Tyrosine Kinase Activity Is Necessary for Growth Factor-Stimulated Rabbit Type II Pneumocyte Proliferation

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ABSTRACT

Tyrosine kinases are important in the signal transduction of a number of growth factors. As shown previously, transforming growth factor (TGF)- α stimulated proliferation of type II cells *in vitro*. The mitogenic effect of TGF- α could be blocked by the addition of the tyrosine kinase inhibitors genistein or tyrphostin. Tyrosine phosphorylation in type II cells exposed to growth factors was examined using an antiphosphotyrosine antibody. After addition of TGF- α , phosphorylation of a tyrosine protein with a molecular mass of 170 kD, presumed to be the epidermal growth factor receptor (EGF-R), peaked by 5 min, returning to baseline by 30 min. As expected, genistein or tyrphostin decreased the TGF- α -induced phosphorylation of the EGF-R. Addition of TGF- β resulted in no newly phos-

phorylated tyrosine proteins. TGF- β decreased the TGF- α -induced phosphorylation of the EGF-R. Previous work has shown that TGF- β blocks the TGF- α stimulation of type II cell proliferation. It appears that TGF- β interferes with TGF- α -induced phosphorylation of the EGF-R. (*Pediatr Res* 36: 481-486, 1994)

Abbreviations

TGF, transforming growth factor
EGF, epidermal growth factor
EGF-R, epidermal growth factor-receptor
TBS, Tris-buffered saline
³H-TdR, tritiated thymidine
PDGF, platelet-derived growth factor

Growth factors are important mediators of cellular functions in many systems, including normal pulmonary development (1-3). In addition, they are thought to play a role in the lung's response to cell injury (4). Type II alveolar epithelial cells are also important in lung injury, because they proliferate and differentiate into pulmonary type I cells during the process of epithelial regeneration (5, 6). The mechanism of this transformation is not well understood, but it is most likely regulated by growth factors.

Growth factors that are involved in pulmonary type II epithelial cell proliferation and differentiation include EGF, TGF- α , and TGF- β . EGF has been shown to accelerate fetal lung maturation *in vivo* (7-9). EGF and TGF- α have 40% homology and have similar biologic effects (10, 11). Both EGF and TGF- α exert their effects by binding to the EGF-R, a 170-kD protein. Upon EGF or TGF- α binding to the membrane-bound EGF-R, the receptor dimerizes and a tyrosine kinase is activated, leading to autophosphorylation of the receptor (12-16). This

is the first step initiating a complex cytosolic signal transduction cascade that ultimately results in gene transcription in the nucleus (17-19).

TGF- β inhibits basal proliferation of type II pneumocytes, as well as the enhanced proliferation of type II pneumocytes, in response to EGF or TGF- α (20). TGF- β signal transduction is not well understood, although its type II receptor is believed to be a serine/threonine kinase (21). The specific mechanism involved in TGF- β antagonism of the proliferative effect of EGF and TGF- α is also unknown, although activation of a serine/threonine phosphatase in a step beyond activation of the TGF- β receptor has been implicated in other systems (22, 23). The present work examines the signal transduction events that occur in type II cells after addition of TGF- α , TGF- β , and tyrosine kinase inhibitors. Cell proliferation was examined using ³H-TdR incorporation with cell counting to corroborate results. The tyrosine kinase inhibitors used were genistein and tyrphostin. Genistein preferentially inhibits ATP binding to a tyrosine kinase rather than serine or threonine kinases, especially at lower concentrations (24). Tyrphostin is a more specific competitive inhibitor of a growth factor substrate binding to a tyrosine kinase (25, 26).

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METHODS

Adult male New Zealand White rabbits were euthanized with approximately 70 mg/kg pentobarbital. New-born rabbits were used for inhibitor concentration curve studies, with results confirmed in adults. Animals were approved for use and handled as instructed by the University Committee on Animal Resources. The lungs were removed, and type II cells were isolated and cultured on collagen-coated 12-well plates at 2×10^6 cells per well using the technique previously described by Finkelstein and Shapiro (27). Using tannic acid staining (28), it has been routine in our laboratory to obtain 92% cell purity of type II cells in culture 22 h after plating (20).

