Indomethacin suppresses the anti-proliferative effects of transforming growth factor- β isoforms on fibroblast cell cultures

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The transforming growth factor- β (TGF β) family of mediators consists of five closely related isoforms, of which three are present in mammals. TGF β_1 has been shown to exert a biphasic effect on the proliferation of several cell types, including fibroblasts, with stimulation at low concentrations and inhibition at higher concentrations. The stimulatory effects are well characterized, but the mechanisms by which TGF β_1 inhibits cell proliferation are incompletely understood. In the present study we have compared the effects of all three mammalian TGF β isoforms on human lung fibroblast proliferation, and have elucidated the role of the TGF β -induced synthesis of prostaglandin E_2 (PGE $_2$) in mediating their actions. All three isoforms stimulated fibroblast proliferation with maximal effects at 5 pg/ml (0.2 pM) and an order of potency of TGF β_3 > TGF β_2

> TGF β_1 . At higher concentrations, proliferation declined, and at 40 pg/ml and above all isoforms inhibited fibroblast proliferation. Again TGF β_3 was the most potent, but there were no significant differences between the inhibitory effects of TGF β_1 and TGF β_2 . Addition of indomethacin, an inhibitor of PGE $_2$ synthesis, did not alter the proliferative activity of any of the TGF β isoforms, but completely overcame their inhibitory effects, restoring the stimulatory actions observed at lower TGF β concentrations. All TGF β isoforms stimulated PGE $_2$ synthesis; TGF β_3 was approximately twice as potent as TGF β_1 and TGF β_2 , each of which had similar effects. These data suggest that the inhibition of fibroblast proliferation at higher concentrations of TGF β isoforms may be mediated by autocrine stimulation of PGE $_2$ synthesis.

INTRODUCTION

The transforming growth factor- β (TGF β) family of mediators consists of five closely related isoforms, TGF β_1 to TGF β_5 , of which TGF β_1 to TGF β_3 have been shown to be present in mammals. They are multifunctional mediators with effects on a wide range of processes, including cell differentiation, proliferation, immune function and extracellular matrix protein metabolism.

The effects of TGF β_1 on cell proliferation are diverse. It inhibits epithelial and endothelial cell proliferation, but in mesenchymal cells both stimulatory and inhibitory effects have been reported. In fibroblasts, smooth muscle cells, osteoblasts and chondrocytes a biphasic effect has been observed, with stimulation at low concentrations and inhibition at higher concentrations of TGF β [1–3]. TGF β_1 and TGF β_2 have been shown to have similar effects on the proliferation of aortic smooth muscle cells [2], TGF β_3 has similar effects to TGF β_1 on osteoblast proliferation [3], and all three mammalian isoforms inhibit the replication of epithelial and endothelial cells [4].

The stimulatory effects of TGF β_1 on cell proliferation have been reported to be mediated via induction of autocrine platelet-derived growth factor (PDGF) synthesis [5,6]. However, the mechanisms by which TGF β_1 inhibits cell proliferation are less well characterized. There is evidence to suggest that TGF β_1 delays or inhibits progression through the late stages of G1 in the cell cycle. It inhibits the expression of G1 cyclins and cyclin-dependent kinases [7], and may also prevent formation of cyclin–kinase complexes [8]. This results in the prevention of phosphorylation of the retinoblastoma tumour suppressor gene product Rb [9] and suppression of *c-myc* gene expression [10–12], leading to inhibition of cell proliferation. The upstream signalling

events between the membrane-bound TGF β receptors and the nuclear events described above are uncertain. In fibroblasts and smooth muscle cells it has been suggested that inhibition of cell growth is partly due to down-regulation of the α subunit of the PDGF receptor, limiting the effect of autocrine PDGF-AA production [2,13]. However, this is unlikely to be a general mechanism, since cells such as epithelial cells do not normally express PDGF receptors and are unresponsive to PDGF [14]. TGF β_1 is also known to stimulate the autocrine production of prostaglandin E_2 (PGE $_2$) by fibroblasts [15]. PGE $_2$ is a potent inhibitor of fibroblast proliferation [16] and could play a potentially important role in mediating the inhibitory effect of TGF β on cell proliferation.

In the present study we have examined the effects of the three mammalian TGF β isoforms on human lung fibroblast proliferation and determined the role of TGF β -induced PGE $_2$ synthesis in mediating these effects. The data suggest that all three TGF β isoforms have a similar profile of stimulatory and inhibitory effects, but TGF β_3 is more potent than TGF β_1 and TGF β_2 . Indomethacin, an inhibitor of prostaglandin synthesis, completely abolished the anti-proliferative effects of all three TGF β isoforms and restored the stimulatory effects observed at lower TGF β concentrations, suggesting that autocrine production of PGE $_2$ is the dominant mechanism by which these TGF β isoforms inhibit fibroblast proliferation.

