Keywords: collagen, transforming growth factor-β1, cardiac fibroblasts, myofibroblasts, gel contraction

Transforming growth factor-β1-mediated collagen gel contraction by cardiac fibroblasts

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Abstract

Objective

Myofibroblasts and transforming growth factor-β1 (TGF-β1) are key elements of cardiac tissue fibrosis development. The aim of this study was to determine whether the ability of TGF-β1 to affect the contractile activity of cardiac fibroblasts depends on their differentiation into myofibroblasts.

Methods

Cardiac fibroblasts (from male adult Wistar rats) from passage two were cultured to confluence and incubated on a hydrated collagen gel with and without TGF-β1 (0, 20, 40, 100, 200, 400 or 600 pmol/L) for one, two and three days in a Dulbecco’s Modified Eagle’s Medium without foetal bovine serum.

Results

TGF-β1 dose-dependently increased the contraction of collagen gel mediated by cardiac fibroblasts, reaching a maximal effect at 100 pmol/L TGF-β1. TGF-β1 also stimulated 3H-thymidine incorporation and total protein content in cardiac fibroblasts in the collagen gel lattice. TGF-β1 dose-dependently induced an increase in α-smooth muscle actin, a marker of myofibroblasts. The TGF-β1-induced reduction of area of the collagen gel was negatively correlated to the TGF-β1-evoked appearance of α-smooth muscle actin in the collagen gel matrix.

Conclusion

Our data demonstrate that TGF-β1 increased the contractile activity of adult cardiac fibroblasts and their ability to differentiate into myofibroblasts. Because contractile activity was correlated with differentiation, the influence of TGF-β1 on cardiac fibroblast-induced collagen gel contraction might depend on the promotion of myofibroblast differentiation.

Introduction

Collagen gel cultures have been used to evaluate the contractile activity of various cell types including cardiac fibroblasts. Cardiac fibroblasts, when cultured in collagen gels, are able to reorganise the collagen fibres to produce a denser arrangement and thus to contract the gels. This system has been used as a model in attempts to determine the mechanisms of extracellular matrix remodelling. Such a collagen lattice model allows the study of the interaction of fibroblasts with the surrounding matrix in three dimensions.

Factors secreted in granulation tissue by macrophages induce fibroblast differentiation to myofibroblasts with the induction of α-smooth muscle actin (α-SMA) expression. Among these α-SMA-inducing factors, TGF-β1 seemed to be the most efficient. TGF-β1 has been shown to induce α-SMA protein and mRNA expression in two-dimensional cultures of human and rat dermal fibroblasts, bovine aortic endothelial cells, breast fibroblasts, cerebral pericytes, lens epithelium, corneal keratocytes, corneal fibroblasts, and cardiac fibroblasts. Petrov et al. has shown that TGF-β1-stimulated collagen production in two-dimensional cultures of adult rat cardiac ventricular fibroblasts cannot be explained by a direct stimulation of the collagen production, either in fibroblasts or in myofibroblasts. Instead, TGF-β1 induces the differentiation of fibroblasts into myofibroblasts, which have a higher activity for collagen production than fibroblasts. The effect of TGF-β1 on the differentiation of cardiac fibroblasts to myofibroblasts in a three-dimensional collagen matrix populates with control or TGF-β1-treated cardiac fibroblasts has, however, not yet been studied.

Fibroblast contraction might be an important feature of tissue reorganisation during wound repair, since contraction of tissue is an essential process in wound healing. In tissues such as the heart, contraction could speed wound closure, minimise scar size and help to maintain the mechanical properties of the heart. The same process, however, might contribute to the abnormal tissue architecture observed in myocardial fibrosis. In this regard, increased numbers of fibroblasts or myofibroblasts have been reported in fibrotic myocardial tissue. Because fibroblasts or myofibroblasts are able to generate traction force, these cells could contribute to the tissue contraction frequently associated with fibrotic processes in the heart. It is believed that TGF-β1 could stimulate in vivo wound healing by recruitment of fibroblasts, by production of extracellular matrix proteins and also by promoting the contractile activity of (myo)fibroblasts or the contraction of the wound matrix. Contraction of newly-formed connective tissue (granulation tissue) by myofibroblasts could be of great clinical importance in reducing the original size of the wound.

The present study therefore aimed to investigate whether TGF-β1 affects the contractile activity of cardiac fibroblasts in a three-dimensional collagen lattice in a dose-dependent manner and...
whether this activity of TGF-β1 depends on their differentiation into myofibroblasts. Therefore, we cultured cardiac fibroblasts on collagen gels in the presence of various concentrations of TGF-β1 for one, two or three days and examined the relationship between the contraction of the collagen gel and the extent of myofibroblast differentiation.

