Regulation of Activin A Expression in Mast Cells and Asthma: Its Effect on the Proliferation of Human Airway Smooth Muscle Cells[1]

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Activin A, a homodimeric protein ($\beta A\beta A$) and a member of the TGF-β superfamily, is involved in the inflammatory repair process. Using cDNA microarray analysis, we discovered strong induction of the activin βA gene in human mast cells (MC) on stimulation with PMA and calcium ionophore (A23187). Activin βA mRNA was also highly induced in primary cultured murine bone marrow MC (BMMC) after stimulation by IgE receptor cross-linking. Secretion of activin A was evident in human mast cell-1 line cells 3 h after stimulation and progressively increased over time. Activin A was present in the cytoplasm of activated but not unstimulated murine bone marrow MC as demonstrated by immunofluorescence studies, suggesting that secretion of activin A by MC was due to de novo synthesis rather than secretion of preformed protein. Activin A also colocalized with human lung MC from patients with asthma by double-immunofluorescence staining. Furthermore, secretion of activin A was significantly increased in the airway of wild-type mice after OVA sensitization followed by intranasal challenge. Secretion of activin A, however, was greatly reduced in MC-deficient WBB6F$_1$-*W/W^v* mice as compared with wild-type mice, indicating that MC are an important contributor of activin A in the airways of a murine asthma model. Additionally, activin A promoted the proliferation of human airway smooth muscle cells. Taken together, these data suggest that MC-derived activin A may play an important role in the process of airway remodeling by promoting the proliferation of airway smooth muscle. *The Journal of Immunology,* **2003, 170: 4045–4052.**

Airway remodeling in asthma is a dynamic process that involves subepithelial fibrosis, extracellular matrix (ECM)[3] deposition, smooth muscle hypertrophy/hyperplasia, and goblet cell hyperplasia in the airways in response to inflammation or injury (1, 2). The inflammatory process underlying asthma results from a highly complex interaction of various cell types (3–5). In addition, ECM interacts with inflammatory cells (6), forms a reservoir for cytokines and growth factors (7, 8), influences trafficking of cells (9), and activates granulocytes to increase mediator release and enhance their survival (10, 11). Conversely, several inflammatory mediators have a known proliferative effect on airway smooth muscle (ASM) and induce the syn-

thesis of ECM and growth factors in fibroblasts and epithelial cells (12, 13). It therefore appears that airway inflammation and remodeling by various cells in the airways are interdependent processes that clearly influence the clinical long term progression of asthma.

Mast cells (MC) may play an important role in pulmonary fibrosis (14, 15). MC promote migration and proliferation of fibroblasts by releasing proinflammatory mediators and growth factors (16, 17). For example, MC tryptase is able to activate and induce proliferation of fibroblasts (18). MC also synthesize and release components of basement membranes such as laminin and collagen IV (19). There are a variety of clinical situations in which fibrosis and MC hyperplasia with activation have been observed (20–22). Increased numbers of MC and evidence of activation have also been observed in animal models of pulmonary fibrosis induced by bleomycin (23) or ionizing irradiation (24). Experimental asbestosis is also associated with MC hyperplasia (25). Furthermore, MC are essential for the full development of silica-induced pulmonary inflammation (26). Collectively, MC appear to be important contributors for the development of airway remodeling in asthma.

The cDNA microarray technology allows monitoring of the expression of multiple genes simultaneously during MC activation (27). Identifying the up-regulated and down-regulated genes involved in ECM synthesis or wound healing may provide a clue as to which genes play a key role in the development of airway remodeling.

Activin A is a member of the TGF-β superfamily which displays structural and functional homology with the different forms of TGF-β (28). Three different forms of activin, homodimeric activin A ($\beta A\beta A$), activin B ($\beta B\beta B$), and heterodimeric activin AB ($\beta A\beta B$) have been described. Recently βC, βD, and βE chains

have been discovered (29). Activin A affects growth and differentiation of many different target cells of various organs (30–32). However, recent studies have provided evidence for a novel and important role of activin A in the inflammatory and repair processes such as cutaneous wound repair, inflammatory bowel disease, liver injury and cirrhosis, and pulmonary fibrosis (33–37).

In the present study, we performed gene expression profiling using a cDNA microarray on HMC-1 cells stimulated with PMA/calcium ionophore (A23187). Among the differentially expressed transcripts, the activin βA gene was chosen for further studies due to its high inducibility and its direct association with tissue remodeling in the lung. We have characterized the expression of activin A in MC in the airways of a mouse asthma model and in the lung of patients with asthma. Furthermore, we investigated the effect of activin A on the proliferation of human airway smooth muscle cells (HASMC).

Materials and Methods

MC culture

HMC-1 cells were a gift of Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN). Exponentially growing HMC-1 cells were maintained in Iscove's medium, 10% FCS supplemented with antibiotics as described (38). The primary cultured human mast cells (PCHMC) were derived from human cord blood CD34$^+$ cells grown in the presence of stem cell factor, IL-6, and IL-10 as described (38). Briefly, human cord blood CD34$^+$ cells (BioWhittaker, Walkersville, MD) were cultured in culture medium, consisting of RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 20% FBS (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, 80 ng/ml stem cell factor, 50 ng/ml IL-6, and 5 ng/ml IL-10. The cell suspensions were seeded at a density of 1–5 $\times$ 10^5 cells/ml, and the cells were harvested for study when >95% of the cells were stained with toluidine blue and anti-tryptase mAb (typically 7–9 wk). Bone marrow MC (BMMC) were derived from C57BL/6J mice and maintained in RPMI 1640 (Invitrogen) supplemented with 10 ng/ml recombinant murine IL-3 (R&D Systems, Minneapolis, MN), 10% FCS, 2 mM glutamine, 50 U/ml penicillin, and 100 μg/ml streptomycin at a concentration of 5 $\times$ 10^5 nucleated cells/ml and cultured at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air. Culture medium was replaced every 7 days. After 5 wk of culture, cells were harvested and suspended in PBS. MC number and purity were assessed by acid toluidine blue staining, and cell viability was determined by trypan blue dye exclusion. BMMC used in experiments consisted of >97% MC and were of 98% or greater viability.