Thymidine incorporation studies. Preliminary studies were performed in A549 cells (a human adenocarcinoma cell line) to estimate the range of concentrations of tyrosine kinase inhibitors needed for type II cell concentration curves. After 22 h in culture in DME/F12 (Sigma Chemical Co., St. Louis, MO) with 10% FCS, incubating at standard conditions of 37°C and 5% CO₂, type II cells were exposed to serum-free DME/F12 and incubated for 2 h. Cells were then pretreated with the tyrosine kinase inhibitors genistein at 0.1–20 µM or tyrphostin at 7.5–30 µM, (GIBCO BRL, Grand Island, NY) in serum-free DME/F12 with 5 µCi/mL ³H-TdR (Amersham, Arlington Heights, IL) for 24 h. Assays for lactate dehydrogenase (Sigma Chemical Co.) were performed in triplicate on cells exposed to 10 µM genistein or 30 µM tyrphostin, the concentrations used for later experiments, to confirm cell viability. Cells were rinsed with PBS four times, 5% trichloroacetic acid two times, and PBS two times. Cells were collected with three 500-µL aliquots of 0.1 M NaOH and added to 10 mL of scintillation fluid (National Diagnostic, Manville, NJ), and 100 µL of 10% acetic acid were added to neutralize the samples. Samples were allowed to equilibrate overnight before counting with a Beckman LS 7500 scintillation counter. After incubating for 22 h in DME/F12 with 10% FCS, cells were incubated in serum-free DME/F12 for 2 h. Cells were then pretreated with genistein (10 µM) or tyrphostin (30 µM) in serum-free DME/F12 for 2 h. Medium was then changed to include inhibitors with or without TGF-α (10 ng/mL) or TGF-β (10 ng/mL) (Collaborative, Bedford, MA) in DME/F12 with 5 µCi/mL ³H-TdR and incubated for 24 h. Samples were collected and counted as previously described. Cell counting was performed in triplicate for several experiments. Cells were counted while plated using a gridded eyepiece (29).

Western blot analysis. Type II cells were isolated and cultured as previously described. Twenty-two hours after plating, cells were placed in serum-free DME/F12 and incubated for 2 h. Cells were pretreated with genistein (10 µM) or tyrphostin (30 µM) for 30 min to 2 h, then exposed to TGF-α (10 ng/mL), TGF-β (10 ng/mL), or both for 30 s to 30 min. Samples were collected in 200-µL Laemmli sample buffer plus 100 µM Na vanadate (Fisher Scientific Co., Fair Lawn, NJ) and 30 mM Na pyrophosphate

(Sigma Chemical Co.) to inhibit dephosphorylation (30), and samples were boiled for 5 min. Protein quantification was performed using bicinchoninic acid analysis (Pierce Chemical Co., Rockford, IL). Approximately 200 µg of protein were loaded per well on an SDS (ICN, Cleveland, OH) polyacrylamide (National Diagnostics, Manville, NJ) gel. The positive control used was phosphorylated EGF-R from A431 cells (UBI, Lake Placid, NY). The molecular weight markers were Rainbow (Amersham, Arlington Heights, IL). Electrophoresis was performed over 3 to 4 h at 35 mA per gel using a Hoefer apparatus (Hoefer, San Francisco, CA). Proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) at 2.5 mA/cm² for 40 min using an ISS Multiblotter (Enprotech, Natick, MA) dry blotter. Protein transfer was verified using 0.2% Ponceau S (Sigma Chemical Co.) staining, which was removed using 0.1 M NaOH. Nonspecific binding sites were blocked using a blocking solution composed of 5% casein in TBS with 1 mL of Tween 20 and 10 µL of Antifoam A (Sigma Chemical Co.) per liter for 1/2 h at room temperature. The blots were then incubated overnight at 4°C in 10 mL of 1 µg/mL monoclonal mouse antiphosphotyrosine antibody (UBI) (31). Blots were washed with blocking solution for 2 × 5 min, then 1 × 15 min, and then incubated for 1 h at room temperature in 10 mL of 1 µg/mL biotinylated goat anti-mouse IgG (Cappel, Durham, NC). The blots were washed in blocking solution as before, then washed in TBS 2 × 5 min. The blots were then incubated at room temperature for 40 min with Vectastain horseradish peroxidase (Vector, Burlingame, CA) in TBS, then washed with blocking solution and TBS as before. The blots were incubated in enhanced chemiluminescence (Amersham) for 1 min and then exposed to x-ray film (Kodak x-omat, Rochester, NY) for 3–45 min. A Western blot was performed similarly on 200 µg of rabbit type II cell protein as previously described using 10 mL of 1 µg/mL sheep anti-human EGF-R (UBI) as the primary antibody and 10 mL of 1 µg/mL biotinylated rabbit anti-sheep IgG (Chemicon, Temecula, CA) as the secondary antibody. A Western blot without the primary antibody was performed to identify nonspecific binding.

