

Amphiregulin expression in human mast cells and its effect on the primary human lung fibroblasts

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Background: Amphiregulin is a member of the epidermal growth factor family and has been shown to stimulate the proliferation of human keratinocytes in an autocrine manner. **Objective:** The aim of the present study was to examine the expression change of growth factors, especially amphiregulin, in human mast cells induced by IgE cross-linking. **Methods:** Microarray analysis and RT-PCR were used to analyze the gene expression profile of human cord blood–derived mast cells (CBMCs) stimulated with IgE cross-linking. Protein secretion in the supernatants of CBMCs was measured by means of ELISA. Double-immunofluorescence staining was used to analyze the expression in the lung mast cells. **Results:** Of the 64 different growth factor genes analyzed, 5 were found to be substantially upregulated. Among them, amphiregulin mRNA was induced by 44-fold in CBMCs on activation through IgE cross-linking. Secretion of amphiregulin protein was evident in CBMCs 8 hours after stimulation. Amphiregulin was also expressed in human lung mast cells from patients with asthma, as demonstrated by means of double-immunofluorescence staining. Amphiregulin promoted the proliferation of the primary human lung fibroblasts, and amphiregulin-treated primary human lung fibroblasts showed a marked increase in the expression of *c-fos*, a proto-oncogene that facilitates or is required for the proliferation of a wide variety of cells. **Conclusion:** Human CBMCs secreted amphiregulin on IgE cross-linking, and the amphiregulin induced proliferation of primary human lung fibroblasts. These data suggest that local release of amphiregulin by human mast cells could play an important role in lung fibrosis by promoting the proliferation of primary human lung fibroblasts. (*J Allergy Clin Immunol* 2005;115:287-94.)

Key words: Mast cells, amphiregulin, IgE cross-linking

It is well known that human mast cells play critical roles in the pathogenesis of many allergic diseases, such as asthma, atopic dermatitis, and other allergic diseases.¹ The mediators released by mast cells are responsible for most of the early events in allergic reactions and contribute to

Abbreviations used

CBMC: Cord blood–derived human mast cell
C_t: Threshold cycle
EGF: Epidermal growth factor
HB-EGF: Heparin-binding EGF-like growth factor
OVA: Ovalbumin
PDGF: Platelet-derived growth factor α
VEGF: Vascular endothelial growth factor

the expression of late-phase reactions and chronic allergic inflammation through cytokine production and other mechanisms.² Increasing evidence suggests that mast cells also play an important role in fibrosis.³ In fibrotic lung disorders the number of mast cells in the tissue section was much larger than numbers seen in control patients, and mast cells were present in thickened and fibrous alveolar septa.⁴ The density of the cutaneous mast cell population in the skin of patients with early stages of scleroderma was also shown to be significantly increased.⁵ In animal models of pulmonary fibrosis induced by asbestos,⁶ bleomycin,⁷ or ionizing irradiation,⁸ mast cells accumulated in the cutaneous sites of fibrotic lung tissues, and it was evident that the mast cells at the lesion sites had been activated. *In vitro* studies showed that the supernatants of activated mast cells promoted the expression of fibroblast collagen genes and the migration and proliferation of fibroblasts.⁹ These effects are mediated by proinflammatory mediators and growth factors released by mast cells.¹⁰ Therefore mast cells contribute to tissue fibrosis associated with chronic allergic diseases and other disorders by releasing mediators on activation.

Amphiregulin is a polypeptide growth factor that belongs to the epidermal growth factor (EGF) family, which mediates its biologic function through the EGF receptor.^{11,12} The binding of amphiregulin to EGF receptor has been shown to increase EGF receptor tyrosine phosphorylation.¹³ Amphiregulin displays bifunctional properties in that it promotes the growth of fibroblasts, tumor cells, and cultured human epidermal keratinocytes¹⁴ but inhibits the growth of some normal and neoplastic cell lines.¹⁵ Amphiregulin has also been implicated to play important roles in the inflammatory and repair processes, such as cutaneous wound repair, tumor cell growth, and psoriasis.¹⁶

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TABLE I. Transcripts of human growth factor expressed by human mast cells*

