Tranilast attenuates cardiac matrix deposition in experimental diabetes: role of transforming growth factor-β

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Abstract

Objective: The pathological accumulation of extracellular matrix is a characteristic feature of diabetic cardiomyopathy that is directly related to a loss of function. Tranilast (n-[3,4-anthranilic acid), used for the treatment of fibrotic skin diseases, has also been shown to inhibit transforming growth factor-β (TGF-β)-induced matrix production in kidney epithelial cells.

Methods: To investigate the effects of tranilast in the diabetic heart, we examined its effects in cultured cardiac fibroblasts and then assessed its effects in (mRen-2)27 diabetic rats with established disease (8 weeks after streptozotocin).

Results: In vitro studies demonstrated a 58% reduction in TGF-β1-induced 3[H]-hydroxyproline incorporation with tranilast 30 μM (p < 0.01). At 16 weeks, diabetes in the Ren-2 rat was associated with increased cardiac fibrosis and evidence of TGF-β1 activation, as measured by the abundance of phosphorylated Smad2. Despite persistent hyperglycaemia and hypertension, tranilast attenuated cardiac fibrosis by 37% (p < 0.05) in association with reduction in phospho-Smad2 (p < 0.01).

Conclusion: These findings indicate that tranilast has antifibrotic actions in the Ren-2 model of experimental diabetic cardiac disease by mechanisms that might attributable to reduced TGF-β activity.

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Keywords: Fibrosis; Diabetes; Growth factors; Extracellular matrix

I. Introduction

Diabetic subjects have a two- to fivefold increase risk of developing heart failure [1]. In addition to ischemic heart disease, heart failure in diabetes is also associated with a cardiomyopathy, independent of coronary artery disease [2]. This so-called “diabetic cardiomyopathy” is characterized histologically by myocardial fibrosis with reduced myocardial elasticity, impaired contractility and overt cardiac dysfunction [3–6]. Accordingly, strategies that reduce the pathological accumulation of extracellular matrix have been advocated as potential therapies for the treatment and prevention of heart failure in both diabetic and nondiabetic states [7].

Tranilast (n-[3,4-anthranilic acid) is used in Japan for the treatment of hypertrophic scars [8] and scleroderma [9], skin disorders associated with an excessive fibrotic response. Although the precise mechanisms underlying the antifibrotic effects of tranilast are incompletely understood, its known actions include inhibiting the actions of transforming growth factor-β (TGF-β) [10,11], a profibrotic growth factor that has been pathogenetically linked to the excessive accumulation of collagenous matrix in a wide range of organs and disease states, including the diabetic heart [7,12]. In experimental animal studies, tranilast has been shown to reduce pathological matrix accumulation in...
hypertensive heart disease [13] and in diabetic nephropathy [14], although its effects in the diabetic heart have not been previously examined.

In the present study, we sought to determine the effects of tranilast on diabetes-associated cardiac fibrosis with both cell culture and in vivo studies. Having determined that tranilast abrogated the profibrotic effects of TGF-β in cultured cardiac fibroblasts, we then examined the effects of tranilast in the diabetic (mRen-2)27 rat. This transgenic animal, that has the entire mouse renin gene (Ren-2) inserted into the genome of a Sprague–Dawley rat, develops hypertension and many of the structural and functional characteristics of human diabetic complications when diabetes is induced with streptozotocin [15]. An experimental design was chosen to mimic, in part, the clinical context with drug therapy initiated in established disease and in the presence of persistent hyperglycaemia and hypertension.

2. Materials and methods

2.1. Cell culture studies

To determine the effects of tranilast on cardiac collagen production in vitro, fibroblasts were isolated from the hearts of Ren-2 control and diabetic rats, as previously described [16]. To replicate the in vivo context, cells from control rats were maintained at 5 mM, whilst those derived from diabetic animals were cultured in 25 mM glucose. Cells were passaged twice and then seeded at a density of 50,000/well in DMEM (Gibco™; Invitrogen, Grand Island, NY). After 24 h, fibroblasts were serum starved in either 5 (control) or 25 mM glucose (diabetic) DMEM supplemented with 0.5% bovine serum albumin (BSA), 150uM L-ascorbic acid (Sigma-Aldrich), and 1% antibiotic/antimycotic mixture (Gibco™, Invitrogen). After 44 h, media was replaced with DMEM nutrient mix F12 (Gibco™, Invitrogen), 0.5% BSA L-ascorbic acid in either 5 or 25 mM glucose, as above. Tranilast 30 μM was then added to the wells, followed 4 hours later by [3H]-proline (1 μCi/well) and TGF-β 2.5 ng/ml (R&D Systems). Fibroblasts were harvested 48 h poststimulation, washed four times with PBS, solubilised in 200ml 0.2 M NaOH and then neutralised with 200 ml 0.2 M HCl. Incorporation of exogenous [3H]-proline (L-[2,3,4,5-3H]-proline; Amersham Biosciences, Piscataway, NJ), was then measured using a liquid scintillation counter (Wallac 1410; Amersham Biosciences). Cell viability was assessed by trypan blue exclusion.