**Methods**

**Isolation of cardiac fibroblasts**

All animal procedures were in accordance with the laws, regulations and administrative provisions of the Member States of the European Community (Council Directive 86/609/EEC of November 24, 1986) regarding the protection of animals for experimental and other scientific purposes. This research protocol was also approved by the Ethical Committee for Animal Experiments of the Katholieke Universiteit Leuven (K.U.Leuven), Belgium.

Cardiac ventricular fibroblasts were obtained from male Wistar rats, seven to eight weeks old and weighing about 200 g, using a slightly modified isolation procedure as described by Brilla et al.19 In brief, rats were heparinised (625 units/kg body weight), anaesthetised intraperitoneally with nembutal, 50 mg/100 g body weight. The hearts were removed and placed immediately in sterile Joklik’s medium. The extirpated hearts were flushed to rinse blood out of the heart. The hearts were then preincubated at 37°C in humidified air and the aorta was connected with a cannula. A syringe was filled with a few ml of basic salt solution containing NaCl (130 mM), KCl (3 mM), KH2PO4 (1.2 mM), MgSO4·7H2O (1 mM), CaCl2·1.25 mM, glucose 10 mM, Na2-hydroxyethyl) piperazine-2’-(2-ethanesulfonic acid) (HEPES) 10 mM, pH 7.2 and the cannula was flushed to rinse blood out of the heart. The hearts were then perfused via the ascending aorta according to the Langendorff method with Joklik’s medium for five minutes. Then, by recirculating Joklik’s medium containing 0.02% collagenase A and 2% bovine serum albumin (BSA) for 35 minutes with a flow of 5 ml/minute. Thereafter, the atria and vessels were removed and the ventricular tissue placed in Joklik’s medium containing 1% BSA and 0.02% collagenase A for an additional 10 minutes (37°C). The tissue was then minced and filtered through a 200 µm mesh net.

**Cell cultures**

The coarse cell/desintegrated tissue suspension was settled for 15 minutes and its supernatant was then centrifuged at 350 g for 10 minutes. The pellet was resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% of penicillin/streptomycin solution and seeded in an 80 cm² tissue culture flask. The cell cultures were then preincubated at 37°C in humidified air (humidity 95%) with 5% CO2 for four hours. The medium with unattached cells was aspirated and the fresh DMEM supplemented with 10% FBS and penicillin/streptomycin added. The cultures were examined daily using phase contrast microscopy. The medium was replaced every two to three days and the cells were grown to confluence and then passaged with trypsin-EDTA. Under these conditions, coronary smooth muscle and endothelial cells are already rarely seen in the primary cultures. Myocytes do not survive the isolation method, since oxygenation is not provided. In the present study, only cardiac fibroblasts from passage two were used.

For the second passage, cells were transferred to six-well dishes in DMEM with 10% FBS at a density of 2,600 cells/dish for three days until confluence. The cells were then harvested and washed; 100,000 cells were then added to the hydrated collagen with or without TGF-β1 (0, 20, 40, 100, 200, 400 or 600 pmol/L) in 24-wells and incubated for one, two and three days.

**Collagen gel contraction assay**

The contraction of a hydrated collagen lattice by cardiac fibroblasts in serum-free conditions was performed as previously described.21

**Measurement of DNA content in the cardiac fibroblasts inoculated in the gels**

The DNA content of the cardiac fibroblasts incubated in the collagen lattice for three days was measured fluorometrically with the Hoechst dye 33258 (bisbenzimide), using an excitation wavelength of 360 nm and an emission wavelength of 460 nm, as previously described.21 Cell protein content was measured according to the method of Bradford.25

**3H-Thymidine incorporation in the cardiac fibroblasts populated within the gels**

After the collagen gels had been cultured with cardiac fibroblasts for two days, 1 ml 3H-thymidine (1 mCi/ml) per 1 ml of medium was added and further incubated for 18 hours. The medium was then aspirated and the gels were washed three times with 1 ml of 0.01 M phosphate-buffered saline containing NaCl (138 mM), KCl (2.7 mM), pH 7.4, plus 50 µM thymidine.

The gels were then solubilised by the addition of 200 µl of collagenase A. After incubation for 90 minutes, the cell suspension was pipetted in an Eppendorff tube and centrifuged at 2,000 g for 10 minutes.

The supernatant was discarded, 500 µl NaOH (0.25 N) was added to the pellet and 500 µl of the resuspended material was added to 5 ml scintillation fluid. The Eppendorff tube was rinsed with 250 µl NaOH and the rinsate was also added to the scintillation fluid. The radioactivity was measured in a liquid scintillation counter (Tri-Carb Model A, Canberra Packard, Benelux NV) and the 3H-thymidine incorporation was expressed in dpm/10⁶ cells.

