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Suppression of activin A in autoimmune lung disease associated with anti-GM-CSF

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Abstract

Pulmonary alveolar proteinosis (PAP) is an autoimmune disorder characterized by neutralizing autoantibodies to granulocyte-macrophage colony stimulating factor (GM-CSF). Surfactant metabolism is severely dysregulated in PAP, resulting in a foam cell appearance of alveolar macrophages. Microarray analysis of RNA from PAP bronchoalveolar lavage (BAL) cells to explore autoimmune-related genes yielded evidence of a deficiency of activin A, a cytokine implicated in regulation of B-cell proliferation and reduction of foam cell formation. Subsequent studies confirmed a severe deficiency of activin A gene expression and protein secretion in PAP BAL cells and marked reduction of activin A protein in PAP BAL fluids compared to healthy controls. PAP cells, however, like those of healthy controls, were capable of elevated activin A production in response to GM-CSF. Treatment with activin A in vitro suppressed proliferation of PAP peripheral blood B-cells in a receptor-dependent manner and also reduced secretion of anti-GM-CSF autoantibody. These studies are the first to suggest that activin A may play a role in autoimmune disease.
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1. Introduction

Activin A, a cytokine of 28,000 Da, is a member of the transforming growth factor-beta (TGF-β) superfamily based upon structural and functional homology [1,2]. The molecule, which was originally isolated as a gonadal hormone affecting secretion of follicle-stimulating hormone in the pituitary, is composed of two disulfide-linked inhibitin βA chains which form a homodimeric glycoprotein. Activin A has been found to be widely synthesized throughout the body including lung epithelium, smooth muscle cells, mast cells, and alveolar macrophages [3,4].

In biological fluids, activin A is often complexed with follistatin which binds to activin A with high affinity and blocks biological effects [5]. In order for the activin to become functionally active it requires dissociation from follistatin and activation which is regulated by several mechanisms including GM-CSF [6,7]. The range of activities regulated by activin A is diverse and includes erythroid differentiation, neuronal cell survival and immunoregulation through suppression of B lymphocyte proliferation [8] and macrophage foam cell formation [9]. The focus on activin has been on its involvement in embrogenesis, especially since activin A-deficient mice develop to term but die shortly after birth [10].

Pulmonary alveolar proteinosis (PAP) has been recently recognized to be an autoimmune disorder characterized by neutralizing autoantibodies to granulocyte-macrophage colony stimulating factor (GM-CSF). PAP patients have a functional GM-CSF deficiency which ultimately results in dysregulated pulmonary surfactant metabolism and the appearance of

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foamy alveolar macrophages [11,12]. The autoimmune nature of PAP, the deficiency of GM-CSF and the presence of foam cells led us to hypothesize that activin A is critical to pulmonary homeostasis. To address this hypothesis, we investigated the status of activin A synthesis and responsiveness in PAP bronchoalveolar lavage and peripheral blood cells.

2. Materials and methods

2.1. Study population

This protocol was approved by the Institutional Review Board and written informed consent was obtained from all subjects. Healthy control individuals ($n = 17$, aged 36 ± 10 years, seven females, 10 males) had no history of lung disease and were not on medication. The diagnosis of PAP was established by histopathological examination of material from open lung or transbronchial biopsies showing the characteristic filling of the alveoli with eosinophilic amorphous material with preserved lung architecture and absence of inflammation, and exclusion of secondary etiologies by negative lung cultures or occupational history [13–16]. All PAP ($n = 19$, aged 46 ± 12 years, five females, 14 males) patients had neutralizing antibody to GM-CSF in their sera and were symptomatic with dyspnea, hypoxemic on room air and had typical alveolar infiltrates on radiographs. None of the PAP patients were receiving GM-CSF therapy at the time of sample collection.