Stimulation conditions

HMC-1 cells were adjusted to a density of 5 $\times$ 10^5 cells/ml in medium containing PMA (Sigma-Aldrich) at 50 ng/ml and A23187 (Sigma-Aldrich) at 0.5 mM. The cells were stimulated at 37°C before harvest, and the cell pellet was washed for mRNA extraction. The mRNA was extracted using the manufacturer's procedure (Invitrogen). Unstimulated cells were used as the resting control for the microarray analysis. BMMC and PCHMC were stimulated by IgE receptor cross-linking as previously described (38).

cDNA microarray analysis

Purified resting and stimulating mRNAs (200 ng) were reverse transcribed and labeled with either Cy3 or Cy5 fluorescent dyes. The two-color competitive hybridization was then performed on an Incyte human gene chip, UniGEM-V cDNA microarray. This gene chip is designed to give a broad view of human gene expression, using genes and expressed sequence tags from the public domain UniGene database. All the clones, containing 7075 elements, have been sequence verified. Expression levels were analyzed with the Incyte GemTool program to categorize the genes into meaningful categories, such as wound healing and ECM synthesis.

Northern blot analysis

RNA (20 μg total) was isolated and then subjected to electrophoresis in 1.5% agarose-formaldehyde gels and transferred to nylon-reinforced nitrocellulose membranes (Micron Separations, Westboro, MA). Prehybridizations were performed at 37°C for 30 min. Hybridizations were performed at 37–42°C for 18–24 h in 50% formamide, 5$\times$ Denhardt's solution, 0.55 M NaCl, 0.09 M NaPO$_4$ (pH 7.0), 0.4 mM Na$_3$EDTA, 0.09 M sodium pyrophosphate, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. Human activin βA cDNA was generated by RT-PCR. The 2-kb human β-actin cDNA was purchased from Clontech Laboratories (Palo Alto, CA). The cDNAs were labeled by random hexamer using protocols suggested by the manufacturer (Stratagene, La Jolla, CA).

Quantitative real time RT-PCR analysis

Two sets of oligonucleotide primers (5'-ACAGCCAGGAAGACACT GCA and 5'-CAGGTCACTGCCTTCCTTGG) were selected from the mouse activin βA nucleotide sequences using Primer Express 2.0 (Applied Biosystems, Foster City, CA) and used in real time RT-PCR to monitor the expression of activin βA. RNAs were isolated using Trizol according to the manufacturer's protocol (Invitrogen). Quantitative real time RT-PCR was performed with the ABI Prism 7900 (Applied Biosystems) sequence detection system, using Taqman reagents, according to the manufacturer's instructions (Applied Biosystems). Equal amounts of each of the RNAs were reverse transcribed into first-strand cDNA and used as PCR templates in reactions to obtain the threshold cycle (C_t), and the C_t was normalized using the known C_t from 18S RNAs to obtain ΔC_t. To compare the relative levels of gene expression of activin βA in different samples, $\Delta\Delta C_t$ values were calculated by using the lowest expression levels as the base. The $\Delta\Delta C_t$ values were then expressed as the real fold increase in expression.

ELISA for activin A

Supernatants from stimulated HMC-1 cells, BMMC, PCHMC, or bronchoalveolar lavage fluids (BALF) from mice were assayed in duplicate for human or murine activin A using sandwich ELISA kits (R&D Systems). The lower limits of detection for human and murine activin A assays were 5 and 10 pg/ml, respectively.

Immunofluorescence intracytoplasmic localization of activin A

Intracytoplasmic staining of BMMC for activin A was performed by the method previously described with slight modifications using anti-activin A polyclonal Ab (39). Briefly, the cells were stimulated overnight by IgE receptor cross-linking and centrifuged onto slides by using the Cytospin 3 (Shandon, Pittsburgh, PA) and fixed with 100% cold methanol for 5 min. The slides were washed with PBS and then permeabilized with PBS containing 0.3% Triton X-100 (v/v). The cells were then blocked with 10% normal rabbit serum for 40 min. After a washing with PBS, incubation with 5 μg/ml goat anti-activin A (R&D Systems) was conducted in a humidified chamber overnight at 4°C. The slides were then washed and incubated with FITC-conjugated rabbit anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h. The slides were washed again and then mounted with a fluorescent-preserving medium. Control cells were stained with an irrelevant rabbit IgG (Oncogene, San Diego, CA).

Confocal microscopy

Specimens were imaged on a Leica TCS-SP confocal microscope (Leica, Heidelberg, Germany) equipped with an argon laser for 488 nm blue excitation as previously described (40). Confocal fluorescence images were collected using Leica confocal software and then processed to make maximum projection through-focus images. Differential interference contrast images were also scanned using the 568 nm laser line for excitation and a transmitted light detector on the system for image capture. Confocal microscopy was performed in the Carol Moss Spivak Cell Imaging Facility in the UCLA Brain Research Institute.