2.2. Animals

Thirty-two, female, heterozygous Ren-2 rats, aged 6 weeks were randomised to receive either 55 mg/kg of streptozotocin (STZ) or citrate buffer and studied for a further 16 weeks. Diabetic animals received 2–4 units of isophane insulin (Humulin NPH, Eli Lilly, NSW, Australia) three times per week to promote weight gain and to reduce mortality. Separate groups of control and diabetic Ren-2 rats received tranilast (400 mg/kg/day by gavage in 1% NaHCO3 administered as 200 mg/kg twice daily) from week 8 to week 16 while untreated rats were gavaged with vehicle. Each week, rats were weighed and blood glucose was determined using an AMES glucometer (Bayer Diagnostics, Melbourne, Australia). Every 4 weeks, systolic blood pressure (SBP) was recorded in preheated conscious rats by tail cuff plethysmography [17]. At 16 weeks of study, rats were anaesthetised (Nembutal 60 mg/kg body wt., i.p., Boehringer–Ingelheim, Australia). Hearts were removed from the animal, weighed, sliced transversely, fixed in Bouin’s fixative (Pathtec Diagnostics, Victoria, Australia), or neutral buffered formalin and then paraffin-embedded for light microscopic evaluation. Experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia’s Code for the Care and Use of Animals for Scientific Purposes, in accordance with those of the NIH, and were approved by the Bioethics Committee of the University of Melbourne. All studies were performed with the observer masked to study group to which the animal had been assigned.

2.3. Histopathology and immunohistochemistry

Changes in cardiac structure were assessed in a masked protocol in at least 25 randomly selected tissue sections from each group studied. Sections were stained with Masson’s modified trichrome to demonstrate collagen matrix. Tissue expression of TGF-β was assessed immunohistochemically using a polyclonal anti-TGF-β antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Activity of TGF-β was assessed by quantifying the tissue expression of a specific downstream signalling pathway intermediate, phosphorylated Smad2, using a rabbit anti-phospho-Smad2 antibody (Cell Signalling Technology, Boston, MA). In brief, sections were incubated with antibody overnight at 4 °C. The following day the sections were thoroughly washed in PBS and incubated with rabbit anti-goat (1:200) biotinylated IgG (DAKO, Carpinteria CA, USA) followed by avidin–biotin peroxidase complex (Vector, Burlingame, CA, USA). Localization of the peroxidase conjugates was achieved using diaminobenzidine tetrahydrochloride as a chromagen. Sections incubated with 1:10 NGS, instead of the primary antiserum, served as the negative controls.

2.4. Quantitation of matrix deposition, TGF-β, and phospho-Smad2 expression

The accumulation of matrix, and the extent of immuno-staining for TGF-β and phospho-Smad2 were quantified.
using computer-assisted image analysis, as previously reported [18,19]. Briefly, five random nonoverlapping fields from 6 rats per group were captured and digitised using a BX50 microscope attached to a Fujix HC5000 digital camera. Digital images were then loaded onto a Pentium III IBM computer. An area of blue on a trichrome-stained sections (for matrix) or brown on immunostained sections (TGF-β, phospho-Smad2) were selected for their color ranges. Calculation of the proportional area stained blue (matrix) or brown (TGF-β) were then determined using image analysis (AIS, Analytical imaging Station Version 6.0, Ontario, Canada). For phospho-Smad2, the number of positive nuclei (brown), also quantified using image analysis software, were expressed per mm².

2.5. Statistics

All data are expressed as means±S.E.M. unless otherwise stated. Statistical significance was determined by a two-way ANOVA with a Fisher’s PLSD post hoc comparison. Analyses were performed using Statview II+Graphics package (Abacus Concepts, Berkeley, California) on an Apple Macintosh G4 computer (Apple Computer, Cupertino, California). Results are expressed as ratios of mean values. A p value of <0.05 was taken to indicate statistical significance.