Data analysis. Analysis of variance with Newman-Keul's post-hoc test was used to compare ³H-TdR incorporation of control samples with that of growth factor- and tyrosine kinase inhibitor-treated samples. A *p* value of <0.05 was considered statistically significant.

RESULTS

Treatment of type II cells with genistein at a range of 0.1–20 µM established that incubation with <1 µM genistein resulted in no inhibition of ³H-TdR incorporation. Maximal effect was achieved at 10 µM, with approximately 60% inhibition of ³H-TdR incorporation compared with control (Fig. 1A). Treatment with tyrphostin at a range of 3–30 µM established that treatment with <10 µM tyrphostin resulted in no inhibition of

^3H -TdR incorporation, and treatment with 30 μM resulted in optimal inhibition, approximately 60% inhibition compared with control (Fig. 1B). Assays for lactate dehydrogenase confirmed no change in cell viability at 10 μM genistein and 30 μM tyrphostin (data not shown).

Exposure of type II cells to genistein (10 μM) or tyrphostin (30 μM) resulted in significant inhibition of basal ^3H -TdR incorporation ($p < 0.05$) (Fig. 2), as well as blocking of the increased ^3H -TdR incorporation seen in response to TGF- α ($p < 0.05$) (Fig. 3). Cell counting confirmed ^3H -TdR incorporation results, with 60–70% inhibition of both ^3H -TdR incorporation and cell number (insets, Figs. 2 and 3).

Figure 1. A, Effects of genistein concentration on ^3H -TdR incorporation in isolated rabbit type II cells. Type II cells were isolated and plated for 22 h. They were then pretreated for 2 h with 0.1–20 μM genistein and incubated for 24 h. Values are expressed as a percentage of control. Experiments were performed in triplicate; $n = 2$. B, Effect of tyrphostin concentration on ^3H -TdR incorporation in isolated rabbit type II cells. Type II cells were isolated and plated for 22 h. They were then pretreated for 2 h with 7.5–30 μM tyrphostin and incubated for 24 h. Values are expressed as a percentage of control. Experiments were performed in triplicate; $n = 1$.

Figure 2. Tyrosine kinase inhibitors decrease basal ^3H -TdR incorporation and cell proliferation in isolated rabbit type II cells. Type II cells were isolated and plated for 22 h. They were then pretreated for 2 h with 10 μM genistein or 30 μM tyrphostin and incubated for 24 h. All values are expressed as a percentage of control (mean $\pm$ SEM). Experiments were performed in triplicate with $n = 4$. Basal ^3H -TdR incorporation inhibition was statistically significant (*, $p < 0.05$ by analysis of variance). Inset shows cell number as a percentage of control, performed in triplicate; $n = 1$ –2.

