Uteroglobin Suppresses SCCA Gene Expression Associated with Allergic Asthma*

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Uteroglobin (UG), the founding member of the Secretoglobin superfamily, is a potent anti-inflammatory protein constitutively expressed at a high level in the airway epithelia of all mammals. We previously reported that the lungs of UG-knock-out (UG-KO) mice express elevated levels of Th2 cytokines (e.g. interleukin (IL)-4 and IL-13), which are augmented by allergen sensitization and challenge leading to exaggerated airway inflammation. Notably, these responses are suppressed by recombinant UG treatment (Mandal, A. K., Zhang, Z., Ray, R., Choi, M. S., Chowdhury, B., Pattabiraman, N., and Mukherjee, A. B. (2004) J. Exp. Med. 198, 1317–1330). Recent reports indicate that human orthologs of murine squamous cell carcinoma antigen-2 (SCCA-2/serpinb3a), a serine protease-inhibitor, are overexpressed in the airways of asthmatic patients. We report here that compared with wild type littermates, UG-KO mouse lungs express markedly elevated levels of SCCA-2 mRNA and protein, which are augmented by allergen-challenge. Most importantly, these effects are abrogated by recombinant UG treatment. We further demonstrate that treatment of cultured human bronchial epithelial cells with IL-4 or IL-13 stimulates phosphorylation of STAT-1 and STAT-6 leading to SCCA-1 (SERPINB3) and SCCA-2 (SERPINB4) gene expression. We propose that: (i) IL-4- and IL-13-stimulated SCCA gene expression is mediated via STAT-1 and STAT-6 activation, and (ii) by suppressing the production, and most likely by interfering with the signaling of these cytokines, UG inhibits SCCA gene expression associated with airway inflammation in asthma.

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The abbreviations used are: UG, uteroglobin; UG-KO, uteroglobin-knock-out; WT, wild type; OVA, ovalbumin; IL, interleukin; HRP, horseradish peroxidase; SCCA, squamous cell carcinoma antigen; HBEC, human bronchial epithelial cells; RT, reverse transcription; STAT, signal transducers and activators of transcription.

and immunomodulatory properties (reviewed in Ref. 1). The founding member of the secretoglobin superfamily of proteins (2), UG, is constitutively expressed at a high level in the pulmonary mucosal epithelial cells of virtually all mammals including mice. We previously reported that compared with wild type (WT) littermates, the lungs of the UG-knock-out (UG-KO) mice express markedly higher levels of Th2 cytokines and manifest exaggerated airway inflammatory response to allergens marked by elevated levels of eosinophil infiltration (3, 4). All of these are characteristically found in human airway inflammatory diseases such as bronchial asthma in which sensitivity to allergens play a critical pathogenic role.

Recently, it has been reported that the expression of squamous cell carcinoma antigens (SCCA), cysteine, and chymotrypsin proteinase inhibitors of the ovalbumin/serpin family (reviewed in Ref. 5; Ref. 6) is markedly elevated in the airway epithelia of patients with bronchial asthma (7). Consistent with these findings, it has also been reported that SCCA protein targets a potent allergen and an extrinsic cysteine proteinase in house-dust mites, which is thought to play a pathogenic role in allergic asthma (8), although the physiological role(s) of SCCA is yet to be defined.

In humans, SCCA-1 and SCCA-2 genes encode cysteine and chymotrypsin proteinase inhibitors, respectively. These genes have also been designated SERPINB3 and SERPINB4, respectively, according to the new nomenclature guidelines (reviewed in Ref. 6). The SCCA locus in the mouse contains 4 genes of which Serpinb3a, also known as SQN-5 and SCCA-2, is clearly related to both SCCA-1 and SCCA-2 in humans. Mouse serpinb3a is both a cysteine- and trypsin-like serine proteinase inhibitor (9), and it is expressed in tissues in which human SCCA-1 and SCCA-2 are also expressed. Thus, Serpinb3a, which is referred to as SCCA-2 in this paper, is the mouse ortholog of human SCCA-1 and SCCA-2 (6).