2.2. Cell collection and culture

Alveolar macrophages were derived from bronchoalveolar lavage (BAL) obtained by fiberoptic bronchoscopy as previously described [17]. Differential cell counts were obtained from cytospins stained with a modified Wright's stain. For PAP patients, the mean percentage of alveolar macrophages was $86 \pm 3\%$ (range 67–98%) and lymphocytes was $8 \pm 2\%$; for healthy controls, the alveolar macrophages was $94 \pm 1\%$ and lymphocytes was $4 \pm 1\%$. Mean viability of lavage cells was greater than 95% as determined by trypan blue dye exclusion. For culture, BAL cells were plated into 24-well plates (300,000 alveolar macrophages per well) or chamber slides (60,000 cells/well) in RPMI 1640 medium supplemented with 5% human AB serum (Gemini, Calabasas, CA), L-glutamine, and antibiotics. The cell yields from an individual patient or control were not sufficient to permit all experiments on each specimen.

2.3. RNA purification and analysis

Total RNA was extracted from BAL cells and PBMC according to the manufacturer's directions by RNAeasy protocol (Qiagen, Valencia, CA) and PAX tubes (Becton Dickinson, Franklin Lakes, NJ) respectively. Expression of mRNA was determined by real time RT-PCR using the ABI Prism 7000 Detection System (TaqMan; Applied Biosystems, Foster City, CA) described previously [18,19]. RNA specimens were analyzed in duplicate using primer sets for a housekeeping gene (GAPDH) (Cat # 4310884E), activin A (Cat # Hs00170103) and follistatin (Cat # Hs 00246260) (ABI). Threshold cycle (CT) values for genes of interest were normalized to GAPDH and used to calculate the relative quantity of mRNA expression in PAP samples relative to healthy control values or in GM-CSF-treated samples relative to untreated.

2.4. Gene array

Analysis was carried out by the Gene Expression Array Core Facility at the Comprehensive Cancer Center at Case Western Reserve University. Specimens of total RNA extracted from three PAP and three healthy control BAL specimens were analyzed using the Affymetrix HG-U133A chip. Amplified labeled cRNA was generated from 5–8 μg of total RNA by SuperScript kit (Invitrogen, Carlsbad, CA) and a High Yield RNA transcription labeling kit which was hybridized to arrays according to the manufacturer's instructions. Data were analyzed by the MICROARRAY suite 5.0 software (Affymetrix, Santa Clara, CA) in which PAP patient values were compared to healthy controls.

2.5. ATP assay

Lymphocyte activation was determined by measurement of ATP using CD19 and CD4 magnetic bead assays (Cylex Incorporated, Columbia, MD). Peripheral blood specimens from PAP patients and healthy controls were processed according to the manufacturer's specifications and stimulated with 5 μg/ml PHA in the presence and absence of 0.01 μg/ml human activin A (PharMingen, Franklin Lakes, NJ). Neutralization studies were done with 3.0 μg/ml goat anti-human Activin Receptor IIA (R&D Systems, Minneapolis, MN) or irrelevant goat IgG. Cells were incubated for 18 h prior to the addition of either CD19 or CD4 magnetic beads. After 30 min bead/cell preparations were lysed and evaluated with a proprietary luminescence reagent on a luminometer with CD19 or CD4 specific ATP calibrators. Additional samples of peripheral blood mononuclear cells (PBMC) exposed to activin A were examined for viability by trypan blue exclusion.

2.6. Activin A and follistatin ELISA assays

Levels of activin A and follistatin in BAL fluids or cultured BAL cell conditioned media were determined by ELISA assays (Serotec, Raleigh, NC; R&D, Minneapolis, MN). Briefly, supernatants and BAL fluids were pre-treated with SDS, heated to 100 °C for 3 min to dissociate activin from follistatin, then neutralized with hydrogen peroxide. Samples together with assay diluent were added to anti-activin or anti-follistatin coated microtiter plates followed by biotinylated monoclonal antibodies. The plates were incubated overnight at room temperature and bound protein was detected with streptavidin-alkaline phosphatase at 492 nm.

