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Nerve growth factor secretion by human lung epithelial A549 cells in pro- and anti-inflammatory conditions

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Abstract

Nerve growth factor (NGF) has recently been presented as a possible effector of inflammation and bronchial hyperresponsiveness. However, the production of NGF in human airways as well as the regulation of its expression by inflammatory cytokines and glucocorticoids have received little attention. A549 epithelial cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, and starved for 24 h. The effect of the pro-inflammatory cytokine interleukin-1β (1–30 U/ml), and of the glucocorticoid dexamethasone (1 μM) on NGF secretion was studied and quantified by enzyme-linked immunosorbent assay (ELISA). In addition, NGF production within the cells was visualized by immunocytochemistry. Under basal conditions, A549 cells produced and secreted NGF (12.6 ± 2.0 pg/ml). Stimulation by interleukin-1β for 24 h induced a dose-dependent increase in NGF production (maximal at 10 U/ml with $59.6 \pm 3.5\%$ increase, $P < 0.05$). Dexamethasone (1 μM) markedly reduced the constitute NGF secretion by 44.9% (7.0 ± 2.1 pg/ml, $P < 0.001$). In addition, the interleukin-1β-stimulated NGF secretion was inhibited to approximately the same low level (8.5 ± 2.5 pg/ml, $P < 0.001$). In conclusion, we here report that human airway A549 epithelial cells are capable of producing NGF. This production is positively regulated by the pro-inflammatory interleukin-1β, and negatively regulated by dexamethasone.

Keywords: Airway; Glucocorticoid; Dexamethasone; Interleukin-1β; NGF (nerve growth factor); Neurotrophin; Inflammation; Asthma

1. Introduction

The neurotrophin nerve growth factor (NGF) is well known for triggering nerve survival and growth (for review: Levi-Montalcini et al., 1996; Aloe et al., 1997). NGF has also a profound effect on cells of the immune system, many of which are important in the development of bronchial inflammation and asthma-related symptoms (for review: Aloe et al., 1999; Olgart and Frossard, 2000; Olgart and Frossard, in press). For example, NGF modulates immunological and inflammatory processes by increasing the number of mast cells in peripheral tissues (Aloe et al., 1997), by promoting mast cell differentiation (Matsuda et al., 1991), and by enhancing survival and/or activation of mast cells (Bullock and Johnson, 1996), neutrophils (Kannan et al., 1991), lymphocytes (Otten et al., 1989) and eosinophils (Hamada et al., 1996; Solomon et al., 1998). NGF also enhances mediator release from various inflammatory cells including mast cells (for review: Olgart and Frossard, in press).

Recent studies suggest that NGF may play an important role in asthma. Increased levels of the neurotrophin are found in the serum of patients with allergic diseases and asthma (Bonini et al., 1996). Increased levels of NGF protein are also present in the bronchoalveolar lavage fluid of patients after segmental allergen challenge (Virchow et al., 1998). As well, enhanced levels of NGF mRNA are reported in the bronchial mucosa after repeated inhalation of allergen at low dose (Kassel et al., in press). In allergic rhinitis also, increased levels of NGF are found in the nasal lavage fluid after allergenic provocation (Sanico et al., 1999). NGF may be synthesised and secreted by structural cells, in particular fibroblasts (Yoshida et al., 1992; Olgart and Frossard, 2001b) and smooth muscle cells from various origins (Clemow et al., 2000; Ueyama et al., 1993; Freund et al., 2001). However, little is known

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about secretion and regulation of NGF expression by airway epithelial cells.

In the present study, we have hypothesised that A549 epithelial cells may be a source of NGF, and have studied the regulation of NGF secretion by the pro-inflammatory and asthma-associated cytokine, interleukin-1β. Since NGF expression is known to be regulated by glucocorticoids (Emmett et al., 1997; Olgart and Frossard, 2001b) and since glucocorticoids generally resolve airway inflammation in asthma and allergic respiratory diseases, we have also studied the effect of the anti-inflammatory glucocorticoid dexamethasone on the constitutive and interleukin-1β-stimulated secretion of NGF by A549 cells.

2. Material and methods

2.1. Cell culture

A549 human lung epithelial cells (American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium supplemented with F-12 (DMEM/F-12), foetal bovine serum (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml) (all products from Gibco BRL, Cergy Pontoise, France). Culture was carried out in 75-cm^2 flasks (Costar, Cambridge, MA, USA) in a humidified chamber at 37 °C in air containing 5% CO_2 with a medium changed every other day. Confluent cells were split 1:4 using trypsin-EDTA (Gibco BRL) as a dissociating agent. Cells were then replated 1:12 into 25-cm^2 flasks to study interleukin-1β and dexamethasone effect on NGF secretion, or in 24-well plates (Costar) for immunocytochemical studies.

