Acta Pædiatrica ISSN 0803–5253

REGULAR ARTICLE

Growth factors gene expression in the developing lung

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Keywords
Asthma, Bronchopulmonary dysplasia, Lung development, Growth factors, Real-time PCR

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Received
3 February 2007; revised 26 March 2007; accepted 29 march 2007

DOI:10.1111/j.1651-2227.2007.00332.x

Abstract

Aim: This is the first systematic study using quantitative real-time PCR to analyze and compare the expression profiles for critical members of the epidermal growth factor (EGF), transforming growth factor β (TGFβ), and vascular endothelial growth factor (VEGF) families in developing rat lungs.
Methods: mRNA expression was quantified at embryonic (E) day 15, 17, 19, 21, and postnatal age 1 day, 2 weeks, 12 weeks.
Results: EGF and EGFR increased during gestation and development, then decreased in adulthood, whereas TGFα was highest at birth and remained unchanged afterwards. All TGFβ isoforms increased slightly during pregnancy, reached highest expression during development, and returned to neonatal levels in adulthood. TGFβRI and TGFβRII patterns were similar to TGFβ2 and TGFβ1 respectively, whereas TGFβRIII expression was lowest at the postnatal time points. $VEGF_{164}$ and $VEGF_{120}$ showed a steady increase up to 2 weeks and declined at 12 weeks, whereas highest $VEGF_{188}$ expression occurred at 12 weeks. VEGF-A receptors expression paralleled the summation of all three isoforms, increasing steadily with age.

Conclusion: Expression of growth factors in the developing lung is characterized by highly regulated distinctive patterns that may be critical to understand the early origin and progression of pulmonary diseases in childhood as well as in adulthood. Quantitative real-time PCR analysis revealed several differences compared to previously reported expression patterns defined with older methodologies.

INTRODUCTION

Lung development is a complex process involving branching morphogenesis and cell differentiation along with the coordinated distribution of associated vascular, nervous, and supportive tissues (1–3). An integral factor in establishing and maintaining this architectural organization is the response of constituent cells to various soluble mediators, most notably growth factors and cytokines. Consequently, alteration in the normal expression of these mediators can result in abnormal development and pre-modeling of the respiratory tract. Furthermore, there is mounting evidence suggesting important roles for growth factors and their receptors in the remodeling process associated with respiratory diseases such as asthma (4) and bronchopulmonary dysplasia (BPD) (5). In light of such evidence, growth factors are warranting more attention in the study of diseases affecting the lungs during childhood as well as adulthood.

Although a multitude of growth factors exist, much focus has centered on a particular subset with respect to lung development and disease, including members of the epidermal growth factor (EGF), transforming growth factor β (TGFβ), and vascular endothelial growth factor (VEGF) families (6). The EGF family of growth factors includes EGF, TGFα, HB-EGF, amphiregulin, and betacellulin, all of which trigger similar mitogenic responses via a common receptor (EGFR). Several reports support the notion that EGFR represents a key determinant in the developing lung by modulating critical processes, such as type II cell differentiation (7), branching morphogenesis (8), and vascular remodeling (9). In addition, overexpression of both EGFR (10) and its TGFα ligand (11) has been associated with inflammatory processes contributing to severe asthma.

The TGFβ family of growth factors has also garnered much attention in the way of lung maturation and disease. Three known isoforms of TGFβ exist (TGFβ1, TGFβ2, and TGFβ3), each arising from a distinct gene, whose cellular effects are mediated by binding to receptor types I, II, and III. While members of the EGF family tend to stimulate growth and development, TGFβ family members tend to exert the opposite effect. Several reports have shown that TGFβ1 inhibits branching morphogenesis and cell differentiation (12), and a recent study revealed that transient overexpression of TGFβ1 induces interstitial fibrosis and impaired alveolarization consistent with BPD (13). As with EGF, there is also evidence suggesting a role for TGFβ in the development of childhood asthma (14).