2.7. Anti-GM-CSF autoantibody determination

The presence of anti-GM-CSF was determined using a microparticle based assay developed by our laboratory and described in detail elsewhere [20]. Briefly, PBMCs were collected from CPT tubes (Becton Dickinson) and cultured for either 4 or 7 days in the presence and absence of 5 μg/ml pokeweed mitogen (PWM). Supernatants were incubated with beads coupled with 25 μg/ml human GM-CSF. Captured antibody was detected by biotin-labeled, goat anti-human Ig. Strepavidin-phycoerythrin was used as a conjugate followed by evaluation in a Luminex (Austin, TX) instrument.

2.8. Statistics

Data were analyzed by one-way analysis of variance (ANOVA) and Student's *t*-test using Prism software (GraphPad, Inc., San Diego, CA.). Significance was defined as $p \leq 0.05$.

3. Results

3.1. Activin A is deficient in PAP

Global analysis of RNA from three PAP and three healthy control BAL specimens was carried out to search for autoimmune-related genes. Activin A was found to be dramatically decreased in PAP. Analysis of gene array data indicated that healthy control BAL expressed significantly ($p = 0.001$) more activin (1370 ± SEM fold higher) than PAP BAL. Further evaluation of RNA using real-time PCR validated activin A data obtained by gene array (Fig. 1A, $n = 3$, $p = 0.0001$). Follistatin, an inhibitor of activin A, was not found to be different in PAP compared to controls.

To examine activin A secretion, PAP and control alveolar macrophages were cultured in vitro with and without GM-CSF. Activin A was constitutively secreted by control alveolar macrophages ($n = 3$) at levels significantly higher ($p = 0.02$) than those of PAP macrophages ($n = 4$) (Fig. 1B). In both control and PAP macrophages, however, activin A production increased in response to GM-CSF treatment. Analysis of BAL fluids also indicated significantly lower ($p = 0.009$; $n = 5$) levels of activin A in PAP compared to healthy controls (Fig. 1C). Follistatin was not detectable in BAL fluids from either PAP or healthy controls.