Double-immunofluorescence study

After written informed consent was obtained, bronchoscopy was performed in all asthma subjects, and one to two biopsy specimens were taken from the carina and right middle and upper lobe bronchi. Tissue specimens from two fatal asthma, one severe persistent asthma, and one moderate persistent asthma patients were obtained, and the severity of asthma was determined based on the criteria set in the Global Initiative for Asthma guidelines (41). They had received inhaled β_2-agonists and/or oral steroid. Normal bronchial tissues from three lung transplantation donors who had no asthma or other lung disease history were also obtained. Bronchial biopsy specimens and normal bronchial tissues were processed immediately and stored at −80°C before use for immunofluorescence study. The tissues were fixed in neutral buffered formalin, embedded in paraffin, and cut into 4-μm sections. Simultaneous double-immunofluorescence staining was performed as previously described (42). Briefly, nonspecific binding was blocked with 5% donkey or rabbit serum (according to the species of the secondary Abs; Jackson Immunoresearch Laboratories). After two washes with PBS, primary Abs against activin A (1/20; R&D Systems), MC tryptase (1/500;

Chemicon International, Temecula, CA), MC chymase (1/5000; Chemicon), or CD68 (1/50; DAKO, Carpinteria, CA) were added, and the sections were incubated overnight at 4°C. The sections were washed in PBS twice for 10 min each and then incubated for 1 h at room temperature with the appropriate secondary Abs. The rhodamine-labeled donkey anti-goat Abs (Chemicon) were diluted 1/100, and FITC-labeled rabbit anti-mouse Abs (DAKO) were diluted 1/20. After a nuclear stain with 4′,6-diamidino-2-phenylindole, dihydrochloride (Molecular Probes, Eugene, OR) stain, the coverslips were mounted and then examined using an Eclipse E400 microscope (Nikon, Melville, NY) equipped with a triple-band filter cube (Exciter Filters 402, 496, 571; Barrier filters 462, 531, 627; Nikon 96166; 4′,6-diamidino-2-phenylindole, dihydrochloride, FITC, and rhodamine).

Experimental animals

MC-deficient WBB6F$_1$-*W/W^v* (*W/W^v*) and wild-type (WT) mice, age 6–10 wk, were obtained from The Jackson Laboratory (Bar Harbor, ME). All experimental procedures complied with the requirements of the Animal Care and Use Committee of the Harbor-University of California, Los Angeles Research Education Institute.

Immunization and intranasal challenge with OVA

Mice were housed in autoclaved cages on autoclaved bedding in an air-conditioned room on a 12 h light/dark cycle. The sensitization and challenge procedures were performed as described (38). Briefly, mice were first sensitized by i.p. injection of 1 mg of alum-precipitated chicken egg OVA (grade V, 98% pure; Sigma) in 0.1 ml of PBS. Mice were lightly anesthetized by inhalation of methoxyflurane (Metofane) and subjected to a single intranasal challenge with 100 μl of 1% OVA on the 10th day. Mice were killed by pentobarbital overdose 24 h after the last OVA challenge.

BALF collection

The trachea of a freshly killed mouse was exposed, and a 19-gauge angio-catheter was inserted and tied in place with a suture. The lungs were lavaged three times by slowly instilling 1 ml of sterile PBS at 37°C followed by gentle aspiration. The BALF was centrifuged (1000 × *g* for 10 min at 4°C), and the supernatant was removed and stored at −80°C. Total protein in the BALF supernatant was measured with the Micro-BSA protein assay reagent kit (Pierce, Rockford, IL).

HASMC culture and proliferation assay

HASMC were obtained from Clonetics (San Diego, CA) and used for up to four additional passages as recommended. HASMC were grown to con-fluence using smooth muscle cell basal medium containing 0.5 ng/ml recombinant human epidermal growth factor (hEGF), 5 μg/ml insulin, 2 ng/ml recombinant human fibroblast growth factor, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, and 5% FCS (Clonetics) at 37°C in a 95% air, 5% CO_2 incubator. Proliferation studies were performed on confluent, growth-arrested HASMC. Cells were seeded on 96-well plates and growth arrested by incubating the cultures in serum-free medium. Confluent, growth-arrested cells were used because cells can be synchronized in the G_0-G_1 phase of the cell cycle (43). After 24 h in serum-free medium, the cells were stimulated with either recombinant human activin A (1, 10, or 100 ng/ml) (R&D Systems) or hEGF (100 ng/ml) (R&D Systems). Cell number was quantified using a hemocytometer after enzymatic detachment of cells. The proliferation of incubated cells was also quantified after a 24-h stimulation with recombinant human activin A or hEGF using a BrdU assay. BrdU incorporation during cellular S phase, as assessed by BrdU colorimetric cell proliferation ELISA (Roche Biochemicals, Indianapolis, IN), was used as a direct readout for cell proliferation rates. This assay is a nonradioactive alternative to the [^{3}H]thymidine-based cell proliferation assay with comparable sensitivity (44).

Statistics

Statistical significance assay was determined by Student's unpaired sample *t* test (two-tailed) or one way ANOVA, and significance was determined with *p* values < 0.05.