Perhaps one of the most well-studied growth factor families is that of VEGF. Members of this gene family include, VEGF-B, VEGF-C, VEGF-D, placenta growth factor, and the parental VEGF-A. There are several isoforms of VEGF-A which, unlike TGFβ, originate from a single gene by way of alternate mRNA splicing events. Generally believed to be the most important and widely expressed of these isoforms are $VEGF_{188}$, $VEGF_{164}$, and $VEGF_{120}$. The different isoforms are biologically distinguished from one another primarily by their ability to bind heparan-sulfate proteoglycans, which thereby reflects the extracellular

matrix (ECM) binding and solubility of each. Although the precise functions for each isoform have yet to be determined, VEGF in general is known to be a potent mitogenic factor for vascular endothelial cells (15), where its receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) are predominantly found. In the lung, VEGF was recently shown to control vascular patterning during embryonic development (16), and has been implicated in arrested alveolarization due to hyperoxic injury (17). Finally, new lines of evidence have demonstrated that VEGF has neurotrophic activity (18), although what role, if any, such activity plays in lung development and remodeling has yet to be determined.

We have demonstrated previously that the expression of neurotrophic factors and receptors declines progressively with postnatal age (19). The complex and multifactorial nature of airway premodeling and remodeling has led us to consider additional growth factors that, due to their importance in the overall development and morphogenesis of the lung, may be involved in the pathogenesis of respiratory disorders in early life. To this end, our goal was to obtain a better understanding of the expression patterns for various growth factors in the lung from late fetal life to adulthood. Specifically, we sought to characterize the developmental expression profiles for TGFα, EGF, TGFβ1, TGFβ2, TGFβ3, $VEGF_{188}$, $VEGF_{164}$, and $VEGF_{120}$, as well as their respective receptors, in the lungs of Fischer 344 (F-344) rats. Expression of mRNA levels for each target gene was measured using quantitative real-time polymerase chain reaction (PCR) at different embryonic (E) and postnatal stages: gestational days E15, E17, E19, E21; and 1 day, 2 weeks (weanling rats), 12 weeks (adult rats) after birth. To our knowledge, this is the first study using this methodology for a systematic analysis of the gene expression of growth factors and their receptors in the developing rat lung.

MATERIALS AND METHODS

Animals

Timed pregnant, pathogen-free Fischer 344 rats (Charles River Laboratories, Wilmington, MA) were used for obtaining fetal lungs at E15, E17, E19, and E21. Additional postnatal time points were obtained by harvesting lungs at 1 day, 2 weeks, and 12 weeks of age. All animals were born in our facility and housed in polyester cages supplied with positive individual ventilation with class-100 air to each cage at a rate of 1 cage change of air/min (Maxi-Miser, Thoren Caging System, Hazleton, PA).

For fetal harvests, dams were first anesthetized by intramuscular injection of a cocktail consisting of ketamine (42.8 mg/kg), xylazine (8.6 mg/kg), and acepromazine (1.4 mg/kg) and then subjected to a rapid hysterotomy. Fetuses were delivered one at a time and the uterus clamped to prevent delivery of neighboring fetuses. The fetus was then weighed, pithed through the mouth, and the lung was quickly identified and removed under a dissecting microscope. The entire lung was immediately snap-frozen in liquid nitrogen for subsequent RNA isolation. All experimental procedures used in this study were approved by the institutional Division of Veterinary Resources.

