Regulation of fibroblast procollagen production

Transforming growth factor-β1 (TGFβ1) initiates a series of signalling events resulting in diverse cellular responses including stimulation of extracellular matrix protein production. In this study we have investigated the role of pertussis toxin-sensitive G-proteins in mediating the effects of TGFβ1 on fibroblast procollagen metabolism. TGFβ1 stimulated human fetal lung fibroblast procollagen synthesis and production in a dose-dependent manner which was maximal at 0.5 ng/ml. TGFβ1 also decreased the proportion of newly synthesized procollagen degraded intracellularly. Pertussis toxin, a G-protein inhibitor, further stimulated TGFβ1-induced procollagen synthesis and production, but alone it had no effect on fibroblast procollagen metabolism. Addition of indomethacin also potentiated the TGFβ1-induced increase in procollagen synthesis and production. The effects of pertussis toxin and indomethacin were not additive. Pertussis toxin and indomethacin did not affect the proportion of newly synthesized procollagen degraded intracellularly, either alone or in combination, by control cells. The TGFβ1-induced decrease in intracellular procollagen degradation was maintained but not further affected by pertussis toxin or indomethacin. TGFβ1 increased prostaglandin E2 (PGE2) compared with PGE2 production by control cells. Addition of pertussis toxin or indomethacin blocked the TGFβ1-induced increase in PGE2 production. The TGFβ1-induced increase in PGE2 preceded the increase in procollagen production. These results demonstrate that TGFβ1-induced procollagen synthesis by lung fibroblasts is modulated by production of PGE2. Pertussis toxin and indomethacin block the production of PGE2 and enhance the effect of TGFβ1 on procollagen synthesis. From these data we conclude that the effects of TGFβ1 on PGE2 production but not procollagen synthesis are mediated via a receptor linked to a pertussis toxin-sensitive G-protein.

INTRODUCTION

Transforming growth factor β1 (TGFβ1) is one of a family of related polypeptide mediators consisting of five isoforms, three of which have been identified in mammals. The TGFβs are pluripotent mediators, having amongst their many functions the ability to modulate cell growth and stimulate the production and deposition of connective tissue matrix components including collagens, fibronectin and proteoglycans. Altered expression of TGFβs has been implicated in a number of diseases. Increased expression has been observed in fibrotic disorders of the lung [1–3], skin [4], kidney [5] and liver [6]. It has also been suggested that loss of TGFβ expression in the skin and skin tumours is associated with hyperproliferation and a high risk of malignancy [7]. It is therefore important to understand the mechanisms by which TGFβs mediate their biological effects.

The effect of TGFβ1 on collagen deposition culminates from a network of interlinked actions. It increases procollagen synthesis via an increase in mRNA [8–10] and decreases the proportion of newly synthesized procollagen degraded intracellularly [11]. TGFβ1 also maximizes its stimulation of collagen deposition by stimulating production of antikollagenases and decreasing the production of collagenase [12–14]. The effect of TGFβ1 on collagen synthesis is known to involve binding of transcription factors to elements in the promoter regions of procollagen genes, resulting in upregulation of gene transcription [15–17]. However, the receptor and signal transduction pathways which lead to these effects are poorly understood.

There are at least seven cell surface receptors/binding proteins for TGFβ1 (for review see [18]). The type I and II receptors have been cloned and these as well as the type V receptor have been found to contain transmembrane serine/threonine kinase signalling motifs [19–22]. However, the type I receptor also contains regions with sequence similarity for tyrosine kinase and protein kinase C activation [22]. The type III receptor has also been cloned and its product is a membrane-anchored proteoglycan with no obvious signalling motif [23, 24]. There is co-operativity between the type I and II and the type II and III receptors, with formation of heteromeric complexes [25–28]. Type I and II receptors have been shown to be involved in all effects of TGFβ1 that have been investigated [25, 29–32]. The signalling motifs and functions of the other TGFβ1 receptors/binding proteins have still to be determined and little is known of the intracellular signal transduction pathways.

