BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **244,** 126–130 (1998)
ARTICLE NO. RC988223

Interferon-γ Inhibits the Growth of Human Bronchial Epithelial Cells Independently of Transforming Growth Factor-β-1 and Nitric Oxide (NO)

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Received January 30, 1998

It has been emphasized that epithelial injury is closely correlated with airway hyperresponsiveness, which is one of the important pathophysiological characteristics of bronchial asthma. Growth of epithelial cells is important in the mucosal repair processes and is believed to be regulated by growth factors produced by inflammatory and immune effector cells as well as epithelial cells themselves. We studied the role of T cell-derived lymphokines IFNγ and IL-4 on the proliferation of human bronchial epithelial cell line BEAS-2B. IFNγ, but not IL-4, showed a dose-dependent growth inhibitory activity *in vitro*. Its activity was via its specific receptors on the cells, was augmented by TNFα, and was independent of the activity of endogenous TGFβ and nitric oxide. These results suggested that Th-1 T cells-derived lymphokine IFNγ might be involved in the repair processes after mucosal injury found in bronchial asthma.

It has been accepted that airway mucosal injury is closely linked to the airway hyperresponsiveness, which is an important pathophysiological phenomenon found in bronchial asthma(1). Once the epithelial injury occurs, its recovery is largely dependent upon the replication of bronchial epithelial cells(2). It is also known that combinations of cytokines and growth factors play a crucial role in the regulation of proliferation of these cells(3,4). Other cell types in the lung can influence the growth of bronchial epithelial cells, and it is reported that pulmonary macrophages and fibroblasts stimulate cell proliferation of bronchial epithelial cells in vitro(5,6). However, it remains unclear whether or not lymphocytes and their derived factors may have any effect on the replication of airway epithelial cells. IFNγ is a potent lymphokine which is produced by activated T cells, especially Th1 type T cells and plays important roles in the airway inflammatory and immune reactions by antiviral, antiproliferative, immunoregulatory and pro-inflammatory activities(7,8). IFNγ levels are elevated in brochoalveolar lavage fluid (BALF) from patients with asthma(9,10). IFNγ downregulates the induction of IgE secreting B cells(11). It is also a negative growth factor for Th2 lymphocytes(12) and thus counteracts against Th2-mediated allergic reactions. This Th1-derived lymphokine has further activity to inhibit the growth of some nonlymphoid cells and cell lines(13,14,15), but the exact role in the regulation of airway repair is unknown.

In the present study, we investigated the roles of IFNγ in the growth regulation of human bronchial epithelial cell line BEAS-2B, and found that exogenously added IFNγ had an inhibitory effect on the proliferation of BEAS-2B via its specific receptor in vitro. This activity was independent of that of TGFβ and production of nitric oxide(NO) by these cells. We also studied the effect of IL-4, one of Th2-derived cytokine, on proliferation of these cells.

MATERIALS AND METHODS

Cytokines and antibodies. Recombinant human IFNγ, TGF-β1, IL-1α, IL-1β, TNF-α and IL-4 were purchased from INTERGEN COMPANY (NY, USA). Monoclonal mouse anti-human IFNγ receptor blocking antibody (IgG) was obtained from Genzyme, MA. Polyclonal mouse anti-TGFβ blocking antibody was from R&D Systems, Inc., Minneapolis, MN(16).

Cell culture. A human bronchial epithelial cell line BEAS-2B (17)(a kind gift from Dr. J.F. Lechner and Dr. C.C. Harris, National Cancer Institute, Bethesda, MA) was cultured on collagen-coated tissue culture plates (Iwaki, Tokyo, Japan). BEAS-2B cells were routinely propagated in monolayer culture in humidified incubator at 37°C in a 5% CO_2/95% air atomosphere. Cells were grown in hormonally supplemented Ham's F-12 medium (HD-F12) as reported previously(16). HD-F12 contained 1% penicillin-streptomycin, 5 μg/ml insulin (GIBCO), 5 μg/ml transferrin (GIBCO), 25 ng/ml epidermal

0006-291X/98 $25.00

growth factor (Collaborative Research Corp., Lexington, MA), 15 μg/ml endothelial cell growth supplement (Collaborative Research Corp.), 2×10^{-10}M triiodothyronin (GIBCO), and 10^{-7}M hydrocortisone (GIBCO)(16,17). Immunohistochemical analysis had shown that these cells were positive to keratin stain, but negative to vimentin, showing that they were epithelial cells(data not shown).

Cytotoxicity of interferon-γ. We first examined the cytotoxicity of IFNγ to BEAS-2B cells. The BEAS-2B were cultured in 24-well collagen-coated tissue culture plates until confluency. Different concentrations of IFNγ(0.1 - 100ng/ml) were added and incubated for 18hrs. Then, the cells were treated with 0.25% trypsin-EDTA, and cell viability was determined by trypan blue dye exclusion test. We also evaluated the cytotoxicity of IFNγ to BEAS-2B cells by measuring lactic dehydrogenase (LDH) activity in the supernatants from IFNγ-treated BEAS-2B cells to show no significant changes in comparison to controls(data not shown).