GenBank accession no.	Mast1 rest	Mast1 act	Fold difference† (act/rest)	Mast2 rest	Mast2 act	Fold difference† (act/rest)	Average fold difference (act/rest)	Gene description
M13436	A	2250	315	A	18318	256	286	Activin β A
NM_001657	A	865	17	161	8731	79	44	Amphiregulin
NM_001729	A	A	NC	A	A	NC	NC	Betacellulin
NM_006129	A	A	NC	A	A	NC	NC	Bone morphogenetic protein 1
NM_001200	A	A	NC	A	394	4.3	2.7	Bone morphogenetic protein 2
NM_001201	A	A	NC	A	A	NC	NC	Bone morphogenetic protein 3
D30751	A	A	NC	A	A	NC	NC	Bone morphogenetic protein 4
NM_021073	A	A	NC	A	A	NC	NC	Bone morphogenetic protein 5
NM_001718	A	A	NC	A	A	NC	NC	Bone morphogenetic protein 6
AL157414	A	A	NC	A	A	NC	NC	Bone morphogenetic protein 7
NM_001720	A	A	NC	A	A	NC	NC	Bone morphogenetic protein 8
NM_014482	102	A	NC	A	167	NC	NC	Bone morphogenetic protein 10
NM_001330	A	A	NC	A	A	NC	NC	Cardiotrophin 1
NM_001953	654	636	NC	484	605	NC	NC	Endothelial cell growth factor 1
NM_001963	A	A	NC	A	A	NC	NC	EGF
X59065	A	A	NC	A	A	NC	NC	Fibroblast growth factor 1
NM_002006	A	A	NC	A	A	NC	NC	Fibroblast growth factor 2
NM_005247	A	A	NC	A	A	NC	NC	Fibroblast growth factor 3
BF510715	A	A	NC	A	A	NC	NC	Fibroblast growth factor 4
NM_004464	A	A	NC	A	A	NC	NC	Fibroblast growth factor 5
NM_020996	A	A	NC	A	A	NC	NC	Fibroblast growth factor 6
NM_002009	A	A	NC	A	A	NC	NC	Fibroblast growth factor 7
NM_006119	A	A	NC	A	A	NC	NC	Fibroblast growth factor 8
NM_002010	A	A	NC	A	A	NC	NC	Fibroblast growth factor 9
NM_004465	A	A	NC	A	A	NC	NC	Fibroblast growth factor 10
AL528824	A	A	NC	A	A	NC	NC	Fibroblast growth factor 11
AL119322	A	A	NC	A	A	NC	NC	Fibroblast growth factor 12
NM_004113	A	A	NC	A	A	NC	NC	Fibroblast growth factor 12B
NM_004114	A	A	NC	A	A	NC	NC	Fibroblast growth factor 13
NM_004115	A	A	NC	A	A	NC	NC	Fibroblast growth factor 14
NM_003868	A	A	NC	A	A	NC	NC	Fibroblast growth factor 16
NM_003867	A	A	NC	A	A	NC	NC	Fibroblast growth factor 17
NM_003862	A	A	NC	A	A	NC	NC	Fibroblast growth factor 18
AF110400	A	A	NC	A	A	NC	NC	Fibroblast growth factor 19
NM_019851	A	A	NC	A	A	NC	NC	Fibroblast growth factor 20
NM_019113	A	A	NC	A	A	NC	NC	Fibroblast growth factor 21
NM_020637	A	A	NC	A	A	NC	NC	Fibroblast growth factor 22
NM_020638	A	A	NC	A	A	NC	NC	Fibroblast growth factor 23
AL565812	A	A	NC	A	A	NC	NC	Heparin-binding growth factor 8
NM_001945	A	4453	15	A	1760	14	15	Heparin-binding EGF-like growth factor
M60718	A	A	NC	A	A	NC	NC	Hepatocyte growth factor
NM_004494	3221	3675	NC	2185	1237	0.9	1.0	Hepatoma-derived growth factor
M37484	A	A	NC	A	A	NC	NC	Insulin-like growth factor I
NM_000612	A	A	NC	A	A	NC	NC	Insulin-like growth factor 2
NM_000899	A	A	NC	A	A	NC	NC	KIT ligand
AF063020	904	826	0.7	593	346	0.8	0.8	Lens epithelium-derived growth factor
M57399	A	A	NC	A	A	NC	NC	Nerve growth factor
NM_002506	A	A	NC	A	A	NC	NC	Nerve growth factor, β polypeptide
NM_013959	176	A	0.6	A	A	NC	0.8	Neuregulin 1
NM_013957	A	A	NC	A	A	NC	NC	Neuregulin 2
NM_012277	198	200	NC	A	386	1.4	1.2	Pancreatic β cell growth factor
NM_002607	A	756	6.5	A	318	4.9	5.7	PDGF α
NM_016205	1321	874	0.7	1465	1045	0.8	0.8	PDGF C
NM_025208	160	145	NC	259	186	0.8	0.9	Spinal cord-derived growth factor-B
NM_003212	A	A	NC	A	A	NC	NC	Teratocarcinoma-derived growth factor
NM_003236	489	210	0.4	609	608	NC	0.7	TGF- α
BC000125	1804	2880	1.6	506	1257	2.5	2.0	TGF- β 1
NM_003238	A	A	NC	A	A	NC	NC	TGF- β 2
J03241	A	A	NC	A	A	NC	NC	TGF- β 3

TABLE I. Continued

GenBank accession no.	Mast1 rest	Mast1 act	Fold difference† (act/rest)	Mast2 rest	Mast2 act	Fold difference† (act/rest)	Average fold difference (act/rest)	Gene description
NM_003240	A	A	NC	A	A	NC	NC	TGF-β4
AF022375	542	1357	2.5	416	2831	5.7	4.1	VEGF
NM_003377	272	A	NC	318	A	NC	NC	VEGF B
U58111	A	A	NC	A	148	1.3	1.2	VEGF C
NM_004469	A	A	NC	A	A	NC	NC	VEGF D

Act, Activation.