3. Results

3.1. In vitro studies

In cultured rat cardiac fibroblasts, basal ³H-proline incorporation was greater in cells cultured in 25 mM glucose compared with those cultured in 5 mM glucose (Fig. 1). The addition of TGF-β led to a significant increase in ECM production, as measured by ³H-proline incorporation, at both glucose concentrations. Tranilast 30 µM attenuated TGF-β-induced hydroxyproline incorporation in both low and high glucose environments (Fig. 1).

3.2. Clinical data

In comparison with control animals, diabetic rats had reduced body weight which was unaffected by treatment with tranilast (Table 1). Plasma glucose was elevated to a similar extent in treated and untreated diabetic rat groups (Table 1). After 16 weeks, diabetes was associated with reduced total body weight and increased cardiac/body weight with the latter being significantly reduced with tranilast treatment (p<0.05, Table 1). All rats, both diabetic and nondiabetic, were hypertensive with elevated SBP that was not altered by tranilast treatment (Table 1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nondiabetic</th>
<th>Nondiabetic + tranilast</th>
<th>Diabetic</th>
<th>Diabetic + tranilast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>7.3±0.3</td>
<td>7.0±0.2</td>
<td>27.9±1.0</td>
<td>25.1±0.9</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>314±6</td>
<td>321±7</td>
<td>269±4</td>
<td>261±6</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.18±0.03</td>
<td>1.18±0.03</td>
<td>1.24±0.1</td>
<td>1.09±0.04</td>
</tr>
<tr>
<td>Heart/body weight</td>
<td>3.8±0.1</td>
<td>3.6±0.1</td>
<td>4.5±0.3</td>
<td>4.1±0.2*</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>192±4</td>
<td>180±9</td>
<td>204±7</td>
<td>201±4</td>
</tr>
</tbody>
</table>

Data are expressed as mean±S.E.M.  
* p<0.05 versus untreated diabetic  
† p<0.01 versus nondiabetic.
3.3. Histology and immunohistochemistry

At 16 weeks duration of disease, trichrome-stained sections showed increased matrix in hearts from diabetic with matrix accumulation noted in both perivascular and interstitial regions. (Figs. 2 and 3). Similarly, tissue labeling for TGF-β was also increased in the hearts of diabetic animals as was nuclear phospho-Smad2 (Figs. 4–7). Matrix deposition, TGF-β, and phospho-Smad2 immunostaining were all reduced with tranilast treatment, to levels similar to those of control animals (Figs. 4–7).

4. Discussion

Tissue fibrosis is a pathological hallmark of diabetic complications. In the present study, experimental diabetes was associated with increased deposition of collagenous matrix and evidence of TGF-β activation in the hearts of animals with experimental diabetes. The antifibrotic agent, tranilast was found not only to attenuate collagen production in cell culture but also to reduce the pathological fibrosis that develops in the diabetic heart in the in vivo setting.

Much of the increased risk of heart failure in diabetes has been attributed to macrovascular (coronary artery) disease. However, emerging data from experimental, autopsy and epidemiological studies have all highlighted the high prevalence of diabetic cardiomyopathy, independent of atherosclerotic disease [20]. Indeed, like nephropathy, with which heart failure in diabetes is closely associated [21], diabetic cardiomyopathy is also characterized by increased extracellular matrix deposition, a close correlate of both cardiac and renal dysfunction [22]. In the present experimental setting, excessive matrix deposition was noted in the interstitium of diabetic rat hearts.

While hyperglycemia is a sine qua non of diabetic complications, other components of the diabetic milieu such as hypertension and the renin–angiotensin system (RAS) are also thought to contribute to their development. Indeed, the importance of these factors is thought to underlie the beneficial effects of both blood pressure lowering and drugs.
that interrupt the RAS in diabetic nephropathy [23], retinopathy [24] and heart failure [25]. In contrast to most other rodent models of diabetes, the diabetic (mRen-2)27 rat like the diabetic human, is hypertensive and shows increase activity of the RAS. Moreover, in addition to developing diabetic kidney [15] and eye disease [26], the present study also shows that, akin to humans, the diabetic Ren-2 rat also develops cardiac fibrosis.