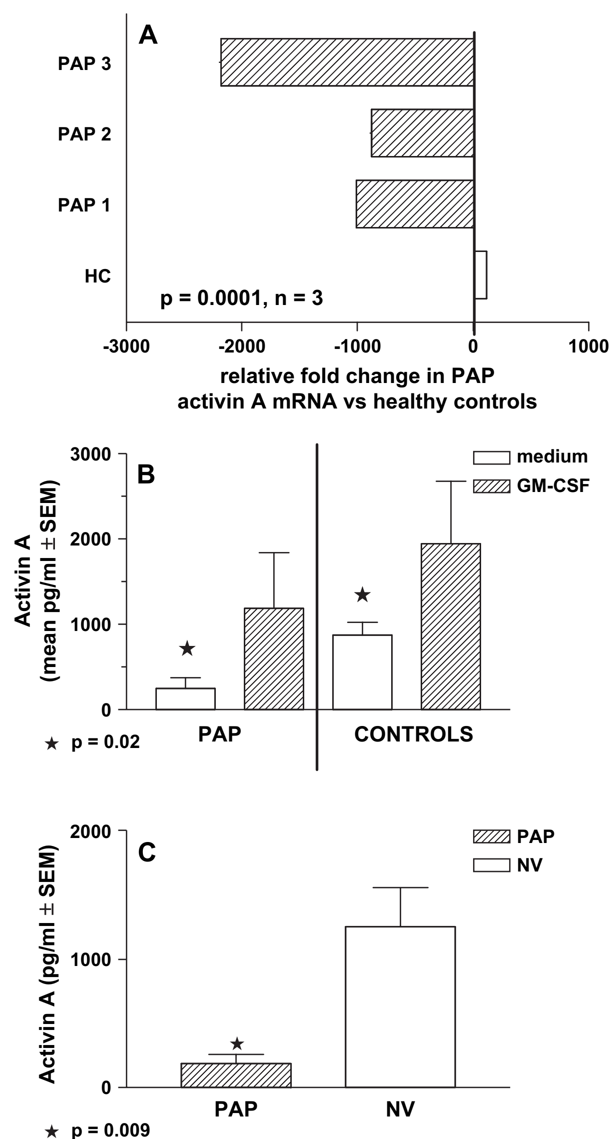

Fig. 1. (A) Activin A mRNA expression is severely depressed in PAP. Total RNA samples from PAP ($n = 3$) and healthy control ($n = 3$) BAL cells were evaluated for activin A expression by quantitative PCR. Activin A mRNA was significantly less in PAP ($p = 0.0001$) than in controls. (B) Activin A secretion is reduced in PAP. Supernatants from PAP ($n = 4$) and healthy control ($n = 3$) alveolar macrophages cultured in the presence and absence of GM-CSF were evaluated for activin A protein by ELISA. Constitutive secretion of activin A was significantly less ($p = 0.02$) in PAP than in healthy controls. GM-CSF stimulated activin A secretion in both PAP and control alveolar macrophages. (C) Activin A is decreased in PAP BAL fluid. BAL fluid from PAP patients ($n = 5$) contained significantly less ($p = 0.009$) activin A protein than healthy control BAL fluid ($n = 5$) as evaluated by ELISA assay.

3.2. Activin A suppresses PAP B-cell proliferation and autoantibody production

Utilizing ATP as an indicator of proliferative activity, PAP and healthy control B-cells demonstrated comparable and significant proliferative activity in response to the mitogen, PHA (Fig. 2A, $n = 3$, $p = 0.001$). Addition of activin A, however, significantly suppressed PHA-stimulated B-cell proliferation in both PAP and healthy controls ($p = 0.026$) (Fig. 2A). Conversely, the proliferative effect of PHA on CD4 proliferation was not altered by the addition of activin A (data not shown). Activin A-mediated suppression was not due to toxicity since 18-h cultured PBMC remained viable as determined by trypan blue exclusion. Polyclonal antibody directed against the activin A RII receptor significantly ($p = 0.021$) blocked activin A suppression of B-cell proliferation, whereas irrelevant polyclonal IgG had no effect on activin A suppression (Fig. 2B; $n = 4$). PAP PBMC cultured with PWM also secreted anti-GM-CSF autoantibody which was significantly ($p = 0.013$) reduced in the presence of activin A (Fig. 2C). Anti-GM-CSF was not detectable in the absence of PWM.

