Transcription activity in fibroblasts from the adult tight skin (TSK) mouse

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ABSTRACT

Objective: To assess if adult tight-skin mouse (TSK) skin fibroblasts have a parallel increase in transcription of collagen and non-collagen genes.

Methods: One-year-old TSK/+ and +/+ (normal littermate) dermal fibroblasts were transfected (lipotransfection) with plasmid constructs containing Chloramphenicol Acetyl Transferase (CAT) gene, directed by promoters of mouse α1(I) α2(I) and α1(III) collagen genes, and by viral enhancers of Simian virus 40, Rous sarcoma virus and an LTR from a Syrian hamster tumour retrovirus. Syrian hamster derived tumour cell lines MF2B and GRI, and fibroblast cell line 3T3/NIH were used as controls. In some experiments, transfected cells were treated with hormones as transcription activating factors. Mixing experiments of tumour cells and TSK/+ or +/+ fibroblasts were done to study potential inhibitors.

Results: Collagen genes promoters failed to induce transcriptional activity in TSK/+ or +/+ fibroblasts, even in the presence of hormone treatment. Mixing experiments did not reveal inhibitor factors acting in these fibroblasts. Viral enhancers induced 2 to 5 times more transcription activity in TSK/+ than in +/+ fibroblasts.

Conclusion: Increased transcription of viral enhancers and not of collagen genes in adult TSK fibroblasts, suggests the presence of transcription activating factors independent of collagen gene activation.

Introduction

Tight skin (TSK) mouse is a genetic model for scleroderma, resulting from a mutation on the fibrillin-1 gene in mouse chromosome 2 (1, 2). Homozygous TSK die in utero (2). Heterozygous TSK/+ mice develop early postnatal skin fibrosis with a consistent temporal sequence (3). At the initial stages of scleroderma development, TSK/+ fibroblasts have shown both in vivo and in vitro increased transcription of several collagen genes, as well as other connective tissue proteins, that persist to a lesser extent in adult life (3, 4). Excessive fibroblast proliferation and increased transcription of collagen genes have both been proposed as mechanisms for the fibrosis development in scleroderma (5, 6). No defects in apoptosis or in the proliferation of fibroblasts have been found in the TSK/+ fibroblasts (7), suggesting that transcriptional activation should be more relevant to fibrosis development in this model.

Dr. Carwile E. LeRoy was fascinated with this mouse model which, as he used to say, provides the opportunity to study, step by step, the development of skin fibrosis from the beginning of the process, a thing almost impossible in human scleroderma. Early after the description of the TSK model, he conducted several studies in order to gain more knowledge about scleroderma. When molecular biology appeared as a tool to study physiological and pathological processes, Dr. LeRoy was one of the first to incorporate the new techniques with his own background. As the profound thinker he was, he believed that the study of the behaviour of collagen genes in the TSK model could yield clues regarding the mechanisms leading to fibrosis in human scleroderma. His ideas and enthusiasm about the TSK model were for us the main inspiration to study the activity of collagen genes in this mouse model.

The aim of the present study was to investigate whether adult skin TSK/+ fibroblasts cultured from the skin of mice with established scleroderma show an increase in the transcription of collagen genes, and if this increase occurs parallel to an increase in the transcription of non-collagen genes.
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Material and methods

Cell cultures

Dermal fibroblasts from one-year-old TSK/+ and +/- (normal littermate) mouse were obtained from the dorsal skin. Skin biopsies were minced and treated with bacterial collagenase/dis- pase (Boehringer Mannheim) as previously described (4). Fibroblasts were then cultured in Dulbecco’s modified minimal essential medium (DMEM) with 10% foetal calf serum (FCS) at 37°C in 95% air with 5% CO₂, until confluent, and maintained for several passages. For the experiments, only low passage (2 to 6) fibroblasts were used. The established fibroblast cell line 3T3/NIH and two tumour cell lines derived from a Syrian hamster ductus deferens smooth muscle tumour (DDT, MF-2 and GR1) (8) were used as controls. 3T3/NIH fibroblasts were grown in the same conditions than TSK/+ and +/- fibroblasts. Tumour cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% FCS, at 37°C in 95% air with 5% CO₂.