Current treatment of chronic heart failure focuses on the modulation of the neurohormonal activation that typically develops in response to the evolving functional abnormalities. However, despite such therapy, frequently used in combination, cardiac dysfunction continues to progress in the majority of patients. Given the importance of pathological fibrosis in adverse cardiac remodeling, a potential role of antifibrotic agents has been suggested [27]. Studies conducted over more than a decade have consistently indicated a major role for the prosclerotic growth factor, transforming growth factor-β (TGF-β) in organ fibrosis and dysfunction [28], such that blockade of its expression and action represent an important therapeutic target. In the present study, the ability of TGF-β to induce collagen production was increased in a high glucose environment, suggesting that the overexpression of TGF-β may be particularly injurious in the diabetic milieu.

Tranilast (n-[3,4-dimethoxycinnamoyl] anthranilic acid) is an antifibrotic agent used in Japan for the treatment of fibrotic skin disorders such as keloids [8] and scleroderma [9]. Although the precise mechanisms mode of action are incompletely understood, its ability to inhibit ERK phosphorylation [29], a major intermediate in the TGF-β signaling pathway, may underlie its antifibrotic effects, with known actions of tranilast including the inhibition of TGF-β-induced extracellular matrix production synthesis in a range of cell types [10,11,14,30]. The present study, extends these findings by showing that tranilast also attenuates TGF-β-induced collagen synthesis in cardiac fibroblasts. In the in

Fig. 4. Immunostaining for TGF-β in hearts of control (A), control+tranilast (B), diabetic (C), and diabetic+tranilast rats (D). In control animals, there is minimal TGF-β immunostaining, while diabetes is associated with increased immunostaining in interstitial areas. Treatment of diabetic rats with tranilast was associated with a reduction in TGF-β immunostaining. Magnification: ×320. Nuclei were stained blue with hematoxylin.

Fig. 5. Transforming growth factor-β in cardiac tissue as assessed by the proportional area of tissue showing positive immunolabelling. *p<0.01 versus nondiabetic controls; †p<0.01 versus untreated diabetic rats.
vivo setting, tranilast has been shown to block TGF-β-induced fibrosis in the hypertensive heart [13], injured vessels [31], and diabetic kidney [14]. In addition to its effects on fibroblasts, tranilast has also been shown to stabilize mast cells [32]. These bone marrow-derived cells are rich sources of numerous growth factors, including TGF-β [33] and have been implicated in the pathogenesis of fibrotic diseases including those in the heart [34]. In the present study, rather than examining its effects on disease prevention, a late intervention approach was used. In contrast to the extensive fibrosis in placebo-treated diabetic rats, animals that received tranilast showed less cardiac matrix deposition despite persistent hyperglycaemia and hypertension.

Transforming growth factor-β is synthesized as a 391 amino acid precursor molecule with little biological activity, requiring cleavage of its N-terminal latency-associated peptide (LAP) to give rise to its active form. In addition, the biological effects of TGF-β may also be modified by the presence of the proteoglycan decorin [35] and the scavenging protein α2-macroglobulin [36]. Thus, increased TGF-β1 mRNA or protein may not necessarily reflect parallel changes in TGF-β1 activity. In the present study, in addition to examining TGF-β protein, we also assessed its biological effects by examining one of its specific intracellular actions, the phosphorylation of the TGF-β receptor-activated protein, Smad2. In contrast to non-diabetic rats, TGF-β protein was increased in conjunction with prominent nuclear staining of phosphorylated Smad2 in diabetic rat hearts, consistent with both increased TGF-β protein and activation of its signalling pathways [37]. Treatment of diabetic Ren-2 rats with tranilast led to a relative diminution of both TGF-β protein and nuclear phosphorylated Smad2 immunostaining, suggesting that the beneficial effects of this drug treatment may be attributable to both reduced TGF-β production and activity.
4.1. Limitations

In the present study, late intervention with tranilast attenuated the pathological collagen accumulation in the diabetic heart, despite persistent hyperglycaemia and hypertension. While in general, the extent of fibrosis correlates inversely with cardiac function, further studies will be necessary to examine tranilast's effects on cardiac diastolic and systolic function. In addition, combination studies using tranilast with an agent that blocks the renin–angiotensin system will be needed to assess the potential clinical usefulness of tranilast in the treatment of diabetic cardiac disease since both angiotensin converting enzyme inhibitors and angiotensin receptor blockers are not only cardioprotective but have also been shown to reduce TGF-β expression [38].

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