G-proteins act as membrane bound intermediates between cell surface receptors and intracellular effector systems (for review see [33, 34]). G-proteins are heterotrimeric consisting of α-, β- and γ-subunits. They are cyclically regulated, activation occurs by binding of GTP to the α-subunit which is hydrolysed to GDP and P, resulting in deactivation followed by dissociation of GDP prior to binding more GTP. The involvement of G-proteins in transduction pathways and identification of their α-subunits is aided by the ability of certain bacterial exotoxins to specifically ADP-ribosylate particular α-subunits and inhibit their activity. Pertussis toxin inhibits α-subunits which have a broad cellular distribution and α-subunits which appear to predominate in neuronal cells. In contrast, cholera toxin inhibits the ubiquitously distributed α-subunits. There is evidence to suggest that some

Abbreviations used: TGFβ1, transforming growth factor β1; PGE2, prostaglandin E2; NCS, newborn-calf serum.

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actions of TGFβ1, including c-sis mRNA expression and mitogenesis in fibroblasts as well as inhibition of epithelial cell proliferation and chemotaxis of interleukin-2-activated natural killer cells, are mediated through receptors coupled to G-proteins [35–39]. Gα1, Gα2 and Gα3 have been implicated in some of these actions [38,39]. In this study we have begun to investigate the mechanism by which TGFβ1 modulates procollagen production by determining the effects of pertussis toxin on TGFβ1-induced fibroblast procollagen metabolism.

MATERIALS AND METHODS

Cell Culture

Human fetal lung fibroblasts (HFL-1) and fetal rat fibroblasts (Rat 2), both obtained from the American Type Culture Collection, (Rockville, MD, U.S.A.) were cultured in 2.4 cm diameter plates with Dulbecco’s modified Eagle’s medium + 5% newborn-calf serum (NCS) in a humidified atmosphere of air containing 10% CO2 at 37°C. When cells appeared confluent they were incubated for a further 24 h. The media was then removed and replaced with 1 ml of pre-incubation medium, which contained 4 mM glutamine, 50 μg/ml ascorbic acid, 0.2 mM proline and 2% (v/v) NCS and incubated for 24 h. The media were then replaced with 1 ml of fresh preincubation medium containing pertussis toxin (Calbiochem Novabiochem, Nottingham, U.K.) at concentrations between 0 and 500 ng/ml, or indomethacin (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) at a concentration of 1 μg/ml, and incubated for 3 h. Finally the media were replaced with media containing appropriate concentrations of pertussis toxin, indomethacin and porcine TGFβ1 (British Biotechnology, Oxford, U.K.) at concentrations between 0.1 and 10 ng/ml (4–400 pM) and incubated for between 2 and 24 h before harvesting.

Sample preparation

For cultures in which procollagen metabolism was to be assessed, the cell layer was scraped into the medium and aspirated. Each well was washed with 1 ml of PBS and the washings were combined with the initial aspirate. Proteins were precipitated by addition of ethanol to give a final concentration of 67% (v/v) and left at 4°C overnight. The precipitated proteins were extracted by vacuum filtration onto polyvinylidene difluoride filters (Millipore, Durapore 0.45 μm) and the ethanol-soluble material in the filtrate retained. The filtrate was evaporated to dryness using a Dri-Block Sample Concentrator (Techne DB-3, SC-3). The dried filtrates and the proteins adhering to the filters were hydrolysed in 2 ml of 6 M hydrochloric acid at 110°C overnight. Hydrolysates were mixed with approximately 70 mg of charcoal and filtered (Millipore, 0.65 μm) prior to chromatography.