In the present study, we sought to determine whether: (a) the level of murine SCCA-2 (Serpinb3a) gene expression is elevated in the lungs of UG-KO mice compared with that of WT littermates, (b) OVA sensitization and challenge of UG-KO mice influence the level of SCCA-2 gene expression, and (c) rUG treatment has any effect on SCCA-2 gene expression in the lungs of UG-KO mice. Our results show that compared with WT mice, the lungs of UG-KO mice express markedly elevated levels of SCCA-2 mRNA and protein, which are further augmented by OVA sensitization and challenge. Most importantly, treatment of the OVA-sensitized UG-KO mice with rUG prior to OVA challenge dramatically suppresses the expression of SCCA-2. Using cultured human bronchial epithelial cells, we further demonstrate that both IL-4 and IL-13 stimulate the expression of SCCA-1 and SCCA-2 genes, which are human orthologs of mouse SCCA-2 (10–12) and that IL-4- and IL-13-induced SCCA expression requires STAT-1 and/or STAT-6 phosphorylation. Since UG inhibits IL-4 and IL-13 production (4) and these cytokines stimulate SCCA gene expression, we propose that by down-regulating the expression and signaling of these Th2 cytokines UG inhibits SCCA gene expression associated with allergic asthma.

EXPERIMENTAL PROCEDURES

Materials—Chicken OVA (grade V), protease inhibitor mixture, phosphatase inhibitor mixture, and leflunomide were purchased from Sigma. Human bronchial epithelial cells (HBEC), the small airway

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epithelial growth medium bullet kit, and SABM basal medium were purchased from Bio-Whittaker, Inc. (Walkersville, MD). Human IL-4 and IL-13 were purchased from Peprotech (Rocky Hill, NJ). Antibodies against STAT-1, phospho-Tyr-STAT-1, STAT-6, phospho-Tyr-STAT-6, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibody for human SCCA-1 and SCCA-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pre-cast SDS-polyacrylamide (4–15%) gels were obtained from Bio-Rad.

Mice—UG-KO mice were generated as described previously (12). Both UG-KO and WT mice were maintained under germ-free conditions, and all experiments were performed according to an institutionally approved animal care and use protocol. The methodology for inducing airway inflammation by OVA is previously reported (3, 4).

Human Bronchial Cell Culture and Treatment with IL-4 and IL-13—Normal HBEC were cultured in small airway epithelial growth medium according to the manufacturer’s protocol. Nearly 60–70% confluent cells were washed twice with SABM basal medium and then cultured in the basal medium in presence or absence of human IL-4 (10 ng/ml) and IL-13 (50 ng/ml) for 24 h as described previously (14). To determine the signaling pathway(s) of IL-4- and IL-13-induced SCCA expression the cells were treated with lefunomide (100 μM-250 μM) in the presence and absence of each of these cytokines.

**RESULTS AND DISCUSSION**

In this study, we demonstrate that SCCA-2 gene expression in UG-KO mouse lungs is markedly higher than in those of WT littermates. In addition, we show that allergen (OVA) sensitization and challenge further augments these levels and, most importantly, that rUG treatment suppresses SCCA-2 expression. Furthermore, using HBEC we demonstrate that IL-4 and IL-13 stimulate the expression of both SCCA-1 and SCCA-2 genes (orthologs of murine SCCA-2), and these cytokines mediate SCCA gene expression via STAT-1 and/or STAT-6 activation.