Results

Scattergram of gene expression from activated HMC-1 cells

It has been noted that increased numbers of activated MC are found in the airways of chronic asthmatics (39). To search for genes induced in activated MC, we performed a DNA microarray analysis in HMC-1 cells. A combination of PMA and A23187 was used to achieve maximum stimulation of MC. The genes fall in the range of differential expression ratio of less than −2 and greater than +2 were considered up-regulated and down-regulated, respectively. The resulting expression profiling showed that expression of the majority of the genes on the microarray varied less than 2-fold, indicating that only a small percentage of genes were significantly up-regulated or down-regulated in response to stimulation for 4 h (Fig. 1*A*). The histogram showed an extensive break-
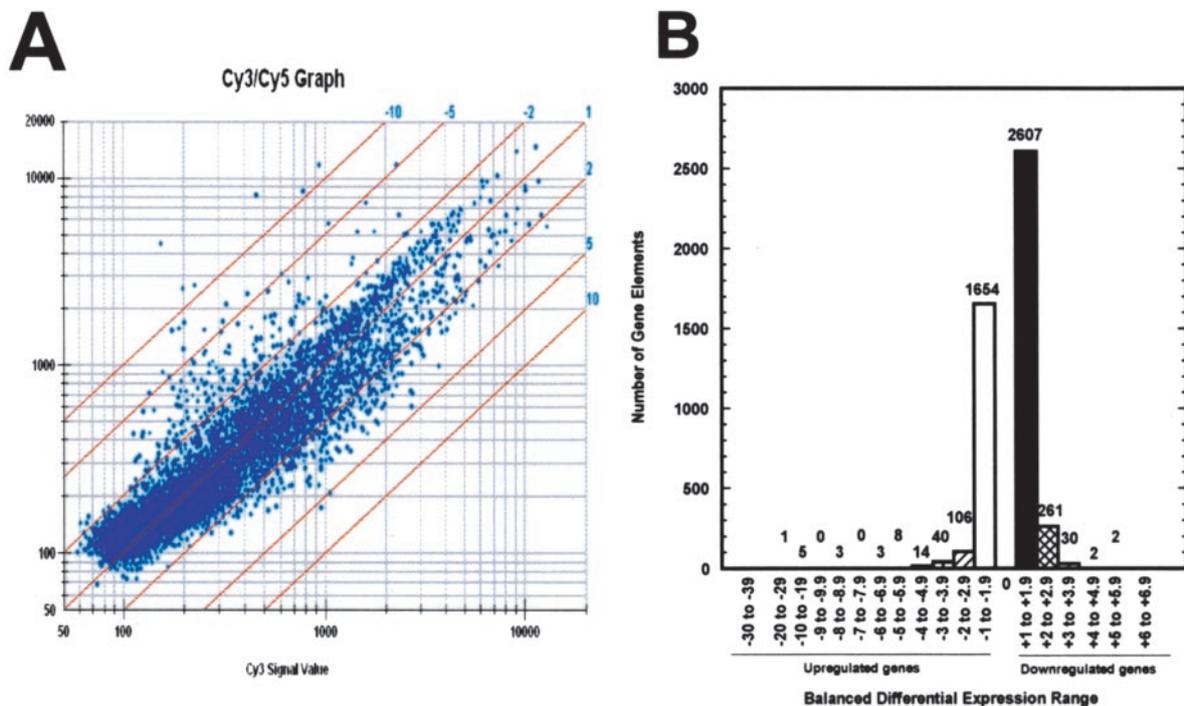
FIGURE 1. *A*, Scattergram of gene expression from HMC-1 cells stimulated with a combination of PMA and A23187, compared with unstimulated cells. The genes with a differential expression ratio of less than −2 or greater than +2 are considered to be either up-regulated or down-regulated. *B*, Histogram of gene expression from activated HMC-1 cells. The numbers above the bars indicate the number of genes in each expression category.
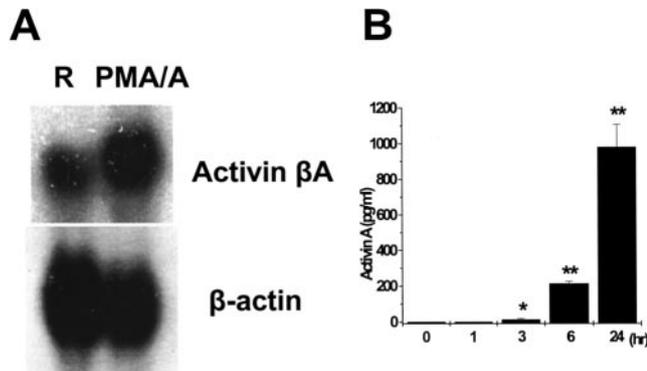
FIGURE 2. *A,* Activin βA mRNA expression in HMC-1 cells. *Top,* Northern blot of activin βA mRNA. Total RNA (20 μg) from unstimulated MC (R) and MC stimulated with PMA and A23187 for 4 h (PMA/A) were loaded onto each lane. *Bottom,* Corresponding β-actin control. Two repeat experiments showed similar results. *B,* Kinetics of activin A secretion by HMC-1 cells. HMC-1 cells were stimulated with PMA and A23187 for the indicated times. Supernatants and cell lysates from the cells were analyzed for the secretion of activin A. Data presented are mean $\pm$ SEM of three independent experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.001$ compared with resting cells.

down of the genes based on differential expression ratio (Fig. 1*B*). In summary, of the 7075 genes tested, 180 genes were up-regulated and 295 genes were down-regulated using a 2-fold cutoff.

Northern blot analysis of the activin βA gene

Among the transcripts, the activin βA gene was of particular interest due to strong induction in HMC-1 cells as well as its direct association with tissue remodeling in the lung. We have also performed a DNA microarray analysis in primary cultured human MC derived from cord blood after IgE receptor cross-linking. The activin βA gene was also found to be highly induced 2 h after IgE

receptor cross-linking (our unpublished observation). To confirm the DNA microarray results that activin βA mRNA was highly induced in MC, we performed Northern blot analysis with total RNA from HMC-1 cells stimulated with PMA and A23187. As shown in Fig. 2*A,* activin βA mRNA was strongly induced in the stimulated cells as compared with unstimulated cells.