*The signal value “A” stands for “absent,” meaning the transcript was not detected; the fold difference “NC” stands for “no change,” meaning the difference of the transcript levels is statistically insignificant.

†The fold difference is calculated by means of signal log ratio algorithm (see the Methods section).

TABLE II. Effect of cyclosporin and dexamethasone on the expression of activated growth factors*

GenBank accession no.	Mast cells		Fold difference† (act/rest)	Dex treatment		CsA treatment		Gene description
	Rest	Act		Signal	Act/Dex	Signal	Act/CsA	
M13436	A	18318	256	10227	1.7	4674	4.0	Activin β A
NM_001657	161	8731	79	12106	NC	8919	NC	Amphiregulin
NM_002607	A	318	4.9	542	0.7	298	NC	PDGF α polypeptide
NM_001945	A	1760	14	1088	2.0	829	2.1	Heparin-binding EGF-like growth factor
AF022375	416	2831	5.7	2280	NC	3132	NC	VEGF

ACT, Activation; Dex, dexamethasone; CsA, cyclosporine A.

*The signal value “A” stands for “absent,” meaning the transcript was not detected; the fold difference “NC” stands for “no change,” meaning the difference of the transcript levels is statistically insignificant.

†The fold difference is calculated by means of signal log ratio algorithm (see the Methods section).

In an effort to systematically examine the growth factor genes that are upregulated on activation in human mast cells, we used microarray analysis to perform gene expression profiling on cord blood–derived human mast cells (CBMCs) activated by IgE cross-linking. We further characterized one of the growth factors, amphiregulin. We examined the expression of amphiregulin in the different tissues and cells and determined its effect on the proliferation of primary human lung fibroblasts.

METHODS

Protein and antibody

Recombinant amphiregulin was purchased from Sigma-Aldrich (St Louis, Mo), and a goat polyclonal antibody to amphiregulin was purchased from R&D Systems (Minneapolis, Minn). Monoclonal antibody (clone G3) to human mast cell tryptase was purchased from Chemicon (Temecula, Calif).

Primary human mast cell culture and activation

CBMCs were derived from human cord blood CD34⁺ cells grown in the presence of stem cell factor, IL-6, and IL-10, as described previously.¹⁷ CBMC purity was assessed by means of acid toluidine blue staining and anti-mast cell tryptase mAb staining. Cells were harvested for study when more than 95% stained positively with toluidine blue and anti-mast cell tryptase. For stimulation by IgE cross-linking, 8- to 10-week-old CBMCs were first incubated with 5 μg/mL IgE and 10 ng/mL IL-4 for 48 hours and then stimulated with ovalbumin (OVA)–gp120 for different times. OVA–gp120 is

a peptide from the HIV envelope protein gp120 that is conjugated to OVA. The IgE used has specificity for the gp120 peptide and therefore can be cross-linked by the OVA–gp120. For microarray analysis, CBMCs were stimulated for 2 hours, and the cell pellet was harvested. For drug inhibition experiments, 500 nM dexamethasone or 500 nM cyclosporin was added to CBMCs for 18 hours before cross-linking. The cell pellet was washed in PBS, and total RNA was extracted with the Qiagen RNA prep kit (Valencia, Calif) according to the manufacturer’s procedure.

Microarray analysis

Total RNAs were isolated from human CBMCs by using the RNeasy Mini kit according to the manufacturer’s instruction (Qiagen, Valencia, Calif). The biotinylated cDNA probes were generated and hybridized to the Affymetrix Genechip Human Genome U133 (HG-U133) Set according to the manufacturer’s protocol (Affymetrix, Santa Clara, Calif). The scanned images were aligned and analyzed by using the Affymetrix GeneChip software MAS 5.0, as per the manufacturer’s instruction. Signal intensity for each cDNA or expressed sequence tags entry (represented as “expression value” in Tables I and II) was calculated as the difference of filtered perfect match probes minus mismatch probes. The signal intensities were normalized to the mean intensity of all the genes represented on the array, and global scaling (scaling to all probe sets) was applied before comparison analysis. In addition, MAS 5.0 was also used to generate detection call, which indicates whether a transcript is reliably detected (present) or not detected (absent), as well as change calls (increase, decrease, and no change) for pairwise comparison. Signal log ratio was calculated by comparing each probe pair on the 2 arrays under comparison, and this number is computed by using a 1-step Tukey biweight method by taking a mean of the log ratios of probe pair

intensities across the 2 arrays. Fold change was obtained by reversing the log scale by $2^{\text{Signal Log Ratio}}$.