**Western blotting**

After the collagen gels had been cultured with cardiac fibroblasts for three days, the medium was aspirated and the gels were washed three times with 1 ml of 0.01 M phosphate-buffered saline containing NaCl (138 mM), KCl (2.7 mM), pH 7.4. The gels were then solubilised by the addition of 200 µl collagenase A. After incubation for 90 minutes, the cell
Figure 1 Dose-dependent stimulation of collagen gel contraction by TGF-β1 in adult rat cardiac fibroblasts incubated directly on the collagen gel for one, two and three days

Table 1 Effect of various doses of TGF-β1 on

<table>
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<tr>
<th>TGF-β1 (pmol/L)</th>
<th>¹H-TdR (dpm/10⁶ cells)</th>
<th>Total protein (µg/well)</th>
<th>DNA content (ng/well)</th>
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<td>0</td>
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<td>10,720±1,700</td>
<td>4.95±1.05</td>
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¹H-TdR = ¹H-thymidine incorporation

Results

Effect of TGF-β1 on collagen gel contraction by cardiac fibroblasts

Control gels containing adult cardiac fibroblasts cultured to confluence and added to a hydrated collagen gel contracted to 38±5%, 43±5% and 45±3%, respectively, of their original gel area after one, two and three days of incubation. The area of the collagen gels measured immediately after the addition of control cardiac fibroblasts averaged 31,000±265 arbitrary units.

TGF-β1 increased (p<0.001) the contraction of collagen gel mediated by cardiac fibroblasts in a dose-dependent manner, when incubated on the collagen gel for one, two and three days (Figure 1). A maximal effect was reached at 100 pmol/L TGF-β1. When neutralising antibody to TGF-β1 (10 µg/ml) was added to the collagen gels, the TGF-β1 (100 pmol/L)-stimulated contraction of the collagen gel by cardiac fibroblasts was abolished, while the control contraction was not significantly affected.

³H-Thymidine incorporation in cardiac fibroblasts incubated in collagen gel

The de novo DNA synthesis, assayed as ³H-thymidine incorporation, in cardiac fibroblasts populated in the collagen gel matrix was dose-dependently increased (p<0.01) by TGF-β1 (Table 1). A maximal effect of TGF-β1 on ³H-thymidine incorporation was obtained at a concentration of 100 pmol/L TGF-β1 and the ³H-thymidine incorporation was increased by 100 pmol/L TGF-β1 by 51±5% (p<0.01).

Cellular protein and DNA content

The total protein content in the cardiac fibroblasts in the collagen gel lattice was increased (p<0.05) by TGF-β1 in a dose-dependent manner (see Table 1) and reached a maximum at 100 pmol/L TGF-β1. The increase in the total protein content by 100 pmol/L TGF-β1 averaged 37±5% (p<0.01).

The DNA content expressed in ng/well or in pg/cell in the fibroblasts in the collagen gel matrix was not significantly affected by TGF-β1 (see Table 1). No cell death occurred in the control or TGF-β1 treated fibroblasts.

TGF-β1 induces α-SMA in the collagen gels populated with cardiac fibroblasts

As shown in Figure 2A, TGF-β1 dose-dependently
induced an increase in α-SMA at concentrations of 20 to 600 pmol/L when incubated on the hydrated collagen gel for three days.

The TGF-β1-induced area reduction of the collagen gel (expressed as % of original area) was negatively correlated to the TGF-β1-evoked appearance of α-SMA (expressed in arbitrary units) in the collagen gel matrix, \( n = 7; y = 3.02 - 0.07 \times, r = 0.79, p<0.02 \).

**Discussion**

The present study showed that TGF-β1 promoted the contraction of collagen gel mediated by cardiac fibroblasts in a dose-dependent manner (see Figure 1). TGF-β1 also stimulated 3H-thymidine incorporation and the total protein content in cardiac fibroblasts in the collagen gel lattice, while the DNA content was not changed. No cell death occurred during TGF-β1 treatment of the cardiac fibroblasts. A possible involvement of polyploidy of the cells may be excluded, since the DNA content per cell did not change after TGF-β1 treatment. However, high tetraploidy levels in granuloma cells was found in chronic wounds. It has previously also been shown by us\(^{26-28}\) that cardiac fibroblasts incubated with TGF-β1 differentiate into myofibroblasts which are larger and contain more protein. This is confirmed in the present study by the increase in protein content after TGF-β1 treatment (see Table 1).