Plasmids

All constructs were pBR322 plasmids containing the Chloramphenicol Acetyl Transferase (CAT) gene, directed by the promoters of mouse α1(I), α2(I) and α1(III) collagen genes (kindly provided by Dr. B de Crombrugghe), or by the viral promoter-enhancers of Simian virus 40 (SV-40), Rous sarcoma virus (RSV) and an LTR obtained from a Syrian hamster smooth muscle tumour retrovirus (LTR-SHR).

Transfection experiments

Transfections were performed by Lipofectin (BRL Life Technologies), following the procedure recommended by the manufacturer. Briefly, 5 ml of cells in culture, at a concentration of 2-5 x 10⁶ cells/ml in DMEM-10% FCS, were plated in 60 mm tissue culture dishes. After 48 hours the cells reached ~80% confluence and were washed 3 times with serum-free DMED, and were transfected with 100 ml of sterile water containing 5-10 mg plasmid DNA and 30 mg Lipofectin Reagent. After 24 hours incubation at 37°C in 95% air with 5% CO₂, the cells were treated with 3 ml of DMEM-10% FCS, containing 0.3% bicarbonate, and incubated for another 48 hours. The cells were then harvested and a CAT assay was performed.

In some of the experiments cells were treated, 24 hours after transfection, with different hormones known to behave as trans-acting factors (Testosterone 10⁻¹ – 10⁴, Diethylstilbestrol 10⁻² – 10⁴, Triamcinolone acetonide 10⁻⁷ – 10⁻⁴ and Dexamethasone 10⁻⁷ – 10⁻⁴). Mixing experiments of tumour cells and TSK/+ or +/- fibroblasts were also run to study the potential presence of inhibiting factors in the fibroblasts. All transfection experiments were repeated at least three times with similar results.

Chloramphenicol Acetyl Transferase (CAT) assay

After transfection, the cells were harvested and disrupted by freezing and thawing several times. Cellular debris was pelleted after centrifugation for 10 minutes at 10,000 rpm at 4°C, and used to obtain the total cellular DNA. In the supernatant, the protein level was normalized for all the samples in every experiment with the Protein Bioassay kit (BioRad), as recommended by the manufacturer. Fifteen mg of protein from each sample were incubated at 37°C for 4 hours with 4 mM acetyl-CoA, 0.25 mCi of ¹⁴C-chloramphenicol and 0.5 M Tris-HCl, pH 7.8. Chloramphenicol was extracted with 1 ml ethyl acetate, and the samples were resolved on thin layer chromatography silica gel plates, using chlorormoph:- methanol 95:5. After 1-12 days, film exposure results were analysed by optical density with a scanner and software Diversity One (PDI Inc, NY).

DNA analysis

To confirm the effectiveness of lipotransfection, DNA was extracted from the pellets of transfected cells after protein extraction. The DNA was then cut with EcoRI, transferred to a nitrocellulose filter and hybridised with a pBR322 labelled plasmid probe.

Results

Collagen genes promoters failed to show any transcription activity in TSK/+, +/- or 3T3/NIH fibroblasts, neither basally nor after treatment with different hormones known to behave as trans-activating factors, at increasing concentrations (Fig. 1). By contrast,
both of the tumour cell lines used in our experiments showed a high transcription rate of the three collagen genes, even without any hormone treatment (Fig. 1). DNA analysis did not show any difference in the efficacy of transfection in any cell line (data not shown).

Mixing experiments of tumour cells and TSK/+ or +/+ fibroblasts did not demonstrate the presence of any potential inhibitor factor in the fibroblasts cell lines, since the transcription rate was proportional to the number of tumour cells present in each transfected plate (Fig. 2).

