Leptin and leptin receptor expression in asthma

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Background: The adipokine leptin is a potential new mediator for bronchial epithelial homeostasis. Asthma is a chronic inflammatory disease characterized by airway remodeling that might affect disease chronicity and severity. TGF-β is a tissue growth factor the dysregulation of which is associated with airway remodeling.

Objective: We sought to determine whether a bronchial epithelial dysfunction of the leptin/leptin receptor pathway contributes to asthma pathogenesis and severity.

Methods: We investigated *in vitro* the presence of leptin/leptin receptor on human bronchial epithelial cells. Then we studied the effect of TGF-β and fluticasone propionate on leptin receptor expression. Finally, the role of leptin on TGF-β release and cell proliferation was analyzed. *Ex vivo* we investigated the presence of leptin/leptin receptor in the epithelium of bronchial biopsy specimens from subjects with asthma of various severities and from healthy volunteers, and some features of airway remodeling, such as reticular basement membrane (RBM) thickness and TGF-β expression in the epithelium, were assessed.

Results: *In vitro* bronchial epithelial cells express leptin/leptin receptor. TGF-β decreased and fluticasone propionate increased leptin receptor expression, and leptin decreased the spontaneous release of TGF-β and increased cell proliferation. *Ex vivo* the bronchial epithelium of subjects with mild, uncontrolled, untreated asthma showed a decrease expression of leptin and its receptor and an increased RBM thickness and TGF-β expression when compared with values seen in healthy volunteers. Furthermore, severe asthma was associated with a reduced expression of leptin and its receptor and an increased RBM thickness with unaltered TGF-β expression.

Conclusions: Decreased expression of leptin/leptin receptor characterizes severe asthma and is associated with airway remodeling features. (J Allergy Clin Immunol 2009;124:230-7.)

Key words: *Leptin, leptin receptor, severe asthma, epithelium, TGF-β, remodeling*

Leptin, a 16-kd adipocyte-derived hormone originally described in metabolism regulation, plays a pleiotropic role in the immune system and inflammation.[1] Leptin exerts its action through the leptin receptor (Ob-R) by activating both phosphatidylinositol-3-OH kinase and mitogen-activated protein kinase signaling pathways.[2,3] Ob-R belongs to the glycoprotein 130 family of cytokine class I receptors and is present in many tissues, including the brain, placenta, hematopoietic cells, liver, heart, and lung.[4] During skin repair,[5] leptin might function as a regulatory link between the endocrine and immune systems and might represent a bridge between inflammation and tissue repair. It also contributes to regulation of the maturation of fetal lung cells[6] and to homeostasis of the endothelium.[7] Leptin is increased during allergic reactions in the airways and might play a role in the relationship between obesity and asthma.[8] The specific role of the leptin/leptin receptor pathway in the bronchial epithelium from asthmatic patients is still largely unexplored. In patients with asthma, there is increasing evidence for an important role played by the epithelium in orchestrating the inflammatory responses and in producing a chronic wound scenario involving tissue injury and aberrant repair leading to airway remodeling.[9] Airway remodeling has been related to the severity of asthma, and consistently, major tissue changes have been observed in patients with fatal asthma.[10] Subepithelial thickening caused by excessive collagen deposition beneath the reticular basement membrane (RBM) is one of the most frequent features of airway remodeling in patients with asthma.[11-14] TGF-β, a cytokine overexpressed in patients with asthma, is considered one of the major fibrogenic factors. It exists in 3 pleiotropic isoforms that play important roles in the regulation of inflammation, cell growth, differentiation, and wound healing. The expression of TGF-β correlates with the degree of subepithelial changes in patients with asthma,[15] and its production is downregulated by corticosteroids.[16] Inhaled corticosteroids and bronchodilators are the mainstay of treatment in persistent asthma, but they are relatively less effective in the treatment of severe asthma.[17,18] It has been assessed that synthetic dexamethasone stimulates both leptin synthesis in preadipocytes[19] and adipocytes[20] and increases leptin secretion and leptin receptor mRNA expression in choriocarcinoma cells,[21] whereas the addition of TGF-β in the presence of dexamethasone blocks cell numbers in preadipocytes culture.[19]

The present study was performed to investigate both *in vitro* and *ex vivo* the role of the leptin/leptin receptor pathway and its relationship with different aspects of airway remodeling and regarding various aspects of human asthma, including the response to corticosteroids.

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Abbreviations used
BECs: Bronchial epithelial cells from brushings
FP: Fluticasone propionate
C: Healthy control volunteer
ICS: Subject with mild asthma controlled with inhaled corticosteroids
NHBE: Normal human bronchial epithelial
RBM: Reticular basement membrane
SDA: Subject with severe uncontrolled asthma treated with inhaled and oral corticosteroids
UA: Subject with mild uncontrolled untreated asthma

METHODS

This study was approved by the local ethics committee, and all patients provided written informed consent.

Cell cultures

The human bronchial epithelial SV40 immortalized cell line 16HBE 14 o-,[22] primary normal human bronchial epithelial (NHBE) cells (Lonza, Brussels, Belgium), and bronchial epithelial cells (BECs) from bronchial brushings were investigated. The detailed methods for cell-line cultures and immunocytochemistry are described in the Methods section of this article's Online Repository, which is available at www.jacionline.org.