2.2. Experimental procedure

Cells in DMEM/F-12 supplemented with 10% foetal bovine serum were seeded in 25-cm^2 culture flasks or in 24-well plates at a density of 4000 cells/cm^2. After 4 days of culture, cells had reached 80% confluency, and were starved for 24 h in culture medium supplemented with 0.3% foetal bovine serum. The effect of interleukin-1β (1–30 U/ml, Boehringer, Mannheim, Germany), dexamethasone (1 μM, Sigma, St Quentin Fallavier, France) or a combination of both agents was then studied by stimulating the cells for 24 h in a low-foetal bovine serum (0.3%) culture medium. Cell supernatants were then collected, centrifuged (+4 °C, 1300 × *g*, 5 min), and stored at −20 °C until NGF analysis. Cells were rinsed in Hank's balanced salt solution (HBSS), dissociated with trypsin, fixed in 2% paraformaldehyde, and counted using a haemocytometer (Casy® 1, Schärfe System, Germany). Cell viability was assessed using trypan blue dye exclusion. Neither interleukin-1β nor dexamethasone had any cytotoxic effect at the concentrations used. All experiments were performed in duplicate.

2.3. Quantification of NGF protein by enzyme-linked immunosorbent assay (ELISA)

NGF secreted into the supernatant of control and treated A549 cells was quantified with a commercially available NGF-specific, highly sensitive, two-site enzyme-linked immunosorbent assay (ELISA) according to the procedure indicated by the manufacturer (Promega, Madison, WI, USA). Briefly, 96-well immunoplates (Maxisorp™, Nunc, Roskilde, Denmark) were coated with a polyclonal goat anti-human NGF antibody in a coating buffer (25 mM carbonate buffer, pH 9.7). After an overnight incubation at +4 °C, plates were washed with a Tris-buffered saline/Tween®-20 solution (TBST, 20 mM Trizma base, 150 mM NaCl, 0.05% (v/v) Tween®-20, pH 7.6, Sigma Aldrich), and incubated in a blocking buffer (5 × TBST solution with gentamicin as a preservative agent, Sigma Aldrich) for 1 h. The supernatants as well as the standards of recombinant human NGF diluted in the low-foetal bovine serum (0.3%) culture medium were incubated into the wells at 37 °C for at least 6 h, and washed. Rat monoclonal anti-NGF antibody (0.25 μg/ml) was added for an overnight incubation at +4 °C, and washed. Anti-rat horseradish peroxidase-conjugated immunoglobulin G (IgG) was added for a 2.5-h incubation period, followed by a 10-min incubation of the enzyme substrate (3,3′,5,5′-tetramethylbenzidine 0.02% and hydrogen peroxide 0.01%). The colorimetric reaction was stopped after 10 min by adding phosphoric acid (1 M), and the optical density was measured in duplicate at 450 nm. The technique allowed detection of NGF in the range of 3.9 to 500 pg/ml.

2.4. NGF immunocytochemistry

After 24-h starvation, cells grown in 24-well plates were washed with Tris-buffered saline (TBS, pH 7.6), and fixed with acetone–methanol (50:50 v/v). Unspecific protein binding was blocked for 30 min at room temperature, using foetal bovine serum (10% v/v, Gibco BRL) and bovine serum albumin (1% w/v, Sigma Aldrich) prepared in TBS. Cells were then rinsed in TBS, and incubated for 90 min with a rabbit anti-human NGF polyclonal antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were then washed three times in TBS, and incubated for 30 min at room temperature with an anti-rabbit IgG directly coupled to alkaline phosphatase (Dako EnVision System alkaline phosphatase, DAKO, Trappes, France). Cells were then rinsed three times with TBS, incubated with Fast Red TR/Naphtol AS-MX (Sigma Aldrich), and coloration was allowed to develop for 5 min at room temperature and obscurity. Cells were then rinsed three times with TBS, followed by distilled water, and dried at room temperature. They were then mounted in gelatin–glycerol (1:1), and analysed under light microscopy. Negative controls were performed by replacing the primary antibody by a normal rabbit serum containing the same

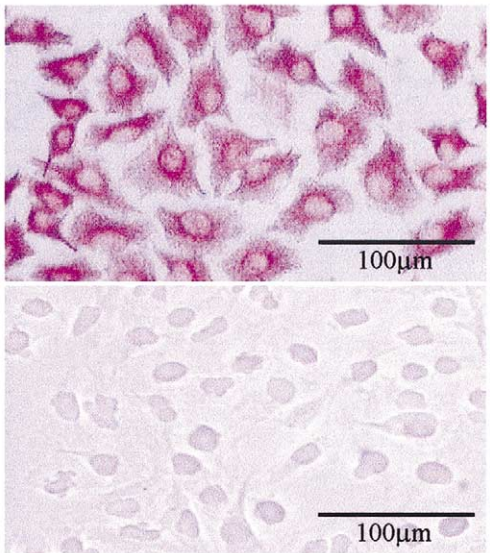

Fig. 1. Immunocytochemical localisation of NGF protein in A549 airway epithelial cells. A representative field of A549 cells stained with a specific human anti-NGF antibody is shown. Magnification: ×400.

rate of IgG, as well as by the blocking buffer alone. All antibodies were diluted in blocking buffer.