MATERIALS AND METHODS

Cell culture

Normal diploid human fetal lung fibroblasts (HFL-1; obtained from American Type Culture Collection, Rockville, MD, U.S.A.)

Abbreviations used: TGF β , transforming growth factor- β ; PGE $_2$, prostaglandin E_2 ; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; NCS, newborn calf serum.

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were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum (NCS) (Imperial Laboratories, Andover, Hants., U.K.). Cells were grown in a humidified atmosphere of 10% CO₂ in air at 37 °C. Experiments were conducted on cells between passages 14 and 20. Cells were regularly tested for mycoplasma contamination by hybridization with a radiolabelled rRNA probe (GeneProbe; supplied by Laboratory Impex Ltd., Teddington, Middx., U.K.).

Proliferation assay

Cell proliferation was assessed using a rapid spectrophotometric assay for counting cells cultured in 96-well microtitre plates [16]. Fibroblasts were seeded (6×10^3 cells/well) in DMEM + 0.4% NCS. After a 24 h preincubation, the medium from each well was replaced with fresh medium supplemented with TGF β isoforms at concentrations up to 160 pg/ml (6.4 pM) or PGE₂ at concentrations up to 64 ng/ml (0.18 μ M). Fibroblasts exposed to DMEM + 0.4% NCS were used as medium controls. In experiments to block PGE₂ synthesis, indomethacin (1 μ g/ml) was added to the cells 30 min prior to addition of TGF β . This protocol has previously been shown to inhibit PGE₂ synthesis in this cell line [15].

Changes in cell number were assessed at various times up to 72 h later. Briefly, medium was removed and the plate immersed in PBS. The PBS was removed by blotting the plate on absorbent paper, and the cell monolayer was fixed by addition of 100 μ l of formal saline [10% (v/v) formalin in 0.15 M NaCl]. The cells were stained with 100 μ l/well Methylene Blue (1% in 0.01 M borate buffer) for 30 min. Excess dye was removed by four washes in 0.01 M borate buffer (pH 8.5). The bound dye was then eluted from the cells by addition of 100 μ l of acidified alcohol (0.01 M HCl/ethanol, 1:1, v/v), and the absorbance was measured at 650 nm using a microplate spectrophotometer. Results were expressed either as mean absorbance or as percentage change in mean absorbance compared with cells exposed to medium alone. Changes in fibroblast cell number expressed in this way were confirmed by direct cell counting.

PGE₂ assay

Fibroblasts were grown to confluence in DMEM + 5% NCS. When cells appeared confluent they were incubated for a further 24 h. The medium was removed and replaced with DMEM + 0.4% NCS and incubated for 24 h. This medium was then replaced with fresh medium containing TGF β isoforms at concentrations between 5 and 160 pg/ml (0.2–6.4 pM) with and without indomethacin (1 μ g/ml). After a further incubation of between 24 and 72 h the medium was removed and PGE₂ was measured using a specific enzyme immunoassay (Amersham International). Results were expressed as pg per well or as percentage change compared with values for cells exposed to medium alone.

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 differences of that magnitude, assuming the null hypothesis to be correct, fell below 5% (i.e. $P < 0.05$). Where mean values are calculated, S.E.M.s are also given.

RESULTS

Figure 1 shows the effects of increasing concentrations of the TGF β isoforms on proliferation of human fetal lung fibroblasts. All three isoforms stimulated fibroblast proliferation maximally at 5 pg/ml (0.2 pM; $P < 0.001$ in all cases). At this concentration, TGF β_3 stimulated proliferation to a greater extent than did TGF β_2 ($P < 0.001$), which was in turn more potent than TGF β_1 ($P < 0.001$). The stimulation induced by TGF β_3 was twice that observed with TGF β_1 . At concentrations above 5 pg/ml, proliferation declined, and at concentrations of 40 pg/ml and above, proliferation was reproducibly inhibited. There were no significant differences between the inhibitory effects of TGF β_1 and TGF β_2 , but at 160 pg/ml TGF β_3 produced a greater inhibition than the other isoforms ($P < 0.001$).