Quantitative real-time RT-PCR analysis

Human tissue RNAs were purchased from BD Biosciences (Palo Alto, Calif). Quantitative RT-PCR was performed, as described previously.¹⁸ Oligonucleotide primers were selected from the amphiregulin nucleotide sequences by using Primer Express 2.0 (Applied Biosystems, Inc, Foster City, Calif). Briefly, RNAs were reverse transcribed into first-strand cDNA and then used as PCR templates in reaction to obtain the threshold cycle (C_t), and the C_t was normalized by using the known C_t from 18S RNAs to obtain ΔC_t . To compare the relative levels of gene expression of amphiregulin in different tissues-cells, $\Delta\Delta C_t$ values were calculated by using the ΔC_t values associated with the lowest expression levels as the bases. The $\Delta\Delta C_t$ values were then transformed to the real fold increase in expression by $2^{\Delta\Delta C_t}$.

Amphiregulin protein secretion determined by means of ELISA

Human CBMCs were stimulated by means of IgE cross-linking, as described above, and supernatants were collected at different time points. Amphiregulin was assayed in triplicate by using a sandwich ELISA kit according to the manufacturer's protocol (R&D Systems). The detection limit of the assay was 5 pg/mL.

Double-immunofluorescence staining of amphiregulin and mast cell tryptase

Bronchoscopy was performed in patients who died from fatal asthma, and tissue specimens were taken from the carina and the right middle and upper lobe bronchi. Normal main bronchial tissues from 2 lung transplant donors who do not have asthma or a history of other lung disease were also obtained. Bronchial tissue specimens were processed immediately and stored at -80°C before use for the study. Tissues were fixed in neutral buffered formalin, embedded in paraffin, and cut into 4- μm sections. Double-immunofluorescence staining with antibodies for amphiregulin and mast cell tryptase was performed as described previously.¹⁷ Briefly, slide sections were first incubated with 5% rabbit serum to block nonspecific binding and then washed in PBS. Slides were incubated with antibodies against amphiregulin (1:100) and mast cell tryptase (1:500) overnight at 4°C . Slides were washed in PBS and then incubated for 1 hour at room temperature with rhodamine-labeled donkey anti-goat antibodies (1:100, Chemicon) and FITC-labeled rabbit anti-mouse antibodies (1:20; Dako, Glostrup, Denmark). After a nuclear stain with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Eugene, Ore) stain, the cover slips were mounted, and slides were examined with an Eclipse E400 microscope (Nikon, Inc, Melville, NY).

Human lung fibroblast culture and proliferation assay

Human primary lung fibroblasts were obtained from Clonetics (Walkersville, Md) and grown in fibroblast basal medium supplemented with 1 ng/mL recombinant fibroblast growth factor and 5 $\mu\text{g}/\text{mL}$ insulin and gentamicin. Cells were seeded onto 48-well plates at a cell density of 400,000/mL and cultured for 24 hours at 37°C . Stimulation conditions were selected on the basis of previous studies with dermal fibroblasts¹⁹ and studies with human airway smooth muscle cells.¹⁷ Cells were then starved in serum-free media for 24 hours, and then recombinant human amphiregulin (1, 10, 50, or 100 ng/mL or 100 ng/mL amphiregulin plus 5 $\mu\text{g}/\text{mL}$ amphiregulin antibody) was added and cultured at 37°C for 24 hours. Tritiated

thymidine (Amersham; 1 $\mu\text{Ci}/200 \mu\text{L}$) was added for 16 hours. The cells were then washed twice with cold PBS, twice with 10% cold TCA, and twice with 95% ethanol and lysed. Cell lysate was counted with a β scintillation counter.

Gene induction by amphiregulin

Human primary lung fibroblasts were seeded onto 60-millimeter individual dishes and cultured at 37°C for 24 hours. Cells were growth arrested in serum-free media for 24 hours and then washed 3 times with serum-free media. Cells were then stimulated with 10 ng/mL amphiregulin in serum-free media for 0 hours (mock), 0.5 hours, 1 hours, 3 hours, or 6 hours or stimulated with 10 ng/mL EGF for 3 hours. Cells were harvested, and RNAs were extracted from the cells. Real-time PCR analysis was then performed with the primers to the following genes: *c-fos*, cyclin D1, amphiregulin, TGF- α , heparin-binding EGF-like growth factor (HB-EGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and GAPDH.

Statistical analysis

Data are presented as means $\pm$ SD unless otherwise indicated. Statistical significance was determined by using the unpaired *t* test, and the change was considered significant if the *P* value of the test was less than .05 (ie, with at least 95% confidence).