Secretion of activin A by human MC

To determine whether activin A secretion is also increased by MC after stimulation, activin A in the supernatants of HMC-1 cells stimulated with PMA and A23187 was measured by ELISA. Minimal amount of activin A was detected in the cultured supernatant 3 h after stimulation (11.5 $\pm$ 6.5 pg/ml), and the level was increased 6 and 24 h after stimulation (212.0 $\pm$ 12.6 and 980.0 $\pm$ 60.3 pg/ml, respectively), whereas activin A was not detected in the supernatants of unstimulated cells or cells stimulated for 1 h (Fig. 2*B*).

Real time RT-PCR and ELISA for activin A in PCHMC

To determine whether or not activin βA is induced in PCHMC by physiological stimuli, we stimulated PCHMC by IgE receptor cross-linking and measured mRNA expression. Human activin βA mRNA was highly induced in PCHMC 2 h after IgE receptor cross-linking (Fig. 3, *A* and *B*). We also measured secreted activin A from PCHMC by ELISA. Activin A was not detected in unstimulated PCHMC. However, after stimulation for 6 h, a significant amount of activin A (144.5 $\pm$ 12.2 pg/ml, $p < 0.001$) was secreted from PCHMC (Fig. 3*C*).

Real time RT-PCR and ELISA for activin A in murine BMMC

To confirm the production of activin A in murine MC, we stimulated murine BMMC by IgE receptor cross-linking and measured activin βA mRNA by real time RT-PCR and secreted activin A by ELISA. Activin βA mRNA was strongly induced in murine

FIGURE 3. *A,* Quantitative real time RT-PCR analysis of activin βA mRNA in PCHMC. PCHMC were stimulated by IgE receptor cross-linking for 2 h. Total RNA from unstimulated PCHMC (resting) and PCHMC stimulated by IgE cross-linking (IgE stimulation) was evaluated by real time RT-PCR as described in *Materials and Methods.* Gene expression was presented as real fold expression difference value. *B,* Specific gene amplicon plots in PCHMC stimulated for 2 h (IgE ST) or in unstimulated PCHMC (resting). *C,* Activin A ELISA in PCHMC. PCHMC were stimulated by IgE receptor cross-linking for 6 h, and supernatants from the cells were analyzed for the secretion of activin A by ELISA. Data presented are mean $\pm$ SEM of four independent experiments performed in duplicate. *, $p < 0.001$ compared with resting cells.
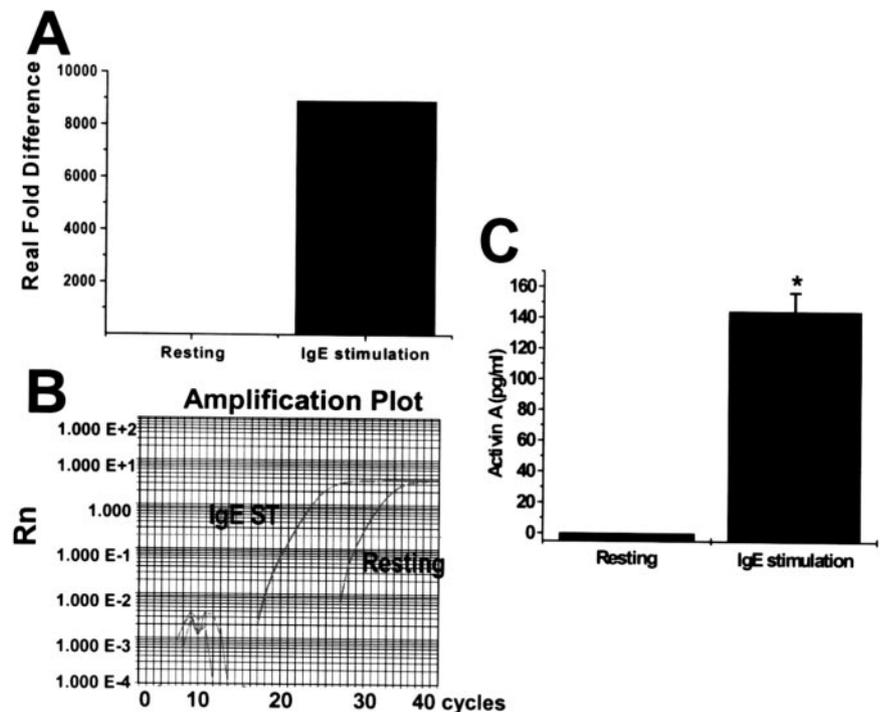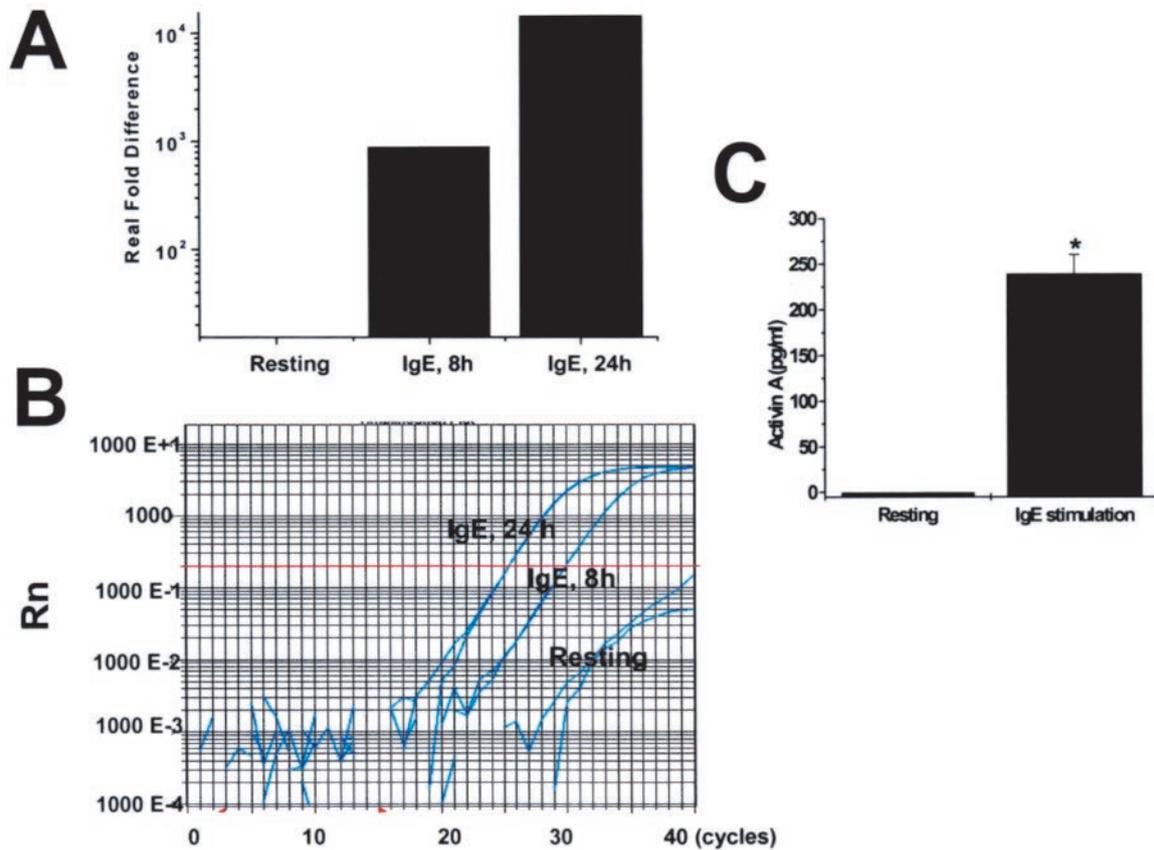
FIGURE 4. *A*, Quantitative real time RT-PCR analysis of activin βA mRNA in murine BMMC. BMMC were stimulated by IgE receptor cross-linking for 8 or 24 h. Total RNA from unstimulated BMMC (resting) and BMMC stimulated by IgE cross-linking was evaluated by real time RT-PCR as described in *Materials and Methods*. Gene expression was presented as real fold expression difference value. *B*, Specific gene amplicon plots in BMMC stimulated for 24 h, for 8 h, or in unstimulated BMMC. *C*, Activin A ELISA in BMMC. BMMC were stimulated by IgE receptor cross-linking for 24 h, and supernatants from the cells were analyzed for the secretion of activin A by ELISA. Data presented are mean ± SEM of four independent experiments performed in duplicate. *, $p < 0.001$ compared with resting cells.