Similar cultures were set up to assess prostaglandin E2 (PGE2) production and cell number by measurement of DNA. At the end of incubation the medium was removed and stored at −40°C prior to measurement of PGE2. The cell layer was scraped into 1 ml of PBS, aspirated into a microcentrifuge tube and centrifuged at 9000 g for 5 min; the clear supernatant was discarded. Any remaining cells were washed from the well with a further 1 ml of PBS, aspirated and combined with the initial cell pellet. After centrifugation the supernatant was discarded and the pellet was stored at −40°C prior to analysis of DNA. PGE2 was measured in 10 and 50 μl aliquots of the cell-conditioned medium using a specific enzyme immunoassay (Amersham International, Buckinghamshire, U.K.) and results were expressed as pg per well. DNA was measured using a modified diphenylamine method as described previously [11].

Measurement of hydroxyproline and calculation of procollagen synthesis and the proportion degraded

Hydroxyproline was isolated and measured by reverse-phase HPLC of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)-derivatized hydrolysates as described previously [11,40]. Hydroxyproline content was determined by comparing peak areas of samples from the chromatogram with those generated from standard solutions, derivatized and separated under similar conditions and run on the same day. As the cell monolayer contains a small amount of procollagen and the serum contained hydroxyproline, the amount of hydroxyproline present in the combined culture medium and cell layer at the start of incubation was determined in both the ethanol-soluble and -insoluble fractions. This background level, which represented between 1.95 and 2.62 nmol hydroxyproline per well in the insoluble fraction and between 0.92 and 1.87 nmol hydroxyproline per well in the soluble fraction, was then subtracted from the sample values.

This represented between 15 and 22% of the total hydroxyproline measured in each sample after a 24 h incubation. The hydroxyproline present in the ethanol-soluble fraction, containing free amino acids and small peptides, from each culture was taken to represent hydroxyproline derived from the degradation of procollagen during the culture period, whilst the hydroxyproline in the ethanol-insoluble fraction was taken to represent hydroxyproline in intact procollagen and was called procollagen production. The proportion of procollagen degraded can be calculated by dividing the hydroxyproline content of the ethanol-soluble fraction by the sum of the hydroxyproline in the ethanol-soluble and -insoluble fractions. The rate of synthesis was obtained from the combined values for ethanol-soluble and -insoluble fractions, and rates of degradation and production were obtained from values for ethanol-soluble and -insoluble fractions respectively and expressed per μg DNA per hour.

Statistical analysis

Statistical evaluation was performed using an unpaired t-test or by one-way analysis of variance for multiple comparisons using the Newman–Keuls procedure. The mean values of various parameters were said to be significantly different when the probability of the differences of that magnitude, assuming the null hypothesis to be correct, fell below 5% (i.e. P < 0.05). Where mean values are calculated, standard errors of the mean (S.E.M.) are also given.

RESULTS

Figure 1 shows the effect of TGFβ1 on fibroblast procollagen metabolism. Production and synthesis increased in a dose-related manner with maximal stimulation obtained at 0.5 ng/ml which remained constant up to 10 ng/ml. The proportion of procollagen degraded intracellularly decreased from 22.80 ± 0.69% in control cultures to 19.28 ± 0.79% in cultures with 1 ng/ml TGFβ1 (P < 0.01). All further studies were carried out with TGFβ1 at a concentration of 1 ng/ml.

The effect of pertussis toxin, at concentrations of 10, 100 and 500 ng/ml, on fibroblast procollagen production is shown in Figure 2. At the two lower doses of pertussis toxin the cells
Figure 1 Effect of TGFβ1 on procollagen synthesis and production by human fetal lung fibroblasts

Procollagen production (solid bars) and synthesis (cross-hatched bars) were measured following incubation of cells for 24 h in the presence of TGFβ1 at the concentrations indicated. Proteins in the combined media and cell layer were precipitated by addition of ethanol to a final concentration of 67% (v/v). Values for procollagen production were obtained from measurements of hydroxyproline in the ethanol-insoluble fraction and values for procollagen synthesis represent the combined measurements of hydroxyproline in the ethanol-soluble and insoluble fractions. Each value represents the mean ± S.E.M. for six replicate cultures.