All the viral promoter-enhancers used (SV-40, RSV and LTR-SHR) induced high transcription activity in tumour cells and moderate activity in 3T3/NIH fibroblasts. All of them induced more transcriptional activity in TSK/+ than in +/+ fibroblasts. Table I shows the peak OD of monoacetylated chloramphenicol spots obtained in the CAT assay, after transfection of TSK/+ and +/+ fibroblasts with the different viral promoter/enhancers. In TSK/+ fibroblasts, viral promoter-enhancers induced 2 to 5 times higher transcription activity than in +/+ fibroblasts (Fig.3, Table I).

Discussion
Our study failed to demonstrate any transcription of collagen genes in adult mouse fibroblasts in culture, even in the presence of different hormones known to act as trans-activating factors in different cell lines (8), whereas two smooth muscle-derived tumour cell lines demonstrated a very high transcription activity of the three collagen genes used, even in the absence of any hormone treatment. On the other hand, previous studies have shown an increased transcription rate of collagen genes in adult TSK in vivo (3). Several possible explanations can account for this difference between the in vitro and in vivo results, where other factors are likely activating fibroblasts to produce increased amounts of collagen and other extracellular matrix proteins, as happens in scleroderma skin (9).

Cultured fibroblasts have shown an increased transcription of human α1(I) collagen gene induced by TGF-β treatment (9), and fibroblasts from TSK/+...
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mice are hyper-responsive to this cytokine (10), that was not included in our experiments but which is present in vivo. An intact T helper 2 cells response is necessary for the development of TSK/+ fibrosis, and skin fibrosis is abrogated by the disruption of IL-4, IL-4Ra or Stat6 genes (11, 12), suggesting a role for these cells and derived cytokines in this model. Finally, many different transcription factors, which perhaps are not induced under in vitro conditions, have been implicated in the regulation of collagen gene expression (6). In the TSK/+ mouse, myocardial fibroblasts show a reduced interaction of a negative regulatory sequence with AP-1 transcription factor. This defect produces a lack of downregulation in the activity of the α1(I) collagen gene promoter, and contributes to the increased α1(I) procollagen gene expression in this cells (13). As 3T3/NIH fibroblasts, an established fibroblasts cell line, did not show any transcriptional activity of collagen genes either, we looked for potential intrinsic inhibitor factors in cultured fibroblasts. Mixing experiments with fibroblasts and smooth muscle tumour cells failed to reveal any soluble inhibitor factors, since neither TSK/+ nor +/- nor 3T3/NIH fibroblasts were able to inhibit the high transcripational activity of the three collagen genes showed by the tumour cell lines.

An interesting result of our study is that three different potent viral promoter-enhancers induced a 2- to 5-fold increase in their transcription rate in TSK/+ with respect to +/- fibroblasts. How the fibrillin 1 gene mutation causes the TSK phenotype is not known. Mouse deficient of fibrillin-1, an accepted model for Marfan syndrome, shows a marked dysregulation of transforming growth factor beta (TGF-β) activation and signalling, resulting in apoptosis in the lung and development of emphysema (14), a characteristic finding in Marfan syndrome and also in the TSK/+ mouse, but the influence of this finding on the fibrotic process has not been studied. Nevertheless, high TGF-β mRNA expression is present in TSK/+ and also in normal mouse dermal fibroblasts during the 2 post-natal weeks. TGF-β expression became undetectable by in situ hybridisation afterwards, whereas there is a persistence of high collagen I and III expression by a subpopulation of fibroblasts in the TSK/+ fibrotic lesions (3), suggesting that alternative pathways should be present in this model. A possible explanation is that the mutation induces a dysregulation in the production of important transcription factors, which results in collagen overproduction in the early stages of the TSK/+ scleroderma mouse model as a secondary event. Our finding of increased transcription of viral promoter-enhancer in TSK/+ dermal fibroblasts in culture suggest the presence of some still unknown transcription activating factors, showing increased activity in TSK/+ compared to +/- fibroblasts, and acting independently of collagen gene activation.

References