BMMC stimulated by IgE receptor cross-linking for 8 h as compared with unstimulated BMMC (Fig. 4, *A* and *B*). The level was further increased after 24 h stimulation. Secreted activin A was not detected in unstimulated BMMC (Fig. 4*C*). However, after stimulation for 24 h, a large amount of activin A was secreted from BMMC (239.7 ± 20.9 pg/ml, $p < 0.001$).

Intracytoplasmic localization of activin A by confocal microscopy

To confirm the intracellular localization of activin A in MC, we stained BMMC with an activin A polyclonal Ab and examined the localization of the protein using confocal laser scanning microscopy. Immunoreactive activin A was expressed in the cytoplasm of stimulated BMMC (Fig. 5, *C* and *D*) but not in unstimulated BMMC (Fig. 5, *A* and *B*).

Activin A secretion in the airways of OVA-challenged WT and MC-deficient mice

To determine whether activin A secretion is enhanced in the airways of a murine model of asthma and whether MC are an important contributor of activin A in the airways of OVA-challenged mice, we compared expression levels of activin A in BALF from MC-deficient *W/W^v* and WT mice. Activin A was not detected in BALF of saline-challenged *W/W^v* or WT mice (Fig. 6). Secretion of activin A was significantly increased in the airway of WT mice after OVA sensitization followed by intranasal challenge. On OVA challenge, however, activin A level in BALF from *W/W^v* mice was

4-fold lower than that of WT mice (84.8 ± 7.4 pg/ml vs 311.8 ± 34.7 pg/ml, $p < 0.001$). These data suggest that MC are an important contributor of activin A production in the airways of OVA-challenged mice.

Activin A expression in human lung MC of patients with asthma

Lung tissues of patients with asthma and normal controls were examined using indirect immunofluorescence to determine whether MC express activin A in human asthma (Fig. 7). A large number of lung MC from asthmatic patients were stained for both tryptase and activin A (Fig. 7, *C* and *D*, red arrows). However, the MC in normal lung tissue were stained for tryptase but not for activin A (Fig. 7, *A* and *B*, white arrows). Chymase-positive MC from asthmatic patients expressed activin A, suggesting that MC containing tryptase and chymase also produce activin A (Fig. 7, *E* and *F*). We confirmed activin A expression in macrophages using the same lung tissues, which was used as a positive control (Fig. 7, *G* and *H*).