**FIG. 1. SCCA-2 gene is overexpressed in the lungs of UG-KO Mice.** A, expression of SCCA-2 mRNA as determined by quantitative real-time RT-PCR using total RNA from the lungs of WT and UG-KO mice. Results are expressed as the mean of at least three experiments ± S.D. B, SCCA-2 protein was detected by Western blot analysis using total protein extract from the lungs of WT and UG-KO mice. C, immunolocalization of SCCA-2 in the lungs of WT (panel a) and UG-KO (panel c) mice using rabbit anti-SCCA-2 antibody, and preimmune sera were used as controls: WT (panel b) and UG-KO mice (panel d).
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Fig. 2. UG suppresses allergen-induced SCCA-2 expression in the lungs. A, expression of SCCA-2 mRNA was determined by quantitative real-time RT-PCR using total RNA from the lungs of WT, OVA-sensitized and -challenged WT (WT + OVA), UG-KO, OVA-sensitized and -challenged UG-KO (UG-KO + OVA), and OVA-sensitized UG-KO mice pretreated with rUG prior to OVA challenge (UG-KO + OVA + rUG). B, detection of SCCA-2 protein by Western blot analysis using the total protein extract from the lungs of WT, WT + OVA, UG-KO, UG-KO + OVA, and UG-KO + OVA + rUG. C, SCCA-2 immunofluorescence in the lungs of WT (panel a), WT + OVA (panel b), UG-KO (panel c), UG-KO + OVA (panel d), and UG-KO + OVA + rUG (panel e). Magnification: ×400.

Fig. 3. Th2 cytokine-induced SCCA expression and STAT phosphorylation in HBEC. A, detection of IL-4- and IL-13-induced SCCA-1 and SCCA-2 gene expression in HBEC by Western blot analysis. B, detection of IL-4- and IL-13-induced phosphorylation on tyrosine residues of STAT-1 and STAT-6 in HBEC by Western blot analysis.
Since both IL-4 and IL-13 stimulate SCCA-2 gene expression (7), it is likely that by inhibiting the levels of IL-4 and IL-13, UG suppresses SCCA-2 gene expression. However, the mechanism of IL-4- and IL-13-induced SCCA-2 gene expression, until now, remained unclear.

To determine the mechanism(s) by which IL-4 and IL-13 stimulate SCCA-2 expression, we treated HBEC with either IL-4 or IL-13 and determined the production of these transcription factors, STAT-1 and STAT-6, as it has been previously reported that the 5′-regulatory region of the SCCA genes contain a STAT-binding site (11, 12). It has also been reported that both STAT-1 and STAT-6 activation require phosphorylation on tyrosine. Accordingly, we studied tyrosine phosphorylation of STAT-1 and STAT-6 by Western blot analysis. Our results show that both IL-4 and IL-13 treatments of the cells stimulate STAT-1 and STAT-6 phosphorylation on tyrosine without altering the STAT-1 and STAT-6-protein levels (Fig. 3B).

To further confirm that STAT-1 and STAT-6 phosphorylation is essential for IL-4- and IL-13-mediated SCCA expression, we performed experiments in which human bronchial epithelial cells were stimulated with IL-4, IL-13, or a combination of IL-4 and IL-13 in the presence and absence of an inhibitor of tyrosine phosphorylation, leflunomide. Our results show that leflunomide inhibits tyrosine phosphorylation of STAT-1 and STAT-6 induced by IL-4 (Fig. 4A), IL-13 (Fig. 4B), and IL-4 plus IL-13 (Fig. 4C). In a dose-dependent manner. Most importantly, leflunomide-mediated inhibition of IL-4-, IL-13-, and IL-4 + IL-13-induced tyrosine phosphorylation in STAT-1 and STAT-6 leads to the suppression of SCCA-1 and SCCA-2 expression (Fig. 4, A–C). Taken together, our results for the first time demonstrate that IL-4 and IL-13 signaling is mediated via STAT-1 as well as STAT-6 and that tyrosine phosphorylation of these transcription factors is critical for IL-4- and IL-13-induced SCCA gene expression.

Although the physiological role(s) of the SCCA proteins are not yet clear, their association with asthma, an inflammatory disease of the airways, and its overexpression in the lungs of mice lacking UG, an anti-inflammatory protein, may suggest that SCCA gene product(s) may have pro-inflammatory effects.

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