Real-time PCR

Total RNA was extracted from whole lung homogenates in 1 mL/100 mg tissue of RNA STAT-60 solution (Tel-Test Inc., Friendswood, TX). Purified total RNA was subsequently treated with DNase I to remove any traces of contaminating genomic DNA. DNase I treatment was performed with the DNase-free kit (Ambion, Inc., Austin, TX) using 10 μg RNA in a 25 μL reaction containing 1× DNase buffer and 2 U of DNase I for 30 min at 37°C. A qualitative agarose gel was run for all DNase I-treated samples to confirm RNA integrity prior to amplification.

cDNA synthesis was accomplished using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Briefly, 1.0 μg of DNase I-treated total RNA was used in a 20 μL final reaction volume containing the following components (final concentrations): 20 mM Tris-HCl, 50 mM KCl, 5 mM MgCl2, 0.5 mM of each dNTP, 0.1 μg random hexamers, and 50 U of SuperScript III RTase. Samples were incubated at 42°C for 50 min followed by a termination step at 85°C for 5 min. A final RNase H step was performed by adding 1 μL of enzyme to each sample and incubating at 37°C for 20 min.

PCR products from target genes were first cloned into the pCR 2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen), and then their sequence was verified. Standard curves were generated from 10-fold serial dilutions of plasmid from 10^9 to 10^4 copies per 2 μL. Prior to performing PCR on experimental samples, specific melting peaks were determined for each product and confirmed by gel electrophoresis.

Real-time-PCR reactions were performed using a LightCycler instrument and the FastStart DNA Master SYBR Green I kit (Roche Applied Science, Indianapolis, IN). Briefly, 2 μL aliquots of the synthesized cDNA (corresponding to 100 ng RNA) were added to a 18 μL PCR mixture containing the following components (final concentrations): 2 mM or 4 mM $MgCl_2$, 0.5 μM of each primer (Table 1), and 1× FastStart Reaction Mix containing Taq DNA polymerase,

Table 1 Real-time PCR primers

Target	Sense	Antisense	Size (bp)	$MgCl_2$ (mM)	Gene Info (identifier)
TGFα	178–199	496–515	338	2	6981645
EGF	2804–2825	3350–3370	567	4	6978796
EGFR	1785–1804	2537–2556	772	4	6478867
TGFβ1	1247–1268	1543–1564	318	4	11024651
TGFβ2	1654–1674	1828–1848	195	4	13592108
TGFβ3	921–941	1157–1177	257	4	6981649
TGFβRI	965–984	1225–1245	281	3	6978442
TGFβRII	330–350	878–896	567	4	13592110
TGFβRIII	1840–1860	2041–2061	222	4	8394445
$VEGF_{120}$	192–215	411–431	240	4	6606565
$VEGF_{164}$	196–219	415–434	239	4	204287
$VEGF_{188}$	192–215	454–472	281	4	6606563
VEGFR-1	931–949	1387–1403	473	4	511662
VEGFR-2	3320–3337	3599–3616	297	4	2098758
GAPDH	170–190	597–614	445	4	204248

reaction buffer, dNTP mix, and SYBR Green I dye. VEGF-A exists in three predominant isoforms that arise from alternate mRNA splicing events ($VEGF_{188}$, $VEGF_{164}$, and $VEGF_{120}$); to distinguish between the different isoforms, we designed primers adapted from Wellmann et al. (20), such that a common upstream primer was used in combination with downstream primers that spanned a splice junction unique for each transcript. Cycling conditions consisted of an initial 10 min denaturation at 95°C, followed by 45 cycles of denaturation at 95°C for 0 sec, annealing at 55°C for 10 sec, and extension at 72°C for 30 sec.

Data analysis

Quantification was performed by obtaining extrapolated CT values using the Second Derivative Maximum Method with the LightCycler software. PCR product specificity was verified following each run by melting curve analysis. All data was normalized by dividing the copy number of the target cDNA for each sample by the corresponding copy number of GAPDH and presented as mean ± SEM ($n = 6$ rats per time point). Statistical analysis was performed by ANOVA using StatView software version 5.0.1 (SAS Institute, Cary, NC). Differences having a P value < 0.05 were considered significant.

RESULTS

EGF family

TGFα mRNA expression (Fig. S1A) increased over 2-fold from E15 to E19 ($P < 0.005$), and then increased nearly 3-fold from E19 to E21 ($p < 0.0001$) before stabilizing at its highest level at postnatal day 1 ($p < 0.0001$) and remaining relatively unchanged at 2 weeks and 12 weeks of age.