Activin A stimulates proliferation of HASMC

It was noted that the number of human MC present in asthmatic ASM is increased as compared with that in nonasthmatic ASM (5, 45), suggesting that mediators released by MC can modulate ASM function in the asthmatic airway. To determine whether MC promote the proliferation of HASMC by releasing activin A, HASMC were treated with recombinant human activin A. Culturing of HASMC in the presence of activin A for 24 h increased (by 214%)
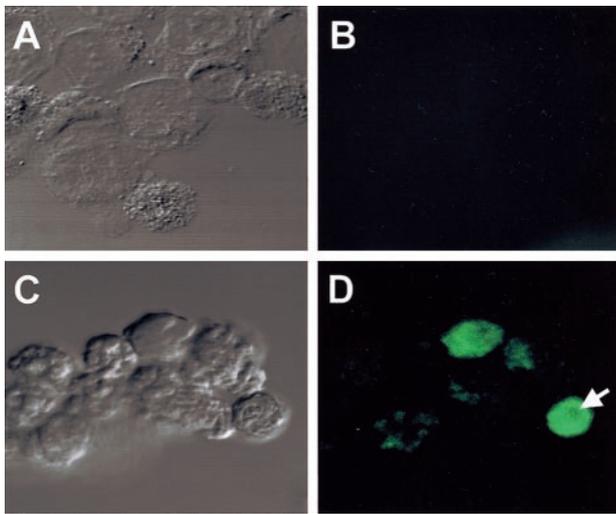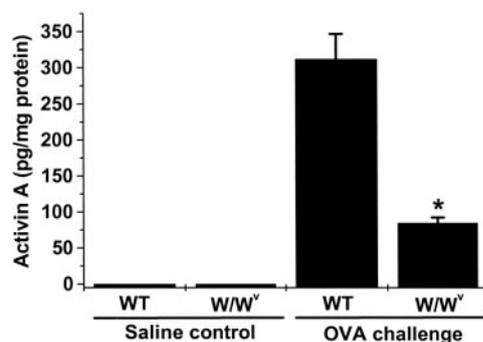
FIGURE 5. Immunofluorescence staining of activin A in BMMC. Confocal differential interference contrast images (*A* and *C*), and fluorescence images (*B* and *D*) of unstimulated cells (*A* and *B*) and cells stimulated overnight by IgE receptor cross-linking (*C* and *D*). Green images represent activin A positive staining. White arrow indicates nuclear sparing of the activin A staining (*D*). The photomicrographs were selected to illustrate the pattern and extent of activin A staining for each slide. All panels are at the magnification of ×400.

FIGURE 7. Double-immunofluorescence labeling of activin A, mast cell tryptase, chymase, and CD68 macrophage surface marker in airways of normal controls (*A* and *B*, original magnification ×400) and asthmatic patients (*C–H*, original magnification ×400). Goat polyclonal Ab directed against activin A was detected with rhodamine-labeled secondary Ab (red; *D, F,* and *H*). Mouse mAb against MC tryptase (*A* and *C*), chymase (*E*), or CD68 (*G*) was detected with FITC-labeled secondary Ab (green). White arrows, tryptase-positive but activin A-negative mast cells; red arrows, double-stained mast cells.
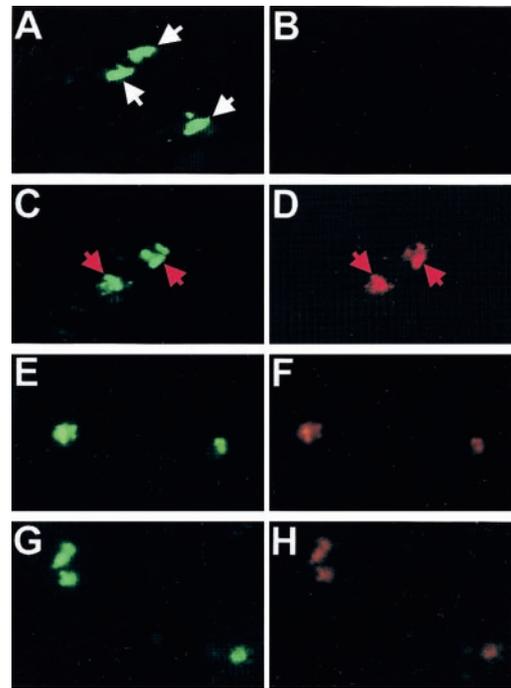
the number of HASMC in a dose-dependent manner as compared with the numbers of HASMC maintained for the same time period under basal, serum-free conditions (Fig. 8*A*). These results were confirmed using the BrdU assay, a colorimetric method that measures the amount of BrdU incorporated into the newly synthesized DNA of replicating cells. Culturing of HASMC in the presence of activin A for 24 h increased DNA synthesis of HASMC (by 191%) in a dose-dependent manner as compared with the numbers of HASMC maintained for the same time period under basal, serum-free conditions (Fig. 8*B*). Activin A was more potent in inducing the proliferation of HASMC than recombinant human EGF which was used as a positive control (Fig. 8, *A* and *B*).

Discussion

In the present study, we used a cDNA microarray to screen for differentially expressed genes that are relevant to airway remodeling. Among the 7075 genes on the microarray, 2.5% were up-regulated and 4.2% were down-regulated more than a 2-fold compared with unstimulated cells. The activin βA gene was highly

FIGURE 6. Activin A secretion into the airways of *W/W^v* or WT mice after OVA challenge. BALF supernatants from saline-challenged mice and OVA-challenged mice were analyzed for activin A protein by ELISA. Data presented are mean ± SEM of five independent experiments performed in duplicate; *, $p < 0.001$ compared with OVA-challenged WT control mice.

up-regulated in HMC-1 cells after stimulation with PMA and A23187 and in PCHMC stimulated by IgE receptor cross-linking. Activin A was also secreted by HMC-1 cells, PCHMC, and murine BMMC after stimulation. The fact that activin A mRNA and protein in MC were strongly induced after IgE receptor cross-linking but were undetectable in unstimulated MC suggests that activin A is newly synthesized and released on stimulation.