Figure 2 Effect of pertussis toxin on normal and TGFβ1-induced procollagen production

Procollagen production by human fetal lung fibroblasts was measured in cultures with and without TGFβ1 (1 ng/ml) at the concentrations of pertussis toxin indicated. Each value represents the mean ± S.E.M. for six replicate cultures. Where no error bars are shown they were within the point.

appeared normal microscopically, and procollagen production was not affected, but at 500 ng/ml some cells lost their characteristic spindle shape and became rounded, a small decrease in procollagen production was also observed. In the presence of TGFβ1 and pertussis toxin, procollagen production was increased. Maximal stimulation of about 22% (P < 0.001) compared with the increase induced by TGFβ1 alone was obtained with pertussis toxin at 100 ng/ml. With 500 ng/ml pertussis toxin the procollagen production was reduced to values similar to those obtained with TGFβ1 alone. Pertussis toxin did not affect the proportion of newly synthesized procollagen degraded intracellularly at any concentration tested (control values in this experiment were 28.0 ± 1.1%). The TGFβ1-induced decrease in intracellular procollagen degradation was maintained in the presence of pertussis toxin. Values at 10 ng/ml pertussis toxin were 29.5 ± 0.64% for pertussis toxin alone and 24.83 ± 1.43% in the presence of pertussis toxin plus TGFβ1. At 100 ng/ml pertussis toxin values were 28.64 ± 0.76% for pertussis toxin alone and 25.35 ± 0.48% in the presence of pertussis toxin and TGFβ1 (P < 0.05 and P < 0.005 respectively).

Similar results were obtained with another fibroblast line (Rat 2). Control rates of procollagen synthesis were 5.24 ± 0.29 pmol hydroxyproline per µg DNA per hour. This was increased by 64% to 8.61 ± 0.33 pmol hydroxyproline per µg DNA per hour (P < 0.001) in the presence of TGFβ1 (5 ng/ml) with a further increase compared with that of TGFβ1 alone of 27% to 10.91 ± 0.51 pmol hydroxyproline per µg DNA per hour in the presence of TGFβ1 and 100 ng/ml pertussis toxin (P < 0.01).

Figure 3 shows the effects of pertussis toxin (100 ng/ml) and the cyclooxygenase (prostaglandin endoperoxide synthase) inhibitor, indomethacin, on fibroblast procollagen production. Indomethacin (1 µg/ml) increased procollagen production by 20% from 34.11 ± 1.72 pmol hydroxyproline per µg DNA per hour to 40.97 ± 0.81 pmol hydroxyproline per µg DNA per hour (P < 0.01). In the presence of TGFβ1, indomethacin further stimulated procollagen production from 44.04 ± 1.21 pmol hydroxyproline per µg DNA per hour to 50.32 ± 1.07 pmol hydroxyproline per µg DNA per hour (P < 0.01). The effects of pertussis toxin and indomethacin were not additive. For cells incubated with pertussis toxin and indomethacin values were 38.74 ± 0.76 pmol hydroxyproline per µg DNA per hour which was similar to the values obtained for each agent alone. In the presence of TGFβ1, as well as pertussis toxin and indomethacin, values were 49.24 ± 0.70 pmol hydroxyproline per µg DNA per hour which was greater than the value for TGFβ1 alone (P < 0.05) but was not significantly different to the values obtained for TGFβ1 in the presence of pertussis toxin or indomethacin. At the dose of indomethacin used, there were no changes in intracellular procollagen degradation. In this experiment, control values were 22.80 ± 0.69%, which decreased to 19.28 ± 0.79% in the presence of TGFβ1 (P < 0.01). Values for indomethacin and indomethacin plus TGFβ1 were 22.41 ± 0.62% and 18.53 ± 0.53% respectively. The effects of indomethacin and pertussis toxin on TGFβ1-induced procollagen synthesis showed similar changes to those for production (Table 1).
Table 1  Effects of pertussis toxin and indomethacin on TGFβ1-induced stimulation of procollagen synthesis by lung fibroblasts