EGF mRNA (Fig. S1B) showed a similar rise in expression from E15 to E19 and a near 3-fold increase from E19 to the E21 and 1 day time points ($p < 0.0001$). However, the highest level of expression was not reached until 2 weeks of age, followed by a 29% decrease in expression measured at 12 weeks of age ($p < 0.0001$).

Expression of the EGF receptor (Fig. S1C) remained relatively stable from E15 to E19 followed by a steady increase of roughly 40% at each time point from E19 to 2 weeks of age ($p = 0.027$). Like EGF, expression decreased for EGFR from 2 weeks to 12 weeks of age, although the magnitude of this decline was greater for EGFR (37%, $P < 0.0001$).

TGFβ family

TGFβ1 (Fig. S2A) demonstrated no significant changes in expression from E15 to E19. However, there was a 4-fold increase in expression from E19 to E21 ($p < 0.0001$) which remained stable at postnatal day 1 and was followed by a peak at 2 weeks of age ($p < 0.0001$) and a return to perinatal levels by 12 weeks of age.

Expression of TGFβ2 (Fig. S2B) increased slightly, although not significantly, from E15 to E19 and was followed by a 3-fold drop just prior to birth at E21 ($p < 0.0001$). By postnatal day 1, TGFβ2 had returned to prenatal levels, and its expression increased over 4-fold at 2 weeks of age ($p < 0.0001$), returning to near-perinatal levels by 12 weeks of age.

TGFβ3 expression (Fig. S2C) remained virtually unchanged during prenatal development and showed a modest increase at postnatal day 1. However, a large increase was evident at 2 weeks with a near 10-fold increase compared to prenatal time points ($p < 0.0001$), followed by a return to near-perinatal levels by 12 weeks of age ($p < 0.0001$).

Analysis of TGFβRI (Fig. S2D) revealed an expression pattern similar to that of TGFβ2 with a decrease in expression at E21 and a peak at 2 weeks of age. By contrast, the expression pattern of TGFβRII (Fig. S2E) more closely paralleled that of TGFβ1, showing small increases in expression from E15 to E19 followed by sustained, significantly higher levels of expression from E21 on with a peak at 2 weeks of age. For TGFβRIII (Fig. S2F), the most pronounced level of expression occurred at E21 with a greater than 4-fold increase from E19 ($p < 0.0001$). However, in contrast to the other receptors which showed the lowest expression levels prenatally, expression of TGFβRIII was lowest at the postnatal time points.

VEGF family

$VEGF_{164}$ and $VEGF_{120}$ demonstrated similar expression patterns showing a steady increase with age up to 2 weeks, followed by a decline at 12 weeks (Fig. S3A). Analysis of $VEGF_{188}$ expression revealed more dramatic changes than the other two isoforms. $VEGF_{188}$ remained at low levels early in development until a nearly 10-fold increase at E21 ($p < 0.0001$), followed by a nearly 3-fold increase at 2 weeks of age ($p < 0.0001$), and reaching the highest level of expression at 12 weeks of age ($p < 0.0001$).

When comparing relative abundance of the isoforms (Fig. S3B), $VEGF_{120}$ showed the highest expression from E15–E19 (~50–60%) followed by $VEGF_{164}$ (~40%) and $VEGF_{188}$ (~10% or less). Thus, the more freely soluble isoforms accounted for nearly all of the VEGF-A present at these time points. At E21 and 2 weeks of age all three isoforms were present in approximately equal amounts, while at 1 day $VEGF_{164}$ and $VEGF_{120}$ again represented higher relative amounts (~40% each) when compared to $VEGF_{188}$ (~20%). By adulthood, $VEGF_{188}$ had reached its highest level representing ~50% total VEGF-A expression while $VEGF_{164}$ and $VEGF_{120}$ were present in roughly equal amounts (~20–25% each).