RESULTS

Growth factors that are induced by IgE cross-linking in CBMCs

To identify growth factors that are induced by IgE cross-linking in CBMCs, we performed 2 independent microarray analyses of human CBMCs from 2 different donors. The CBMCs from donor 2 was first primed with IL-4 to upregulate Fc ϵ RI receptor before IgE cross-linking. Genes that were annotated as growth factors were selected and then expanded to include other members of the same gene family. Of the 64 different growth factors we examined, 9 were detectable (ie, signals above the detection threshold as defined by MAS 5.0) in the resting CBMCs in both donors. These were endothelial cell growth factor 1, hepatoma-derived growth factor, lens epithelium-derived growth factor, PDGF C, spinal cord-derived growth factor B, TGF- α , TGF- β 1, VEGF, and VEGF B (Table I). Five growth factor genes were upregulated by means of IgE cross-linking by at least 4 fold on average (Table I). These genes were activin β A, amphiregulin, HB-EGF, PDGF α , and VEGF upregulated by an average of 286-, 44-, 15-, 5.7-, and 4.1-fold, respectively (Table I). Consistent with the microarray analysis of activated human mast cells published by others,^{20,21} we found that the expression of GM-CSF, IL-3, IL-5, IL-13, MCSF, 4-1BB, IL-8, Mip-1 α , Mip-1 β , MCP-1, and I-309 was upregulated by 39-, 1351-, 37-, 37-, 32-, 8-, 15-, 24-, 24-, 2.3-, and 630-fold, respectively. The genes for tryptase, chymase, and carboxypeptidase were expressed at high levels in unstimulated CBMCs but were not upregulated by stimulation as expected. We also determined the effect of glucocorticoids and cyclosporin A on the expression of these 5 genes. Although the expression of activin β A was suppressed by cyclosporin A by 4-fold, the expression of amphiregulin was not significantly altered by cyclosporin A. The expression of HB-EGF was reduced by 2-fold by cyclosporin A.

Treatment of cells with dexamethasone followed by IgE cross-linking increased the expression of amphiregulin by 1.5-fold but suppressed the expression of activin β A and HB-EGF by 1.7- and 2-fold, respectively (Table II).

Real-time RT-PCR analysis of the expression of human amphiregulin gene

The induction of activin β A, PDGF α , HB-EGF, and VEGF in mast cells has been reported by us and others.¹⁷ However, the expression of amphiregulin has not been reported in human mast cells, and we therefore focused on the characterization of amphiregulin. Real-time RT-PCR was used to measure the expression of human amphiregulin in different tissues and mast cells. Among the 8 different human tissues examined, very low levels of amphiregulin were detected in the lung, trachea, and spleen (Fig 1). Amphiregulin was not expressed in the brain, heart, kidney, liver and thymus (Fig 1). In 2 different batches of the *in vitro* cultured CBMCs, the expression of amphiregulin was low in unstimulated CBMCs but strongly induced by IgE cross-linking in both batches of the human CBMCs (Fig 1), confirming the DNA microarray analysis results that amphiregulin mRNA was highly induced in human CBMCs. Real-time PCR analysis found that cyclosporin at 500 nM inhibited activin β A expression (9-fold) but did not significantly affect amphiregulin expression. Dexamethasone at 500 nM inhibited activin β A expression by 1.7-fold, whereas it enhanced amphiregulin expression by approximately 3.6-fold. These data were consistent with the data generated by means of microarray analysis.

Secretion of amphiregulin protein by human CBMCs

Amphiregulin in the supernatants of CBMCs before and after IgE cross-linking was measured by means of ELISA to determine whether amphiregulin protein secretion is also increased in CBMCs after IgE cross-linking. Amphiregulin was not detectable in the resting CBMCs derived from 3 different donors (Fig 2); the level of amphiregulin increased to 45.42 ± 15.6 , 43.5 ± 7.35 , and 38.63 ± 3.61 pg/250,000 CBMCs, respectively, 8 hours after cross-linking. To determine the kinetics of amphiregulin expression, we stimulated CBMCs by means of IgE cross-linking for 0, 2, 4, 8, and 24 hours and measured amphiregulin by means of ELISA. Amphiregulin was detected in the supernatant 8 hours after stimulation (35 ± 14.5 pg/250,000 CBMCs), and the level increased to 90.6 ± 38.3 pg/250,000 CBMCs 24 hours after stimulation. Amphiregulin secretion is cross-linker dose dependent (Fig 2).