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<tr>
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<th>Procollagen synthesis (pmol hydroxyproline per μg DNA per hour)</th>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td>Pertussis toxin</td>
<td>44.76 ± 1.28</td>
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<tr>
<td>Indomethacin</td>
<td>52.83 ± 1.19*</td>
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<td>Pertussis toxin + Indomethacin</td>
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The effect of TGFβ1 on PGE2 production by human fetal lung fibroblasts is shown in Figure 4. Control cultures contained 239 ± 90 pg of PGE2 per well. In the presence of TGFβ1, the PGE2 production increased about 5-fold to 1175 ± 291 pg per well (P < 0.01). Pertussis toxin and indomethacin alone showed a tendency to decrease basal PGE2 production but this was not significant. However, pertussis toxin and indomethacin completely blocked the TGFβ1-induced increase in PGE2 (P < 0.01 in both cases).

Figure 5 shows the changes in PGE2 (Figure 5a) and procollagen production (Figure 5b) at various times up to 24 h. In control cultures PGE2 levels remained constant over the 24 h incubation period. In the presence of TGFβ1, PGE2 levels were not significantly different compared with control values at 2 h. However, by 4 h PGE2 had increased almost 4-fold [control, 107 ± 10 pg of PGE2 per well; TGFβ1, 403 ± 25 pg of PGE2 per well (P < 0.001)] and continued to increase up to 24 h when values were approximately seven times the control values [control, 116 ± 16 pg of PGE2 per well; TGFβ1, 826 ± 94 pg of PGE2 per well (P < 0.001)]. In contrast there were no consistent differences in procollagen production between control and TGFβ1-treated cells up to 8 h. At 16 and 24 h procollagen production was increased in TGFβ1-treated cells by about 20 and 45%, respectively (P < 0.001 in both cases).

**DISCUSSION**

In these studies we have shown that TGFβ1 stimulates the synthesis and production of procollagen by fetal fibroblasts and that intracellular degradation of newly synthesized procollagen is decreased. Pertussis toxin did not inhibit basal or TGFβ1-induced procollagen metabolism. These findings suggest that TGFβ1-induced procollagen metabolism is not mediated by receptor-coupling to pertussis toxin-sensitive G-proteins. These conclusions are consistent with those of a recent study of steady-state mRNA levels for type I procollagen in bovine adrenal capillary endothelial cells [41]. In contrast to this previous study, in our experiments pertussis toxin reproducibly further stimulated the TGFβ1-induced increase in procollagen synthesis and production.

One interpretation of the pertussis toxin enhancement of TGFβ1's stimulation of collagen synthesis and production is that pertussis toxin may be blocking a TGFβ1-induced inhibitory pathway of procollagen metabolism. One such pathway might involve prostaglandins. Prostaglandins of the E type are potent inhibitors of procollagen metabolism [42-46]. TGFβ1 has been reported to stimulate cyclooxygenase 1 but not cyclooxygenase 2 gene expression and production of PGE2 by lung fibroblasts [46,47]. Furthermore, this increase in PGE2 limits the TGFβ1-induced stimulation of collagen production [46]. To investigate the potential role of PGE2 in modulating the TGFβ1-induced
procollagen synthesis and its potentiation by pertussis toxin, experiments were performed in the presence of indomethacin, and fibroblast PGE$_2$ production was measured. Indomethacin alone stimulated procollagen synthesis, indicating that the cells synthesized prostaglandins under basal culture conditions. In the presence of TGF$_{β1}$, indomethacin also potentiated the increase in procollagen synthesis and production to a similar extent to that induced by pertussis toxin. The effects of pertussis toxin and indomethacin on TGF$_{β1}$-induced procollagen production and synthesis were not additive, suggesting that both agents were acting on the same pathway. TGF$_{β1}$ increased PGE$_2$ production and this increase was blocked by pertussis toxin and indomethacin. These results suggest that the potentiation of TGF$_{β1}$-stimulated procollagen synthesis and production by pertussis toxin is due to inhibition of TGF$_{β1}$-induced PGE$_2$ synthesis and further suggests that TGF$_{β1}$-induced fibroblast production of prostaglandins is mediated either directly or indirectly through a pertussis toxin-sensitive G-protein. PGE$_2$ is also a potent inhibitor of fibroblast proliferation [48] and may be at least partly responsible for TGF$_{β1}$-induced inhibition of cell proliferation observed in many cells.