Confocal laser scanning microscopy

Cytospin preparations were created from *in vitro* differentiated NHBE cells, and cells were fixed and permeabilized as previously described.[2,3] Immunofluorescent staining was performed with the rabbit polyclonal antibody Ob anti-leptin (A-20) and the goat-polyclonal antibody Ob-R anti-leptin receptor against the common part of the short and long isoform (M-18; both 1:40 dilution, overnight at 4°C; Santa Cruz Biotechnology, Santa Cruz, Calif). The primary antibodies were diluted in PBS plus 3% BSA plus 2% normal serum. Nonimmune IgG at the same titer (0.25 μg/mL) as the primary antibodies was used as a negative control. Incubation with appropriate tetramethylrhodamine isothiocyanate– and fluorescein isothiocyanate–conjugated secondary antibodies (both from Jackson Immunoresearch, Immunotech, France) was performed in the dark for 1 hour at room temperature. The antifading agent Slowfade (Molecular Probes, Invitrogen, Carlsbad, Calif) was added, and slides were analyzed by using a confocal laser scanning microscope (SP5 Confocal; Leica, Wetzlar, Germany) equipped with Ar and HeNe lasers at a final magnification of ×630.

Flow cytometric analysis for leptin receptor

16HBE cells, NHBE cells, and BECs were analyzed for leptin receptor expression by using FACS analysis. All the cells were incubated in the antibody Ob-R for 1 hour at 4°C. Nonimmune IgG at the same titer (1 μg/mL) as the primary antibody was used as a negative control. Cells were then washed in cold PBS and incubated with fluorescein isothiocyanate–conjugated polyclonal rabbit anti-goat immunoglobulin (DAKO, Glostrup, Denmark) in the dark for 30 minutes at 4°C. Fluorescence-positive cells were quantified by using a FACSCalibur (Becton Dickinson, Mountain View, Calif) flow cytometer. Percentages of positive cells were determined from forward and sideways scatter patterns. Nonspecific binding and background fluorescence were quantified by analyzing the negative control.

16HBE cell treatment

16HBE cells were cultured in the presence or absence of recombinant human TGF-β1 (2 ng/mL) and fluticasone propionate (FP; 10^{-8} M; Glaxo SmithKline, Greenford, United Kingdom) for evaluating leptin receptor expression at 24 hours (n = 6). Furthermore, 16HBE cells were cultured in the presence or absence of recombinant human leptin (0.5 μmol/L) and recombinant human TNF-α (30 ng/mL) for TGF-β1 release. Experiments were performed from 3 to 6 times at 24 and 48 hours. Anti–TGF-β1 mAb was used to neutralize the TGF-β1 activity, and the pharmacologic inhibitors SB203580 (inhibitor of p38 mitogen-activated protein kinase) and LY294002 (inhibitor of phosphatidylinositol-3-OH kinase; both at 50 μmol/L; Sigma, St Louis, Mo) were used to neutralize the leptin activity.[2,3] The cells were cultured with or without leptin (0.5 μmol/L) for evaluating cell proliferation. All cytokines and antibodies are from R&D Systems (Minneapolis, Minn).

TGF-β1 release by 16HBE cells

Supernatants were recovered, centrifuged, and stored at −80°C until assayed. TGF-β1 concentrations were measured with a commercially available ELISA Kit (RD; Amersham-Pharmacia Biotech, Little Chalfont, Bucks, United Kingdom). The sensitivity of the assay was 4.0 pg/mL.

Clonogenic assay in 16HBE cells

The colony growth of 16HBE cells exposed to leptin for 24 hours was assessed. Detailed methods are described in the Methods section of this article's online repository.

Ex vivo study

The patients were consecutively recruited and enrolled over a 6-month period. Four groups of subjects were enrolled in this study: healthy control volunteers (C; n = 15); subjects with mild uncontrolled untreated asthma (UA; n = 8); subjects with mild asthma controlled with inhaled corticosteroids (ICS; n = 8), and subjects with severe uncontrolled asthma treated with inhaled and oral corticosteroids (SDA; n = 15). Asthmatic subjects were selected according to the last Global Initiative for Asthma guidelines.[23] They were classified as having uncontrolled asthma according to their recent Asthma Control Questionnaire score[24] and their exacerbation rate during the last year.[25] The clinical severity of asthma was assessed by using the Global Initiative for Asthma guidelines and based on the anti-inflammatory treatments used to reach control.[18] All patients and healthy volunteers were nonsmokers. Subjects who had any bronchial or respiratory tract infections during the month preceding the test were excluded from the study.

Bronchoscopy and tissue processing and staining

Fiberscopic bronchoscopy was performed by the same physician as previously described.[26] The detailed methods for biopsy treatments are described in the Methods section of this article's Online Repository.

Statistical analysis

Results are expressed as means ± SDs. Unpaired *t* tests for *in vitro* experiments and Kruskal-Wallis tests for *ex vivo* evaluations were performed. A non-parametric Mann Whitney test was then applied between two groups as the initial Kruskal Wallis test was significant. Correlations were determined with a Spearman rank correlation test. A *P* value of less than .05 was considered statistically significant.

RESULTS

Bronchial epithelial cells express leptin/leptin receptor

NHBE cells, BECs, and 16HBE cells constitutively express leptin and leptin receptor by means of immunofluorescence (NHBE cells) and immunocytochemistry (NHBE cells, BECs, and 16HBE cells). The results are representative of 3 experiments in each cell line (Fig 1).

Flow cytometric analysis for leptin receptor

16HBE cells, NHBE cells, and BECs express leptin receptor (Fig 2) by means of FACS analysis. Furthermore, on 16HBE