To examine further the effects of TGF- α on type II cell proliferation, tyrosine phosphorylation was studied. An antiphosphotyrosine antibody was used to examine the response of type II cells to growth factors. As expected, addition of TGF- α resulted in phosphorylation of a

Figure 3. Tyrosine kinase inhibitors decreased ^3H -TdR incorporation and cell number in isolated rabbit type II cells in response to TGF- α . Type II cells were isolated and plated for 22 h. They were then pretreated for 2 h with 10 μM genistein or 30 μM tyrphostin followed by 10 ng/mL TGF- α and incubated for 24 h. Experiments were performed in triplicate with $n = 4$. All values are expressed as a percentage of control (mean $\pm$ SEM). Inhibition of TGF- α -induced ^3H -TdR incorporation was statistically significant (*, $p < 0.05$ by analysis of variance). Inset shows cell number as a percentage of control, performed in triplicate; $n = 1$ –2.

170-kD protein corresponding to the known size of the EGF-R by 30 s, peaking by 5 min, and returning nearly to baseline by 30 min (Fig. 4). Also as expected, the tyrosine kinase inhibitors genistein or tyrphostin inhibited phosphorylation of the presumed EGF-R previously seen in response to TGF- α (Fig. 5). The inhibition by genistein did not appear to be altered by timing of treatment. The inhibition by tyrphostin was more pronounced when cells were pretreated with tyrphostin for 30 min before addition TGF- α compared with addition of tyrphostin and TGF- α simultaneously. An anti EGF-R antibody confirmed the presence of the EGF-R at 170 kD (Fig. 6).

Exposure of type II cells to TGF- β resulted in no newly tyrosine-phosphorylated proteins within 30 min (Fig. 7). Pretreatment with TGF- β for 30 min resulted in decreased phosphorylation of the presumed EGF-R previously seen in response to TGF- α (Fig. 8).

DISCUSSION

Previous work has established the importance of protein kinases in regulating cellular functions in type II cells. Protein kinase C and cAMP-dependent protein kinase are important in the regulation of surfactant secretion (32–34). cAMP-dependent protein kinase activity has been found to be altered at various states of development in the rat lung, possibly to mediate developmental changes in pulmonary response to hormonal stimuli (35). Actin, a cytoskeletal protein involved in regulating type II cell shape and mobility, is phosphorylated via a cAMP-dependent protein kinase (36). Tyrosine kinase activity in type II cells in response to TGF- α and TGF- β has not yet been studied.

The results of the current work suggest that tyrosine kinases are important in basal type II cell proliferation *in vitro* as measured by ^3H -TdR incorporation and cell counting, inasmuch as known tyrosine kinase inhibitors were capable of reducing basal proliferation. In the rat type II cell *in vitro*, TdR incorporation has not always been associated with a concomitant increase in cell number (37). However, in rabbit type II cell proliferation studies, cell number correlated with ^3H -TdR incorporation (20). In addition to species differences, other factors such as cell density and matrix components may also

Figure 4. TGF- α resulted in rapid phosphorylation of a 170-kD tyrosine protein presumed to be the EGF-R. Rabbit type II cells were isolated and plated for 24 h, then treated with 10 ng/mL TGF- α for 30 s to 30 min. Cells were collected in Laemmli sample buffer and subjected to SDS-PAGE using a 12% gel followed by Western blot analysis with an antiphosphotyrosine antibody. Molecular masses are indicated on the left in daltons.

Figure 5. Tyrosine kinase inhibitors blocked TGF- α -induced phosphorylation of the presumed EGF-R. Rabbit type II cells were isolated and plated for 24 h (control), pretreated for 30 s with 10 ng/mL TGF- α (lane α), pretreated for 30 min with 10 μM genistein or 30 μM tyrphostin (lanes G and T), pretreated for 30 min with 10 μM genistein or 30 μM tyrphostin, then treated with 10 ng/mL TGF- α for 30 s (lanes G $\rightarrow$ α/G and T $\rightarrow$ α/T), or treated with inhibitor plus TGF- α simultaneously for 30 s (lanes α/G and α/T). Cells were collected in Laemmli sample buffer and subjected to SDS-PAGE using a 3–17% gradient gel followed by Western blot analysis with an antiphosphotyrosine antibody. Molecular masses are indicated on the left in daltons.

play a role in the relationship between ^3H -TdR incorporation and cell number.