The time courses for the effects of the three TGF β isoforms on proliferation at one stimulatory (5 pg/ml) and two inhibitory (80 and 160 pg/ml) concentrations are shown in Figures 2(a), 2(c) and 2(e) respectively. After 24 h, the cell number was not significantly different compared with untreated cells for any of the isoforms at any concentration. At 48 and 72 h, all TGF β isoforms at a concentration of 5 pg/ml stimulated fibroblast proliferation (Figure 2a), while at 80 and 160 pg/ml they inhibited proliferation (Figures 2c and 2e). At the higher concentrations, TGF β_3 reduced cell numbers at 48 and 72 h compared with the number present at the time of TGF β_3 addition to the cultures. In cultures exposed to TGF β_1 and TGF β_2 the cell number did not fall significantly below that present at the start of the experiment at any time. At 48 h the magnitude of the effects and the order of potency for the three isoforms were similar to those shown in Figure 1.

The effects of indomethacin on TGF β -mediated changes in fibroblast proliferation are shown in Figures 2(b), 2(d) and 2(f). Indomethacin alone had no effect on fibroblast proliferation (compare control cell growth shown in Figure 2a with that in Figure 2b). Furthermore, indomethacin did not alter significantly

Figure 1 Effects of TGF β isoforms on the proliferation of human lung fibroblasts

Fibroblast proliferation was assessed 48 h after the addition of the TGF β isoforms at the indicated concentrations. Results are expressed as percentage changes compared with cells grown in media alone. Values represent means $\pm$ S.E.M. for six replicate cultures. Where no error bars are shown, they are within the point.

Figure 2 Effect of indomethacin on the proliferative response to TGF β isoforms

Cell numbers were assessed after the indicated periods of incubation with TGF β isoforms at concentrations of 5 pg/ml (**a** and **b**), 80 pg/ml (**c** and **d**) or 160 pg/ml (**e** and **f**), either alone (**a**, **c** and **e**) or in the presence of 1 μ g/ml indomethacin (**b**, **d** and **f**). Cells grown in media alone (**a**, **c** and **e**) and indomethacin alone (**b**, **d** and **f**) are indicated by the broken lines. Values are means $\pm$ S.E.M. for six replicate cultures. Where no error bars are shown, they are within the point.

Table 1 Effect of TGF β_3 on PGE $_2$ production by human lung fibroblasts

Significant differences compared with control: * $P < 0.01$; ** $P < 0.001$.

TGF β_3 (pg/ml)	PGE $_2$ (pg/well)					
	24 h		48 h		72 h	
	— Indomethacin	+ Indomethacin	— Indomethacin	+ Indomethacin	— Indomethacin	+ Indomethacin
5	56.2 $\pm$ 3.9	30.1 $\pm$ 3.0	52.9 $\pm$ 2.4	26.9 $\pm$ 1.8	55.1 $\pm$ 5.9	27.1 $\pm$ 7.5
80	96.7 $\pm$ 7.8*	21.6 $\pm$ 4.8	50.4 $\pm$ 2.4	27.6 $\pm$ 3.3	67.7 $\pm$ 5.3	42.4 $\pm$ 2.4
160	95.4 $\pm$ 5.7**	17.3 $\pm$ 3.5	60.2 $\pm$ 3.9	36.8 $\pm$ 2.9	56.2 $\pm$ 6.0	16.0 $\pm$ 5.2

the proliferative activity induced by the three TGF β isoforms at a concentration of 5 pg/ml, although at 48 h in this experiment the value for TGF β_2 was lower than that without indomethacin. However, in the presence of TGF β isoforms at concentrations of 80 and 160 pg/ml, indomethacin overcame the inhibitory effects, and stimulation of cell proliferation, similar to that seen with TGF β concentrations of 5 pg/ml, was observed.

Levels of PGE $_2$ produced by cells grown in medium alone were similar at the 24 h (55.5 $\pm$ 2.2 pg/well), 48 h (57.8 $\pm$ 3.4 pg/well) and 72 h (52.2 $\pm$ 9.2 pg/well) culture times. In the presence of indomethacin, control PGE $_2$ levels were inhibited by 40–70 % (24 h, 15.9 $\pm$ 5.1 pg/well; 48 h, 22.9 $\pm$ 2.9 pg/well; 72 h, 32.2 $\pm$ 5.4 pg/well). Table 1 shows the effects of the most potent inhibitor of proliferation, TGF β_3 , on fibroblast PGE $_2$ production

Table 2 Effects of TGF β isoforms on fibroblast PGE₂ productionSignificant differences compared with control: * $P < 0.05$; ** $P < 0.001$.