2.5. Expression of results and statistical analysis

Data were expressed as a percentage of NGF production in control cultures. They are shown on figures as means ± S.E.M. of $n = 4$ experiments performed in triplicate. Differences between groups were analysed from raw data using a two-way analysis of variance followed by an unpaired two-tailed Student's *t*-test to compare data from treated cells to their respective controls, or by a Student–Newman–Keuls test when more than two variables were compared. Data were considered as significantly different when $P < 0.05$.

3. Results

3.1. NGF secretion under basal culture conditions

A549 cells in culture produced NGF constitutively. A 12.6 ± 2.0 pg/ml NGF protein secretion was measured in culture supernatants after a 24-h period. This constitutive production of NGF was confirmed in immunocytochemical studies, showing NGF-immunoreactive staining in the cytoplasm of A549 cells (Fig. 1).

3.2. Effect of interleukin-1β on NGF secretion

NGF protein secretion was significantly enhanced after a 24-h stimulation with interleukin-1β ($P < 0.05$) (Fig. 2). This effect was dose-dependent (1–30 U/ml). Increase in NGF was maximal at 10 U/ml interleukin-1β (59.6 ± 3.5 % increase over basal secretion).

3.3. Effect of dexamethasone on NGF secretion

Dexamethasone (1 µM) reduced the constitutive secretion of NGF in control cells by 44.9 ± 10.3% ($P < 0.001$) (Fig. 3). It abolished the interleukin-1β-stimulated NGF secretion to the same low levels (45.6 ± 9.7%, $P < 0.001$).

4. Discussion

Our study clearly shows that airway epithelial cells in culture are capable of producing and secreting NGF and that this secretion is increased in response to the pro-inflammatory and asthma-associated cytokine interleukin-1β. The glucocorticoid dexamethasone decreases both the con-

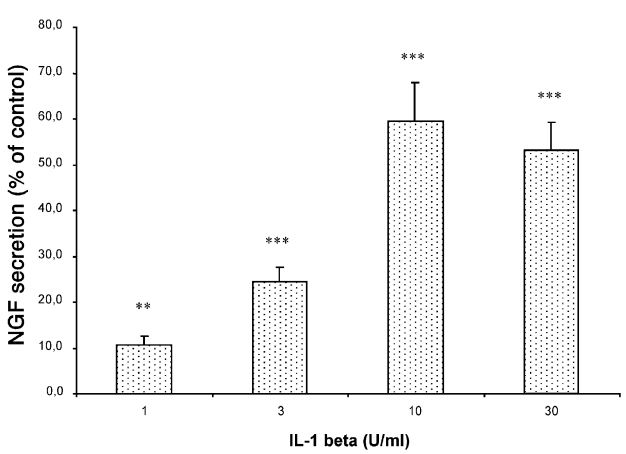

Fig. 2. Dose-dependent effect of interleukin-1β (1–30 U/ml) on NGF secretion by human A549 airway epithelial cells in culture. NGF levels (pg/ml) were assessed by ELISA and expressed as a percentage of control. Values are means ± S.E.M. of $n = 12$ experiments performed in triplicates. $^{**}P < 0.05$, $^{***}P < 0.001$ as compared to control.

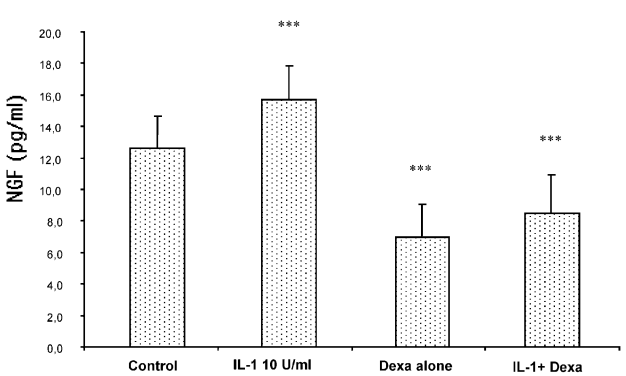

Fig. 3. Effect of dexamethasone (1 µM), of interleukin-1β (10 U/ml), or of a combination of dexamethasone and interleukin-1β on NGF secretion by human A549 airway epithelial cells in culture. NGF levels were assessed by ELISA and expressed in pg/ml of culture supernatant. Values are means ± S.E.M. of $n = 12$ experiments performed in triplicates. Dexamethasone, $^{***}P < 0.001$ as compared to control.

stitutive and the interleukin-1β-induced secretion of NGF by A549 cells.