Amphiregulin expression in human lung mast cells of patients with asthma

Lung tissues from patients with asthma or control patients were examined by using indirect immunofluorescence with antibodies to amphiregulin and mast cell tryptase to determine whether amphiregulin is expressed in the lung mast cells of asthmatic patients. In normal lung tissues we found that mast cells are positive for tryptase

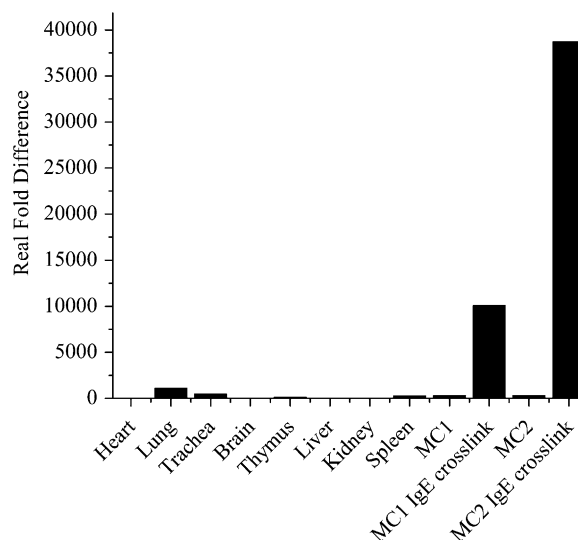


FIG 1. Amphiregulin mRNA expression in human tissues and CBMCs. Quantitative real-time RT-PCR was used to measure the expression levels. MC1 and MC2 are 2 different batches of CBMCs.

staining; however, these cells stained negative for amphiregulin (Fig 3, A and B). In 2 asthmatic patients a number of lung mast cells were both tryptase positive and amphiregulin positive (Fig 3, C-F). We quantified the percentages of amphiregulin-labeled cells by observing at least 50 mast cells for each patient and healthy control subject. Approximately 70% of mast cells from the airways of asthmatic patients were stained for amphiregulin, whereas none of mast cells from the airways of healthy control subjects were stained for amphiregulin.

Effects of amphiregulin on primary human lung fibroblasts and airway smooth muscle cells

Given that the amphiregulin is expressed in the mast cells of the asthmatic patients, and it is known that the number of the human mast cells present in asthmatic airway smooth muscle and fibrotic lung is increased,²² we therefore determined whether amphiregulin can promote the proliferation of either the human lung primary fibroblasts, primary airway smooth muscle cells, or both. Human lung primary fibroblasts or human airway smooth muscle cells were serum starved and then cultured in the presence of different concentrations of recombinant human amphiregulin, and the incorporation of tritiated thymidine into DNA was determined. As shown in Fig 4, amphiregulin increased the tritiated thymidine into the human primary lung fibroblasts in a dose-dependent manner. Amphiregulin increased the DNA synthesis by 123%, 128%, 168%, and 234% at 1, 10, 50, and 100 ng/mL, respectively, compared with that of control subjects. The amphiregulin-induced DNA synthesis can be neutralized by an antibody to amphiregulin (Fig 4). When similar studies were performed with human airway smooth muscle cells, amphiregulin did not consistently induce the DNA synthesis of the airway smooth muscle cells. Activin

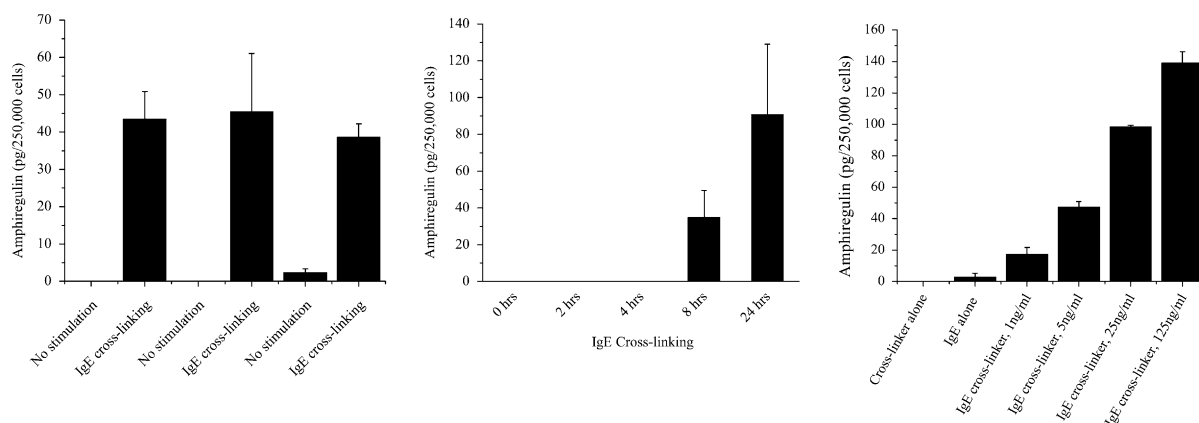


FIG 2. Amphiregulin protein secretion in CBMCs. **A**, CBMCs from 3 different donors were stimulated for 8 hours. **B**, Kinetics of amphiregulin protein secretion. **C**, Dose-dependent response of amphiregulin protein secretion.