[TGF β] (pg/ml)	PGE ₂ (% of control)		
	TGF β_1	TGF β_2	TGF β_3
5	89 $\pm$ 6	100 $\pm$ 6	111 $\pm$ 5
80	119 $\pm$ 6*	139 $\pm$ 14*	182 $\pm$ 13**
160	186 $\pm$ 16**	175 $\pm$ 14**	228 $\pm$ 11**

Figure 3 Effect of exogenous PGE₂ on human lung fibroblast proliferation

Fibroblast proliferation was assessed 72 h after addition of PGE₂ at the indicated concentrations (●). The broken line represents the number of cells present at the time of PGE₂ addition (○). Values are means $\pm$ S.E.M. for six replicate cultures.

over the 72 h culture period. TGF β_3 at a concentration of 5 pg/ml, which had been shown to stimulate proliferation, did not affect fibroblast PGE₂ production at any time. At TGF β_3 concentrations of 80 pg/ml and 160 pg/ml, which inhibited fibroblast proliferation, PGE₂ levels were increased by about 70 % after 24 h. However, by 48 h the PGE₂ levels had declined to control values, and were also unchanged compared with control values at 72 h. Indomethacin prevented the increase in PGE₂ induced by TGF β_3 at 24 h, with levels similar to those in cells incubated in the presence of indomethacin alone. Indomethacin remained active throughout the 72 h culture period, with PGE₂ levels reduced by between 25 % and 70 % compared with levels for cells grown in medium alone.

Table 2 shows the effects of the three TGF β isoforms on fibroblast PGE₂ production at 24 h. All three isoforms stimulated the production of PGE₂ by fibroblasts. PGE₂ production increased with increasing concentrations of TGF β . At 80 pg/ml, TGF β_3 induced a larger increase in PGE₂ than TGF β_1 or TGF β_2 ($P < 0.01$ and $P < 0.05$ respectively). The level of PGE₂ produced in the presence of TGF β_3 at a concentration of 80 pg/ml was similar to that produced by TGF β_1 and TGF β_2 at concentrations of 160 pg/ml. There were no significant differences between the

levels of PGE₂ produced in response to TGF β_1 and TGF β_2 . Consistent with the effects of TGF β_3 shown in Table 1, TGF β_1 and TGF β_2 did not stimulate PGE₂ production at 72 h: levels were 50.2 ± 3.0 pg/well and 61.5 ± 4.4 pg/well respectively compared with the control value of 52.9 ± 9.2 pg/well.

The effects of exogenously applied PGE₂ on fibroblast proliferation are shown in Figure 3. In medium alone the cell number increased by about 55 % over the 72 h culture period. PGE₂ at concentrations of up to 2 ng/ml did not affect fibroblast proliferation (results not shown), but concentrations of 4 ng/ml and above inhibited proliferation in a dose-dependent manner, with complete inhibition at concentrations of 32 ng/ml and above.

DISCUSSION

In this study we have demonstrated a biphasic effect of TGF β_1 on the proliferation of normal human lung fibroblasts, which is consistent with previous studies on marrow and skin fibroblasts [1,2]. Additionally, we have demonstrated that TGF β_2 and TGF β_3 exert similar biphasic effects. The stimulatory effects of TGF β_3 were greater than those of TGF β_2 , which were in turn greater than those of TGF β_1 . The inhibitory effects of TGF β_3 were greater than those of TGF β_1 and TGF β_2 . There were no consistent differences in the potency of TGF β_1 and TGF β_2 with respect to inhibition of proliferation. We have also demonstrated that all three isoforms stimulate fibroblasts to produce PGE₂. Consistent with the anti-proliferative effects of the TGF β isoforms, TGF β_3 was the most potent in this regard, with no differences observed between TGF β_1 and TGF β_2 .

Higher concentrations of TGF β_3 decreased cell numbers at 48 and 72 h (Figures 2c and 2e). This may be related to limited cell death associated with the sparse culture conditions and the presence of a potent inhibitor of proliferation. There was a trend towards decreased cell numbers in the presence of TGF β_1 and TGF β_2 at 72 h, but this was not statistically significant, further demonstrating the greater potency of TGF β_3 in these cells.