One factor to consider in analyzing and interpreting the data using tyrosine kinase inhibitors is that tyrosine kinases are ubiquitous within living cells. By using tyrosine kinase inhibitors, we may be affecting other vital cellular functions (38). These effects may be somewhat mitigated by using tyrphostin, a more specific antagonist of the

Figure 6. The EGF-R was identified in isolated rabbit type II cells at 170 kD. Type II cells were isolated and plated for 24 h. Cells were collected in Laemmli sample buffer and subjected to SDS-PAGE using an 8% gel followed by Western blot analysis with (lane a) or without (lane b) an anti-EGF-R antibody. Molecular masses are indicated on the left in daltons.

Figure 7. Treatment of isolated rabbit type II cells with TGF- β resulted in no newly phosphorylated tyrosine proteins. Type II cells were isolated and plated for 22 h, then treated with 10 ng/mL TGF- β for 30 s to 30 min. Cells were collected in Laemmli sample buffer and subjected to SDS-PAGE using a 12% gel followed by Western blot analysis with an antiphosphotyrosine antibody. Molecular masses are indicated on the left in daltons.

EGF-R tyrosine kinase, and by minimizing exposure times (25, 26). It appears that in our system the cellular function affected is specific, inasmuch as tyrphostin and genistein, with different mechanisms of action, achieve similar effects.

Phosphorylation of the EGF-R is a crucial step in the signal transduction pathway initiated by TGF- α in type II cells. This event peaks by 30 s to 5 min, a time course comparable to EGF studies in other cell systems (17, 18). Pretreatment with tyrphostin resulted in more inhibition of EGF-R phosphorylation than simultaneous addition with TGF- α , whereas timing of genistein had little effect. This may be due to tyrphostin being a more specific tyrosine kinase inhibitor or to the cells not being synchronous in their cell cycles.

We have demonstrated that TGF- β decreases the level of phosphorylation of the EGF-R in response to TGF- α . One possible explanation is that TGF- β affects TGF- α binding to its receptor. In previous studies in our laboratory, TGF- β had no effect on EGF binding to the EGF-R for up to 20 min (39). This is similar to the time course of the EGF-R phosphorylation we studied. Alternatively, TGF- β may enhance dephosphorylation of the EGF-R in response to TGF- α . Fontenay *et al.* (22) have demonstrated a similar effect of TGF- β decreasing the PDGF-induced phosphorylation of the PDGF receptor, another

tyrosine kinase receptor. In their work using human passaged fibroblasts, it appears that TGF- β exerts its inhibitory effects on PDGF via serine/threonine phosphatase activity, resulting in accelerated dephosphorylation of the PDGF receptor. Kim *et al.* (23) have demonstrated that okadaic acid, a protein phosphatase inhibitor, blocks the proliferative effect of TGF- β on mouse fibroblasts, even when added 10 h after TGF- β treatment. This again supports the involvement of a protein phosphatase beyond the initial activation of the TGF- β -receptor complex in the TGF- β signal cascade.

In conclusion, we have demonstrated that tyrosine kinase activity is important in type II pneumocyte proliferation *in vitro*. TGF- α , known to stimulate type II cell proliferation, exerts its effects via phosphorylation of a 170-kD protein, presumed to be the EGF-R. In examining the mechanism by which TGF- β blocks the TGF- α mitogenic effect on type II cells, we have demonstrated that TGF- β decreases the normal TGF- α -induced phosphorylation of the EGF-R. Additional studies are needed to determine the specific mechanism by which TGF- β affects EGF-R phosphorylation.

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Figure 8. TGF- β decreased the phosphorylation of the 170-kD tyrosine protein previously phosphorylated after treatment with TGF- α . Type II cells were plated for 22 h, then pretreated for 30 s with 10 ng/mL TGF- α (lane α), or pretreated for 30 min with 10 ng/mL TGF- β , then treated with 10 ng/mL TGF- α for 30 s (lane $\beta \rightarrow \alpha + \beta$). Samples were collected with Laemmli sample buffer and subjected to SDS-PAGE using a 10% gel followed by Western blot analysis with an antiphosphotyrosine antibody. Molecular masses are indicated on the left in daltons.

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