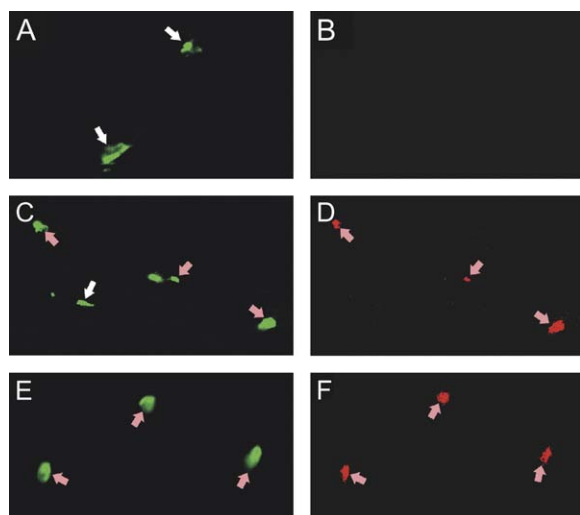


FIG 3. Double-immunofluorescence labeling of amphiregulin and mast cell tryptase. Tissue sections from the lungs of healthy control subjects (**A** and **B**) and 2 asthmatic patients (**C-F**) were stained with antibodies to amphiregulin (**B**, **D**, and **F**) and mast cell tryptase (**A**, **C**, and **E**) and then detected with rhodamine (red) or FITC-labeled secondary antibody (green).

A, another growth factor induced by IgE cross-linking from the human CBMCs that we previously reported to be a mitogen for airway smooth muscle cells, induced DNA synthesis in a dose-dependent manner in this cell type, indicating our assay is working properly (data not shown).¹⁷ The proliferation of human primary fibroblasts cultured in the presence of amphiregulin was also determined by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt incorporation. We found that amphiregulin stimulated the proliferation of the primary lung fibroblasts (data not shown).

Amphiregulin induces the expression of *c-fos* but not cyclin D and other growth factors

To determine whether amphiregulin induces the expression of *c-fos*, cyclin D, and other growth factor genes,

we stimulated human primary lung fibroblasts with 10 ng/mL amphiregulin for 0, 0.5, 1, 3, and 6 hours and isolated total RNA from the cell. Real-time RT-PCR was then performed to assess the expression of a *c-fos*, cyclin D, TGF- α , VEGF, PDGF α , HB-EGF, activin β A, and amphiregulin. Treatment of lung primary fibroblasts with amphiregulin (10 ng/mL) induced the expression of *c-fos* by 6-fold 30 minutes after treatment and reached maximal induction of *c-fos* RNA by 1 hour (16-fold) and returned to baseline by 3 hours of treatment (Fig 5). Amphiregulin-induced *c-fos* expression was dose dependent because amphiregulin at 100 ng/mL induced 11.9-fold more *c-fos* expression compared with stimulation with 10 ng/mL amphiregulin (199.4-fold vs 16.8-fold) in a separate experiment. Amphiregulin did not induce the expression of cyclin D1, TGF- α , VEGF, PDGF α , HB-EGF, activin β A, and amphiregulin.

DISCUSSION

Using microarray analysis on a highly pure population of *in vitro* derived CBMCs, we were able to systematically examine the expression of the human growth factors before and after IgE cross-linking. Of the 64 growth factor genes we analyzed, 8 were expressed at low-to-moderate levels under normal cultured conditions. Interestingly, only 2 of these 8 genes, VEGF and TGF- β 1, were induced further after IgE cross-linking. Among the 5 genes whose expression was induced by 4-fold or more on average in 2 different batches of human mast cells, amphiregulin ranked the second in terms of fold change. Consistent with our previous findings, activin β A was strongly induced in CBMCs after IgE cross-linking.¹⁷ Other growth factors that were activated include HB-EGF, PDGF α , and VEGF. The induction of HB-EGF, PDGF α , and VEGF has been reported in other mast cells.^{19,23,24} The fact that amphiregulin mRNA and protein in mast cells were strongly induced after IgE receptor cross-linking but were undetectable in unstimulated CBMCs suggests that

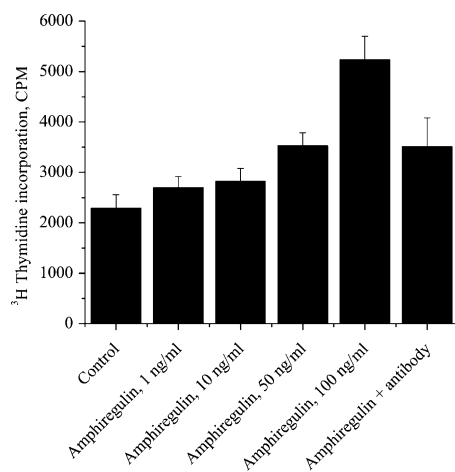


FIG 4. Effect of amphiregulin on the proliferation of human lung primary fibroblasts. Human lung primary fibroblasts were stimulated and tritiated thymidine incorporation was measured as described in the Methods section.