PGE$_2$ has previously been shown to inhibit procollagen synthesis and increase the proportion of newly synthesized procollagen degraded intracellularly by human lung fibroblasts [42,44-46]. In the present studies TGF$_{β1}$-induced PGE$_2$ production, which had an inhibitory effect on procollagen synthesis but did not affect the proportion of procollagen degraded intracellularly. This difference may be explained by the amount of PGE$_2$ produced by the fibroblasts which resulted in concentrations of about 1–2 ng/ml in the media of cells incubated with TGF$_{β1}$. Previous studies have shown effects of exogenously applied PGE$_2$ on procollagen synthesis at concentrations in the range of 10–250 ng/ml [44–46], whereas effects on degradation appear to require concentrations of at least 2 μg/ml [42,44].

In this study we have also shown that the effects of TGF$_{β1}$ on PGE$_2$ production precede the increase in procollagen production. PGE$_2$ production was increased within 4 h of addition of TGF$_{β1}$ but stimulation of procollagen production was not apparent until 16 h. To our knowledge this is the first report of the time-course of TGF$_{β1}$-induced procollagen production. However, procollagen mRNA levels have been reported to be increased in as little as 2 h following treatment of rat kidney fibroblasts with TGF$_{β1}$ [9]. This apparent discrepancy in the time-course of changes in mRNA and protein synthesis could be explained either by differences in cell type or by the increase in PGE$_2$ production, which may negate the effect of increased procollagen mRNA on procollagen synthesis at the early times.

The receptor(s) mediating these effects of TGF$_{β1}$ is(are) uncertain, although it is likely that receptors I and II are involved. TGF$_{β1}$-mediated effects on cell proliferation have previously been attributed to the type I receptor [49] and these effects were shown to be coupled through pertussis toxin-sensitive G-proteins [37]. However, studies demonstrating the cooperative nature of the TGF$_{β}$ type I and II receptors [25] suggest that the anti-proliferative effects of TGF$_{β1}$ are mediated through the type II receptor in conjunction with the type I receptor [30]. Both type I and type II receptors have been shown to contain cytoplasmic serine/threonine kinase signalling motifs and the type II receptor has been shown to require its kinase activity to enable signalling of growth inhibition [25]. However, it is known that receptors may be coupled to multiple signalling motifs which enable a diverse range of responses to receptor activation, and it has been reported that a consensus G-protein activating sequence has been found in the type II receptor (Arg$_{334}$-Lys$_{335}$) but this has yet to be confirmed [39]. Alternatively, it has recently been proposed that the multifunctional nature of responses to TGF$_{β}$ may be due to different isoforms of receptor I [29,31,32]. Whether TGF$_{β1}$-induced prostaglandin and procollagen synthesis are mediated through one of these receptors or another of the less well characterized TGF$_{β}$ receptors/binding proteins remains to be determined.

These results demonstrate that the effects of TGF$_{β1}$ on fibroblast procollagen metabolism are modulated by production of PGE$_2$ which is inhibited by pertussis toxin or indomethacin with a concomitant increase in procollagen synthesis. From these data we conclude that the effects of TGF$_{β1}$ on PGE$_2$ but not procollagen synthesis are mediated via a receptor linked, directly or indirectly, to a pertussis toxin-sensitive G-protein.

The authors are grateful for the financial support of the Arthritis and Rheumatism Council and the British Lung Foundation. We also thank Beeja Vyas for her assistance whilst working in the Biochemistry Unit on a Wellcome Trust Vacation Scholarship.

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Received 17 June 1994/25 November 1994; accepted 6 December 1994