The receptors for VEGF-A (Fig. S4) followed very similar patterns to one another at all time points analyzed and paralleled that of the summation of all three isoforms, each receptor showing a steady increase in expression with age.

DISCUSSION

With regards to analyzing gene expression, quantitative real-time PCR has several advantages over more traditional methods such as conventional reverse-transcription PCR, Northern blot analysis, and RNase protection assays (21). Most important are the greater sensitivity conferred by continuous analysis in real-time and the greater speed at which the reaction is run, as well as the rapid postreaction analysis

(22,23). There is currently a solid and growing body of work concerning growth factor expression in the developing lung. However, given the relatively recent advent of real-time PCR, the vast majority of observations have been made using the comparatively less sensitive and older techniques previously mentioned. In addition, these studies were generally conducted with a small, focused subset of growth factors in varying animal models. It was the goal of the present study to provide the first comprehensive report on the changes in expression of a more encompassing set of growth factors and receptors in the developing rat lung utilizing the highly sensitive technique of real-time PCR. Indeed, our analysis shows several areas of discrepancy with previously published data, which are highlighted in the following paragraphs.

Both TGFα and EGF increased gradually during prenatal development, demonstrating a small change in early pregnancy (from E15 to E19) and the greatest change just before delivery (from E19 to E21). This is in contrast with a previous report showing a decline from E19 to E21 by RIA and Southern blot of PCR products (24). Expression of both genes continued to increase postnatally during the rapid phase of development corresponding to childhood. However, while the highest level of TGFα expression was reached at birth and was maintained virtually unchanged through adulthood, maximal EGF expression occurred later during development (2 weeks) and was followed by decreased expression in adulthood (12 weeks). The fact that EGFR roughly follows expression of TGFα and EGF is consistent with previous data showing colocalization of this receptor and both ligands in developing human lung, suggesting a possible autocrine signaling mechanism (5).

We also examined members of the TGFβ family for their expression during lung development. All TGFβ isoforms increased slightly during pregnancy (from E15 to E19) and diverged just prior to birth (E21) as expression increased for TGFβ1, whereas it decreased for TGFβ2 and remained unchanged for TGFβ3. Expression of these factors reached the highest levels during development (2 weeks) and returned back to neonatal levels in adulthood (12 weeks).

A previous study has also described TGFβ and TGFβR expression in the developing rat lung using Northern blot analysis and *in situ* hybridization (25). The expression patterns for TGFβ1 and TGFβRII shown in that report were fairly consistent with our results at the same time points studied. The only significant discrepancy was found with TGFβ1 expression at postnatal day 1. Our results demonstrated no change from E21 whereas the previous report showed a greater than 2-fold increase from E21 to 1 day of life.

With respect to the developmental expression patterns for TGFβ2, TGFβ3, and TGFβRI, our results differed more profoundly. Zhao and coworkers observed a decline in expression for all three genes during prenatal development. Our data reveal either slight increases (TGFβ2, TGFβRI) or no significant change (TGFβ3) in expression during prenatal development. Given the fact that only two prenatal time points are shared between both studies and also considering other potentially confounding factors related to the rapid maturation rate of the rat (e.g. inherent uncertainty of gestational stage *in utero*, possible variance in timing of lung harvests), it may not be surprising that differences were observed. However, one might expect changes in magnitude more likely than trends in opposite directions. Differences in sample sizes and methodology are other factors that may contribute to some of the conflicting observations. It may be interesting to note, however, that consistent in both reports is the observation that expression of TGFβ1 more closely resembled the expression profile for TGFβRII, while TGFβ2 more closely paralleled that of TGFβRI.

The expression of VEGF in lung maturation has been relatively well documented in several animal models, including the mouse (26,27) and rabbit (28), as well as in humans (29). Such evidence has indicated that the relative abundance of the predominant isoforms is tightly developmentally regulated and critical for the proper development of the lung (30). Our data support these results, showing that $VEGF_{120}$ and $VEGF_{164}$ have higher relative abundance earlier in fetal development, whereas $VEGF_{188}$ is predominantly expressed just prior to birth and postnatally. The receptors show an expression profile similar to that of total VEGF production, increasing steadily over time.