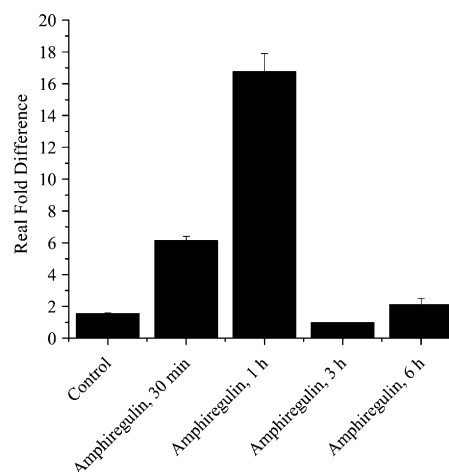


FIG 5. Effect of amphiregulin on *c-fos* gene expression. Human lung primary fibroblasts were stimulated and RNAs were isolated as described in the Methods section. The expression of *c-fos* was evaluated by means of real-time RT-PCR.

amphiregulin is newly synthesized and released on stimulation.

Even though both activin and amphiregulin are highly inducible in the CBMCs, the signaling pathway leading to the induction of these 2 different growth factors might be different. We found that cyclosporin A, a calcineurin inhibitor, inhibited the induction of activin A by means of microarray analysis and by means of real-time RT-PCR. However, in repeated experiments we found that cyclosporin A had no effect on the expression of amphiregulin, indicating that *de novo* synthesis of amphiregulin after IgE cross-linking is independent of the calcineurin pathway. Dexamethasone, a glucocorticoid, weakly inhibited the expression of activin A but weakly enhanced the expression of amphiregulin. These findings were confirmed by our ELISA: dexamethasone at 1 μ M induced the secretion of 10.6 pg/250,000 cells of amphiregulin. Interestingly, dexamethasone has also been shown to induce the expression of plasminogen activator inhibitor-1 in a mast cell line (HMC-1), a molecule that could play an important role in airway remodeling and tissue repair.²⁵

Increased numbers of fibroblasts-myofibroblasts and the deposition of extracellular matrix proteins, such as collagens, have been observed in a number of different clinical disease conditions, including asthma, chronic bronchitis, and skin disorders.²⁶ Mast cell hyperplasia has been associated with these disorders. Given that amphiregulin is released by mast cells on activation and is present in the lung mast cells of asthmatic patients and amphiregulin induces the proliferation of human lung fibroblasts, it is possible that mast cell-derived amphiregulin might promote the proliferation of fibroblasts in the lung and skin and thus contribute to fibrosis. In addition to amphiregulin, other growth factors activated in mast cells by IgE cross-linking have also been demonstrated to exert a mitogenic effect on fibroblasts. For example, activin A

could promote the proliferation of lung fibroblasts and their differentiation into myofibroblasts.²⁷ HB-EGF can stimulate the mitogenesis of lung fibroblasts in culture,²⁸ and PDGF is a potent chemotactic factor for fibroblasts and might play an important role in controlling connective tissue production.²⁹ It can be envisioned that these growth factors released simultaneously on mast cell activation might act synergistically to exert their physiologic or pathophysiologic effects.

Upregulation of the proto-oncogene *c-fos* is one of the earliest transcriptional events associated with growth factors and is often used as a marker for growth response.³⁰ Induction of *c-fos* has been associated with diverse cellular functions, such as cellular transformation, differentiation, and gene regulation, and induction of *c-fos* has been shown to facilitate or is required for cellular proliferation.³⁰ We found that amphiregulin induced *c-fos* expression in lung fibroblasts 30 minutes after treatment, and the level reached maximum at 1 hour. It is possible that the proliferative response of amphiregulin in fibroblasts is mediated in part or in full by *c-fos*. Amphiregulin has been shown to be the major autocrine growth factor in human keratinocyte,¹⁴ and we therefore evaluated whether amphiregulin could induce the expression of itself and several other growth factors. Amphiregulin did not induce the expression of itself and other growth factors in the primary lung fibroblasts. However, it remains to be determined whether amphiregulin induces the expression of other fibroblast growth factors, such as fibroblast growth factor 2.

In summary, we demonstrated for the first time that both amphiregulin mRNA and protein are induced in human *in vitro* derived CBMCs by means of IgE cross-linking. Recombinant amphiregulin can induce the proliferation of primary lung fibroblasts, and the mitogenic effect by amphiregulin is associated with an increase in *c-fos* gene expression. These results suggest that human mast cells

might contribute to the fibrotic process by means of local release of amphiregulin after activation.

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