 (*n* = *10*): direct hernia over 40; I < 40 (*n* = *10*): indirect hernia under 40; I > 40 (*n* = *10*): indirect hernia over (40).

areas of the fascia outside the microvasculature; these areas were less extended in specimens from the older patients. In TF specimens obtained from patients with indirect hernias, expression of the active form of TGFβ1 was similar to that noted in the healthy fascia, and also outlined the microvasculature of the fascia and small areas near the vessels showing weak, diffuse staining (Fig. 1f).

Western blotting

TGFβ1 expression analysed by Western blotting, indicated significantly greater active TGFβ1 expression in the TF of patients with direct hernia (Fig. 3a). The differences were statistically significant when this group was compared with the control group and with the TF of patients with indirect hernia (**P* < 0·05). Including the age factor of the subjects, significant differences only emerged in the younger groups, between the TF of patients with direct hernia and the control group, and between the TF of patients with direct hernia and the TF of patients with indirect hernia (Fig. 3b) (**P* < 0·05). These results confirm the up-regulation of processes involved in activating the growth factor (TGFβ1) in these TF specimens obtained from young patients with direct inguinal hernia, leading to overexpression of the active form of the growth factor.

TGFβ1 expression analysed by Western blotting was similar among control subjects and patients with direct or indirect inguinal hernias (*P* > 0·05) from the older age groups.

Collectively, our data indicate increased levels of the active form of TGFβ1 in the TF of young patients with direct inguinal hernia, indicating that the activation of this growth factor is up-regulated in these patients.

Discussion

Tissue remodelling occurs continuously in almost all tissues and a balance between degradation and synthesis of matrix components is required for homeostasis. However, in several diseases, this balance seems to be upset as occurs in genitourinary prolapse [17] and aneurysm [18], and may give rise to an increased incidence of hernia in patients with these diseases.

Here we demonstrate the overexpression of the active form of TGFβ1 in the transversalis fascia of young patients with direct inguinal hernia, indicating that both age and the type of inguinal hernia could contribute to failure of the TF.

TGFβ1 plays a central role in tissue repair through its modulating effects on cell growth and extracellular matrix synthesis [19,20]. Several authors have reported both positive and negative effects of this growth factor in wound healing, indicating that excessive TGFβ1 at the wound site does not benefit wound healing [21].

TGFβ1 is normally synthesized and secreted in an inactive high-molecular weight precursor form (LAP-TGFβ1). Once secreted by cells in complex tissue *in vivo*, inactive TGFβ1 can either be cleared from the site of secretion, retained locally by binding to extracellular matrix components, or may be activated by proteolysis to release the biologically active 24–25 KDa mature form of TGFβ1 [22].

When we compared healthy and diseased specimens of fascia, no differences were detected in LAP-TGFβ1 expression. However, the active form was found to be significantly increased in patients with direct hernia. This indicates that the activation of the growth factor was up-regulated in the TF of patients with direct hernias. Age also seems to play an important role, in that this up-regulation was restricted to the younger group of patients (20–40 years) with direct hernia.

TGFβ1 has been described as an important modulator of MMPs [11], inhibiting matrix degradation by down-regulation of collagenase-1 (MMP-1) and stromelysin-1 (MMP-3) and through the up-regulation of proteinase inhibitors such as TIMPs [23,24], even promoting extracellular matrix accumulation. However, TGFβ1 also induces the expression of other MMPs. It has been reported that TGFβ1 increases the expression of both 92 and 72 KDa type IV collagenase (MMP-2 and -9) in cultured human keratinocytes [25], and this increase was associated with markedly raised levels of mRNAs for these enzymes. Other authors have suggested the possibility that, under pathophysiological conditions, extracellular matrix digestion by MMPs (MMP-2, -3 and -7) may induce tissue reactions mediated by TGFβ1 released from the connective tissue [26]. Some authors have provided *in vitro* and *in vivo* evidence that BMP1 (bone morphogenetic protein 1), like metalloproteinases, could be involved in TGFβ1 activation completing a fast-forward loop in tissue remodelling [27].

In previous studies [28,29], we were able to demonstrate the overexpression of MMP-2 in the transversalis fascia of young patients with direct inguinal hernia. In effect, this link has been recently related by Abci *et al.* [30] to diminished levels of its tissue inhibitor, TIMP-2. Klinge *et al.* [31] and Zheng *et al.* [32] have also demonstrated the overexpression of MMP-1 and -13 in patients with a recurring inguinal hernia. We can now correlate this finding with the increased active TGFβ1 expression detected here in the same type of specimen. Our results therefore suggest that the degradation observed in this type of hernia could be caused in part by the TGFβ1-mediated enhanced expression of the 72 KDa type IV collagenase (MMP-2).

Other authors have also demonstrated the selective regulation of MMP-2 but not of other MMPs by TGFβ1 at the transcriptional and post-transcriptional levels, reporting increased MMP-2 gene transcription rates in fibroblasts and an increased half-life of MMP-2 mRNA [12]. In addition, TGFβ1 may directly influence collagenase secretion and consequent proteolytic activity both *in vitro* and in animal models that demonstrate enhanced synthesis of type IV collagenase (MMP-2) in response to TGFβ1 release, thus contributing to matrix remodelling and tissue metabolism [15].

In conclusion, the results of our study indicate over-expression of the active form of TGFβ1 in the TF of young patients with direct inguinal hernia. This overexpression reflects an attempt to counterbalance the enhanced matrix degradation process observed in these patients, identifying a subset of patients requiring the use of a prosthetic material for primary hernia repair.

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