TGF β_3 has previously been shown to be more potent than TGF β_1 in stimulating proliferation of mouse embryonic AKR-2B fibroblasts and osteoblasts [3,17]. TGF β_3 has also been shown to be a more potent inhibitor of epithelial cell growth than TGF β_1 or TGF β_2 [4]. Such results would be consistent with TGF β receptors having a higher affinity for TGF β_3 than for TGF β_1 or TGF β_2 . Receptor competition assays suggest that this is the case for osteoblasts [3], but in AKR-2B cells the receptors have a higher affinity for TGF β_1 than TGF β_3 [17], and in epithelial cells similar affinities for TGF β_1 and TGF β_3 have been observed [4]. This suggests that different cells have subpopulations of the TGF β type I and II receptors that have different affinities for the different TGF β isoforms. This has been demonstrated for TGF β_2 : most TGF β type I and II receptors have a lower affinity for TGF β_2 than for the other isoforms, but some cells have also been shown to express a subset of type I and II receptors with high affinity for TGF β_2 [4]. The fact that similar responses were obtained with TGF β_1 and TGF β_2 in the present study suggests that HFL-1 fibroblasts also express these high-affinity receptors for TGF β_2 .

The proliferative effects of TGF β_1 on mesenchymal cells are well characterized and have been demonstrated to be due to induction of autocrine synthesis of PDGF [5,6] and PDGF α receptors [18]. The proliferative response to TGF β observed here occurred after a lag period of approx. 24 h. This lag phase has been observed previously and is consistent with TGF β acting as an indirect mitogen via induction of PDGF synthesis and up-regulation of PDGF-inducible genes such as *c-fos* and *c-myc* [5].

In the present study we have demonstrated that blocking PGE₂ synthesis induced by TGF β overcomes the anti-proliferative effects of high concentrations of TGF β isoforms and restores the proliferative response observed at low concentrations of TGF β . PGE₂ is a potent inhibitor of cell proliferation which is known to decrease levels of *c-myc* mRNA in fibroblasts and macrophages [19,20]. This is consistent with the inhibition of *c-myc* gene expression observed in the presence of TGF β ₁ [10–12].

TGF β ₃ only stimulated PGE₂ production at the 24 h time point, with levels returning to control values by 48 h. We have previously shown that TGF β ₁ stimulates PGE₂ production, with levels increasing up to 8 h and a plateau being attained between 8 and 24 h [15]. The present data suggest that, at some time between 24 and 48 h, either TGF β is inactivated or PGE₂ production in response to TGF β ₃ is down-regulated. However, the increased PGE₂ production induced by TGF β isoforms over this period is sufficient to induce and maintain the inhibition of fibroblast proliferation up to 72 h. Furthermore, basal levels of PGE₂ produced by these cells appear to be insufficient to affect proliferation, since inhibition of PGE₂ synthesis by indomethacin had no effect on proliferation.

Exogenously applied PGE₂ also inhibited fibroblast proliferation, but concentrations approximately two orders of magnitude greater than that produced in response to TGF β were required to elicit the same response. There are a number of possible explanations for this. PGE₂ is rapidly metabolized, and levels measured in culture media in response to TGF β represent steady-state levels rather than total production. Exogenously applied PGE₂ will also be metabolized rapidly, and high initial doses are required to maintain active concentrations for sufficient time to inhibit proliferation. It is also possible that the microenvironment of the cell may allow PGE₂ released by the cell to interact more effectively with its receptor than exogenously applied PGE₂.

Previous studies have demonstrated that the anti-proliferative effects of TGF β on epithelial cells are mediated via pertussis toxin-sensitive G-proteins [12,21]. Furthermore, we have recently shown that the TGF β ₁-induced synthesis of PGE₂ is mediated via a similar mechanism [15], providing further evidence that PGE₂ plays a key role in mediating the anti-proliferative effects of TGF β .

In summary, we have demonstrated that TGF β ₂ and TGF β ₃ share the ability of TGF β ₁ to stimulate or inhibit fibroblast proliferation, depending on their concentration. All three TGF β isoforms stimulate fibroblast synthesis of PGE₂ in a dose-dependent manner, and inhibition of PGE₂ synthesis by indomethacin restores the proliferative response observed at low concentrations of TGF β isoforms. These data suggest that TGF β -induced synthesis of PGE₂ may be responsible for the anti-proliferative effects of TGF β isoforms. These findings have potentially important implications for understanding the mechanisms of disease. For example, it has been suggested that fibroblasts from patients with pulmonary fibrosis have a

decreased capacity to synthesize PGE₂ [22,23], which in the presence of increased levels of TGF β [24] could enhance the fibrotic response. There is also evidence that many tumour cells lose their anti-proliferative response to TGF β , resulting in uncontrolled cell proliferation [25]. A defect in the ability of these cells to synthesize PGE₂ could provide the basis for these observations.

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