The demonstration of NGF secretion by the human A549 airway epithelial cells is in agreement with findings showing that structural cells of the lung, such as fibroblasts (Olgart and Frossard, 2001b) or smooth muscle cells (Freund et al., 2001), are capable of producing NGF in vitro. Secretion of NGF by airway epithelial cells in culture has been previously reported by Fox et al. (1997), and is in accordance with NGF immunolabelling of bronchial epithelium in biopsies from asthmatic, as well as of control patients reported by our group (Olgart et al., 2000; Kassel et al., in press). NGF secretion by airway epithelial cells also extends the findings that epithelial cells from various origins are capable of producing NGF, as shown for intestinal epithelial cells (Varilek et al., 1995), amniotic epithelial cells (Uchida et al., 2000), or keratinocytes (Pincelli and Marconi, 2000).

Additionally, our study shows an enhanced secretion of NGF when A549 airway epithelial cells are stimulated with the pro-inflammatory cytokine interleukin-1β. Such effect of interleukin-1β on NGF secretion was reported in other structural cells, either in primary culture including pulmonary fibroblasts (Olgart and Frossard, 2001b) and airway smooth muscle cells (Freund et al., 2001), or in cell lines such as human astrocytoma and glioblastoma (Emmett et al., 1997). Our finding extends the knowledge on the effect of pro-inflammatory cytokines, such as interleukin-1β, capable of stimulating airway epithelial cells to produce other inflammation-associated mediators or growth factors, such as eotaxin (Lilly et al., 1997; Jedrzkiewicz et al., 2000), the regulated upon activation normal T cell-expressed and secreted protein (RANTES) (Terada et al., 1996), or the granulocyte-macrophage colony-stimulating factor (GM-CSF) (Bedard et al., 1993; Hashimoto et al., 2000).

Excessive amounts of interleukin-1β have been shown to play some important inflammatory role in the lung and airways during various pathological conditions, including asthma (Barnes et al., 1998; Tillie-Leblond et al., 1999). Since the dose of interleukin-1β used in this study was within the biological range of action of interleukin-1β (Brodie et al., 1992), increased levels of NGF might occur in inflammatory conditions in the airways. Indeed, enhanced NGF levels have been reported in the serum of patients with inflammatory allergic diseases, particularly in patients with severe asthma (Bonini et al., 1996). Also, enhanced levels of NGF were detected in bronchoalveolar (Undem et al., 1999; Olgart et al., 2000), or nasal (Sanico et al., 1999) lavage fluids from asthmatic or rhinitic patients, respectively, as compared with control subjects, as well as after segmental allergen challenge in asthma (Virchow et al., 1998). Also, an increased expression of NGF mRNA was observed in patients with allergic asthma after repeated inhalation of allergen at low dose (Kassel et al., in press). Therefore, airway epithelial cells, if capable of producing elevated levels of NGF upon stimulation with interleukin-1β as shown in this study, might participate into the enhanced levels of NGF in the inflamed airways.

Because of the known anti-inflammatory effect of corticosteroids, which contributes to suppress inflammation in the lung and airways, we also determined whether dexamethasone was capable of inhibiting NGF secretion by airway epithelial cells, as was observed in human astrocytoma cell lines (Emmett et al., 1997). Both the constitutive and the interleukin-1β-stimulated secretion of NGF were markedly decreased by the glucocorticoid. These findings are in agreement with results of inhibition of the production of several pro-inflammatory factors from airway epithelial cells by corticosteroids, including eotaxin (Lilly et al., 1997), interleukin-6 and interleukin-8 (Ek et al., 1999), GM-CSF (Marini et al., 1991), or RANTES (Berkman et al., 1996). Our data then add a new target to the anti-inflammatory action of glucocorticoids, inhibiting excessive NGF secretion during inflammatory conditions.

In conclusion, our findings show that the human airway A549 epithelial cells are an important source of NGF. The pro-inflammatory and asthma-associated cytokine interleukin-1β enhances this NGF secretion, whereas the anti-inflammatory glucocorticoid dexamethasone reduces the NGF production triggered by interleukin-1β. This result adds some part in the mechanism of the anti-inflammatory action of glucocorticoids, suppressing NGF oversecretion by airway epithelial cells.

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