The biological function of the different isoforms is putatively associated with the ability to bind heparan-sulfate. The smaller splice variant $VEGF_{120}$ lacks the ability to bind to heparan-sulfate and $VEGF_{164}$ has intermediate binding affinity, whereas $VEGF_{188}$ has strong binding affinity. Previous authors have suggested that the more freely diffusible isoforms are more important earlier in development due to likely roles in angiogenesis and vasculogenesis, whereas the more localized $VEGF_{188}$ is important in contributing to vessel maintenance and specialization (27).

CONCLUSIONS

We have used the highly sensitive method of real-time PCR for an accurate and systematic reanalysis of the expression of various growth factors from late fetal life to adulthood in the rat lung, focusing on members of the EGF, TGFβ, and VEGF families because of their relevance to lung development and disease. General trends emerging from our study indicate: (a) gradual increase of gene expression during fetal development, which accelerates just before birth; (b) maximal expression occurring after birth, during the rapid phase of development corresponding to childhood; (c) progressive decrease with age with return to neonatal levels in adulthood. The postnatal pattern of expression coincides with what we had previously reported for neurotrophic factors and receptors (19). The information provided herein represents essential background information to understand lung development and remodeling, and for future investigations of the early origin and progression of pulmonary diseases.

ACKNOWLEDGEMENTS

The authors would like to thank Cheryl Chapin (University of California at San Francisco, San Francisco, CA) and Eric Bluestein for their technical assistance. Some of the findings reported in this article were presented at the 2005

International Conference of the American Thoracic Society in San Diego, CA. This research was supported in part by a grant from the National Institutes of Health (NHLBI HL-61007) to Dr. Giovanni Piedimonte.

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Supplementary material

The following supplementary material is available for this article:

Figure S1. EGF family: Pre- and post-natal expression of TGFα (A), EGF (B), and EGFR (C) in the lungs of F-344 rats. mRNA expression for each target gene was determined at embryonic (E) day 15, 17, 19, 21, and postnatal age 1 day

(pnD1), 2 weeks (W02), 12 weeks (W12) using real-time polymerase chain reaction (PCR). Quantification was performed by obtaining extrapolated CT values with the Second Derivative Maximum Method. All data was normalized by dividing the copy number of the target cDNA for each sample by the corresponding copy number of GAPDH, and presented as mean ± SEM (n = 6 rats per time point).

Figure S2. TGFβ family: Pre- and post-natal expression of TGFβ1 (A), TGFβ2 (B), TGFβ3 (C), TGFβRI (D), TGFβRII (E), TGFβRIII (F) in the lungs of F-344 rats. mRNA expression for each target gene was determined at embryonic (E) day 15, 17, 19, 21, and postnatal age 1 day (pnD1), 2 weeks (W02), 12 weeks (W12) using real-time PCR. Data is presented as mean ± SEM (n = 6 rats per time point).

Figure S3. VEGF family: Absolute (A) and relative (B) pre- and post-natal expression of the VEGF isoforms $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ in the lungs of F-344 rats. Panel A shows absolute expression; panel B shows relative expression. mRNA expression for each target gene was determined at embryonic (E) day 15, 17, 19, 21, and postnatal age 1 day (pnD1), 2 weeks (W02), 12 weeks (W12) using real-time PCR. Data is presented as mean ± SEM (n = 6 rats per time point).

Figure S4. VEGF receptors: Pre- and post-natal expression of VEGFR-1 (A) and VEGFR-2 (B) in the lungs of F-344 rats. mRNA expression for each target gene was determined at embryonic (E) day 15, 17, 19, 21, and postnatal age 1 day (pnD1), 2 weeks (W02), 12 weeks (W12) using real-time PCR. Data is presented as mean ± SEM (n = 6 rats per time point).

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