Given that activin A is a member of TGF-β superfamily and other members of this family are known to be involved in the tissue repair process, our findings that activin A is strongly induced in activated MC and in the airways of OVA-challenged mice suggest that activin A may play an important role in airway remodeling. These finding are consistent with the observations by Rosendahl et al. (46). Our data from a murine model of asthma using MC-deficient mice also suggest that MC are an important contributor of elevated activin A level in the asthmatic airway. Matsuse et al. (47) demonstrated the elevated levels of activin A in lung tissue of mice after treatment with bleomycin and found a correlation between the up-regulation of activin A expression and fibrotic changes in the lung. They also confirmed these findings in humans, where increased activin A expression was found in various types of pulmonary conditions associated with interstitial pulmonary fibrosis (37). Ohga et al. (48) showed that activin A promotes the proliferation of lung fibroblasts and their differentiation into myofibroblasts. On activation, these fibroblasts and myofibroblasts produce ECM such as fibronectin, tenascin (49), and collagens I, III, and V (50) and elaborate the production of growth factors that stimulate the proliferation of fibroblasts, nerve cells, smooth muscle cells, and vessels (51, 52). As a result, irreversible structural changes and wall thickening may occur in the asthmatic airway

FIGURE 8. Effect of activin A on proliferation of HASMC. HASMC were cultured in the absence or presence of recombinant human activin A (1, 10, or 100 ng/ml) or hEGF (100 ng/ml) at day 2 after addition of the mitogens. *A,* Data are expressed as cell number per well and represent the mean ± SEM of five experiments in triplicates. ∗, Significant compared with control, $p < 0.05$. *B,* Cell proliferation was quantified by BrdU incorporation. Data are expressed as percentage of proliferative response from cells cultured in serum-free control medium and are the mean percentage of change in proliferation ± SEM; there are duplicate per treatment in each experiment. ∗, Significant compared with control, $p < 0.05$.
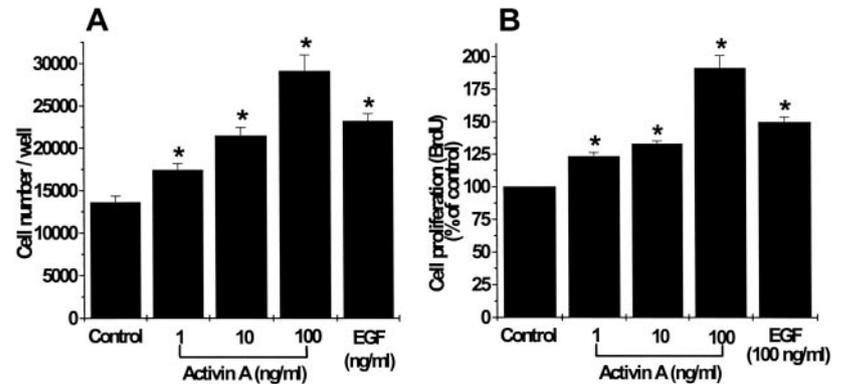
(53). A recent study also showed that activin A stimulates matrix metalloproteinase (MMP)-2 production in macrophages (54). MMP-2 plays an important role in tissue remodeling in the lung, and the MMP-2 levels are significantly elevated in the sputum of asthmatic patients (55). Taken together, elevated activin A may promote tissue remodeling in the asthmatic airway.

Bronchial epithelial cells, ASM cells, and alveolar macrophages are well-known cellular sources of activin A in the lung (37, 47). In the present study, we demonstrated that activin A was induced in human and murine MC after stimulation. There are a variety of clinical situations in which fibrotic lung disorders and MC hyperplasia have been observed (20–22). For example, increased numbers of activated MC have been appreciated in animal models of pulmonary fibrosis, including fibrosis induced by bleomycin (23) and ionizing irradiation (24). We demonstrated that activin A secretion in the airways of MC-deficient mice was considerably lower than in the airways of WT mice after OVA challenge. By double immunofluorescence, we found that activin A was expressed in human lung MC of patients with asthma but not in normal controls. These data suggest that MC-derived activin A has an important pathophysiological role in the asthmatic airway.

Recently, Brightling et al. (5) reported that the number of tryptase-positive MC in the bundles of ASM from subjects with asthma was substantially higher than in subjects with eosinophilic bronchitis or in normal controls. Although asthma and eosinophilic bronchitis have similar inflammatory infiltrates in the submucosa of the lower airway, eosinophilic bronchitis does not have variable airway obstruction or airway hyperresponsiveness, which are characteristic in asthma. This report suggests that interaction between MC and ASM cells plays a unique and important role in the development of asthma. ASM hyperplasia is one of the irreversible structural changes that occur in the airways of asthmatic patients (56). Thickening of the airway wall, partially from ASM hyperplasia, has a potential contribution toward irreversible airway obstruction in patients with severe asthma (57). In the present study, we demonstrated that activin A increased the number and DNA synthesis of HASMC. The mitogenic potency of activin A was even higher than that of EGF in our experiments. These data suggest that MC-derived activin A may promote the proliferation of ASM in the asthmatic airway and thus contribute to airway remodeling.

In summary, we demonstrated enhanced expression of activin A in stimulated MC, in the airways of a mouse asthma model, and in the lung of patients with asthma. Furthermore, we demonstrated the effect of activin A on the proliferation of HASMC, suggesting that increased activin A expression may contribute to the proliferation of ASM in the asthmatic airway. Together our data indicate that MC-derived activin A may play an important role in airway remodeling in patients with asthma at least in part by mediating the proliferation of ASM cells and provide solid ground to further investigate the functional role of activin A in asthma.

Acknowledgments

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