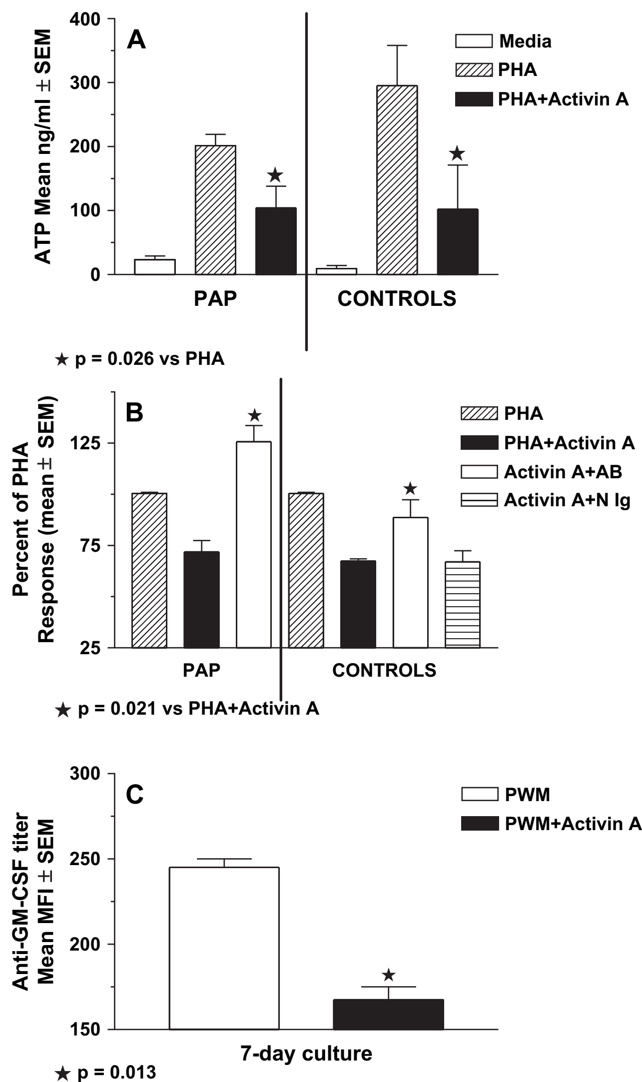

Fig. 2. (A) Activin A suppresses B-cell proliferation. Peripheral blood CD19(+) B-cells from PAP patients ($n = 3$) and healthy controls ($n = 3$) were cultured in the presence and absence of PHA with or without activin A. B-cell proliferation was evaluated by ATP generation. PHA elicited significant and comparable proliferative activity in PAP and healthy control B-cells ($p = 0.001$). Activin A suppressed both PAP and healthy control B-cell proliferation ($p = 0.026$). (B) Antibody to activin A receptor blocks activin A suppression. Activin A suppression of PAP and control B-cell proliferation was blocked by antibody to the activin A RII ($n = 4$, $p = 0.021$) but not by nonspecific polyclonal IgG. (C) Activin A suppresses autoantibody secretion. PBMC from a PAP patient were cultured for 7 days with and without PWM and activin A and anti-GM-CSF autoantibody was determined in conditioned media. In the absence of PWM, no anti-GM-CSF was detected. Anti-GM-CSF secreted in response to PWM (shown as mean fluorescence intensity; [MFI]) was significantly ($p = 0.013$; $n = 2$ replicates) suppressed by activin A.

4. Discussion

Data presented here are the first to demonstrate a deficiency of activin A in autoimmune disease and to show suppression of autoantibody by activin A in vitro. The constitutive expression of activin A mRNA and secretion of activin A protein by healthy human alveolar macrophages is also highlighted by current results. In PAP, an autoimmune lung disease characterized by high levels of circulating autoantibody to GM-CSF, patient alveolar macrophages show a marked deficiency in activin A gene and protein expression compared to healthy controls. Like healthy controls, however, PAP patients are capable of responding to activin A with reduced B lymphocyte proliferation. This reactivity is receptor-dependent as shown by blockade with anti-receptor antibody. Further, PAP production of anti-GM-CSF autoantibody is also suppressed by activin A. Thus PAP patients express activin A RII receptors and are responsive to activin A regulation of B-cell proliferation and antibody secretion.

PAP alveolar macrophages are not intrinsically deficient in activin A but express low levels and are capable of increased production in response to GM-CSF stimulation. Follistatin, a soluble protein that binds to activin A with high affinity and inhibits biological effects [5], is not elevated in PAP and thus cannot account for low activin A levels. Activin A deficiency in PAP however, may be related to the co-existing functional deficiency of GM-CSF caused by high titer neutralizing autoantibodies to GM-CSF. In turn, activin A deficiency may permit continued B-cell expansion and autoantibody production. Among the major producers of activin A in the lung are monocytes, macrophages, mast cells, and epithelial cells [3,6,21,22]. Whether activin A production is equally affected in the various pulmonary cell types remains to be determined.

Of interest to our studies of PAP is the observation that activin A can reduce formation of macrophage foam cells in vitro [9]. PAP lungs contain Oil-Red-O-positive, ''foamy-appearing'' alveolar macrophages which are unable to catabolize surfactant. The link between activin A deficiency and alveolar macrophage foam cell formation in PAP has not been investigated. Documentation of activin A alteration in pulmonary disease is relatively recent and regulatory mechanisms remain to be determined. The overall pathophysiology of PAP together with our observation of severe activin A deficiency, however, suggests that activin A may be important to disease resolution.

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