Such a stimulatory effect of TGF-β1 on collagen gel contraction has also been seen by other cell types, such as retinal pigment epithelial cells,\(^{26,28}\) osteogenic cells,\(^{29}\) glioma cells,\(^{30}\) glomerular mesangial cells,\(^{31}\) bronchial epithelial cells\(^{32}\) and skin, corneal, choroidal or renal fibroblasts.\(^{26,28,33-37}\) However, in rat hepatic lipocytes, TGF-β1 had no effect on collagen gel contraction.\(^{38}\)

In the current study, we have shown, for the first time in adult cardiac fibroblasts, that the three-dimensional collagen gel contraction by these fibroblasts is also stimulated by TGF-β1. This dose-dependent stimulation of the collagen gel contraction by TGF-β1 was accompanied by the induction of α-SMA, an indicator of the differentiation of the cardiac fibroblasts to myofibroblasts. Arora et al.\(^{39}\) also reported that TGF-β1, at a concentration of 400 pmol/L, induced a 1.4-fold increase in α-SMA content in gingival fibroblasts inoculated on a floating collagen gel for three days, as compared to a three- and nine-fold increase when the cells were inoculated on an anchored collagen gel or on collagen-coated plates, respectively.

Correlation analysis also revealed a negative relationship between the TGF-β1-induced collagen gel contraction and α-SMA expression, suggesting a link between the contractile activity of cardiac fibroblasts and the quantity of myofibroblasts in the respective cultures. These findings suggest that the ability of TGF-β1 to promote contractile activity of adult rat cardiac fibroblasts may depend on the myofibroblast differentiation.

It has been shown by Kurosaka et al.\(^{40}\) that TGF-β1 also dose-dependently increased the contraction of collagen gel mediated by bovine corneal fibroblasts during a six day incubation period, starting at 0.4 pmol/L and reaching a maximal effect at 40 pmol/L TGF-β1. The ratio of α-SMA positive cells:total number of cells (the P/T ratio) was also increased by TGF-β1 in a dose-dependent manner; a significant rise in the P/T ratio was already observed with 1.28 pmol/L TGF-β1 and reached a maximum at 400 pmol/L TGF-β1.\(^{26}\)

TGF-β3 also promoted the collagen gel contraction by bovine lens epithelial cells and increased the P/T ratio, an indicator of α-SMA expression, in a dose-dependent manner.\(^{26}\) However, in human myofibroblasts obtained as explant cultures or by collagenase digestion of palmar aponeurosis from patients with Dupuytren’s disease, Vaughan et al.\(^{41}\) has shown that, in a stress-relaxed collagen gel lattice contraction assay, TGF-β1 enhanced first the formation of structural elements including stress fibres, vinculin-containing fibronexus adhesion complexes and fibronectin fibrils. This enhancement occurred prior to and independent of α-SMA expression. Hinz et al.\(^{42}\) has also reported that rat subcutaneous fibroblasts (SCFs), expressing low levels (13%) of α-SMA, caused a 41% collagen lattice contraction compared with a 63% contraction for lung fibroblasts (LFs) expressing high levels (70%) of α-SMA. Treating SCFs with 400 pmol/L TGF-β1 for five days increased lattice contraction by 22% and this was accompanied by an
enhancement of α-SMA (to 69%). TGF-β1 treatment, however, did not show an enhancing effect on lattice contraction or α-SMA level. These data indicate that, during TGF-β1 treatment for five days, a 22% increase in lattice contraction is observed with a 56% rise in α-SMA level. No data are however reported after two, three or four days of treatment.

Because there was a significant negative correlation between the TGF-β1-induced reduction of area of the collagen gel and the amount of α-SMA, the ability of TGF-β1 to affect the contractile activity in our cardiac fibroblasts might depend, at least in part, on changes in expression of α-SMA. The modulation of cardiac fibroblasts’ contractile activity by TGF-β1 might thus be related to an effect on α-SMA or to the appearance of myofibroblasts in the gel matrix through differentiation of the fibroblasts. Indeed, in human gingival and periodontal ligament fibroblasts, Arora et al. have shown that the collagen gel contraction mediated by these cells was inhibited by electroinjection of an α-SMA antibody into the cells. In human articular chondrocytes and meniscal cells, TGF-β1 also regulated the expression of α-SMA and their associated contractile behaviour.

It has been suggested, for dermal fibroblasts, that the ability of TGF-β1 to contract a collagen matrix in vitro represents a fibroblast function that also operates in vivo during the contraction of the wound matrix or the process of wound repair. Our in vitro data in cardiac fibroblasts suggest that one of the main tissue factors governing the process of collagen contraction was TGF-β1, a physiological regulator of wound contraction. TGF-β1 was also the main growth factor responsible for the deposition of tissue collagen and other extracellular matrix proteins leading to the development of cardiac fibrosis. The TGF-β1-stimulated collagen gel contraction mediated by cardiac fibroblasts was due to the TGF-β1-induced differentiation of fibroblasts to myofibroblasts.

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References