Direct enumeration of epithelial cells. BEAS-2B cells (2×10^4cells/well) were plated onto each well of 24 well collagen-coated plates (Iwaki, Tokyo, Japan) with 2 ml of Ham's F-12 medium. Cells were incubated in the absence or presence of human IFNγ for 7 days. On day 3 and day 5, the same fresh medium was changed. On days 3, 5 and 7, 500μl trypsin EDTA (0.25%) was added to each well to detach the cells. After 7 minutes' incubation period, 1500μl of RPMI with 10% FCS was added to each well. Then the cells were harvested and centrifuged at 1000 cpm for 10 min, and stained with trypan blue for cell counting. The cell viability was more than 90% in all samples.

Colorimetric MTT assay. For the evaluation of the effect of IFNγ and other growth factors and cytokines (TGF-β, IL-1α, IL-1β, TNF-α and IL-4) on the growth kinetics of human bronchial epithelial cells, a colorimetric 3-4,5-(dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Boehringer, Germany) was also used as previously reported(16). Briefly, the BEAS-2B cells were plated on 96-well collagen-coated tissue culture plates (Iwaki, Tokyo, Japan) at the density of 0.5×10^4 cells/well in 200μl media. The cells were cultured for 7 days at 37°C, 5% CO2 in the absence or presence of IFNγ and other cytokines. On day 3 and day 5, the same fresh medium was changed. On day 7, the cells were treated with 20μl of the MTT labeling reagent, and incubated for 4 hours at 37°C to yield a dark blue formazan product. Then isopropylalcohol/HCl mixture was added to each well. Absorbance at 650 nm was calculated by an automatic ELISA reader (Minato, Tokyo, Japan). The data were expressed in percentage when the absorbance of the cells cultured in control media was calculated as 100%.

Effect of anti-IFNγ receptor antibodies on cell growth. We evaluated the effect of monoclonal anti-IFNγ receptor antibodies on the activity of IFNγ by using a colorimetric MTT assay as described above. The cells were preincubated for 6 hours with various concentrations of anti-IFNγ receptor antibodies. After preincubation period, 50ng/ml of IFNγ was added to each well. Then the cells were cultured for 7 days, and the growth was examined by using MTT assay. The data were expressed in percentage when the absorbance of the cells cultured in HD-F12 media was calculated as 100%.

Effect of anti-TGFβ blocking antibody and inducible NO synthase inhibitor L-NMMA. To determine if the effect of IFNγ was via the activity of endogenously produced TGFβ (16) and/or NO(18) by the epithelial cells, the cells were treated with anti-TGFβ blocking antibody or NO synthase inhibitor L-NMMA (Sigma Chemicals, St. Louis, MO), and then 50ng/ml of IFNγ was added. The growth of the cells were evaluated by MTT assay after 7 days.

Statistical analysis. Statistical analyses were carried out by using ANOVA test for multiple comparison of the data.

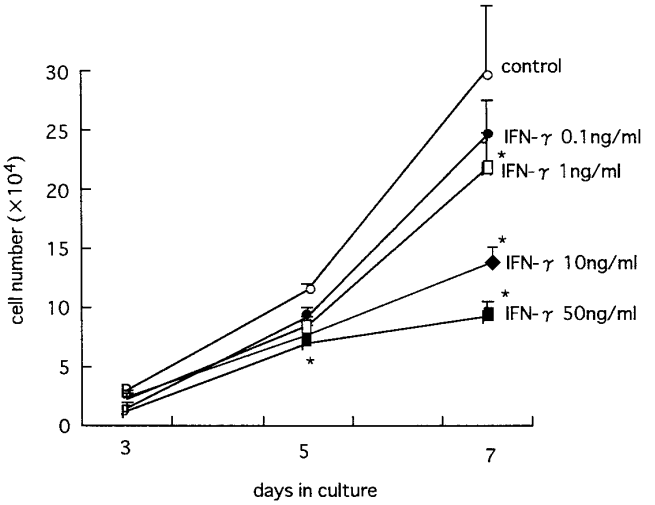

FIG. 1. Human recombinant IFN-γ inhibited the growth of BEAS-2B cells in culture. IFN-γ (0.1-50ng/ml) was added and the effect on cell growth was evaluated by direct counting. IFN-γ showed a dose dependent inhibitory effect on cell proliferation of BEAS-2B cells. The horizontal axis, days in culture; ordinate, actual number of the cells. *$p<0.05$ vs. controls.

RESULTS

Growth Inhibitory Effect of IFNγ on Human Bronchial Epithelial Cells

Exogenously added human recombinant IFNγ inhibited the growth of BEAS-2B cells as shown by counting the cell number(Fig.1). IFNγ inhibited the growth of human bronchial epithelial cells in a dose dependent manner. The effect of IFNγ was not cytotoxic, since cell viability as assessed by trypan blue dye exclusion and LDH release assay showed no significant changes as compared to controls(data not shown). In MTT assay, IFNγ also inhibited the proliferation of BEAS-2B in a dose-dependent fashion when assayed on day 7(Fig.2).

Effect of Anti-IFNγ Receptor Antibodies on IFNγ-Induced Growth Inhibition

To elucidate if IFNγ exerted its effect via its specific receptors on human bronchial epithelial cells in vitro, we evaluated the influence of anti IFNγ receptor antibodies on the growth inhibition by IFNγ.

The preincubation of BEAS-2B with anti-IFNγ receptor antibodies (at the concentration of 0.625, 1.25 and 2.50μg/ml) showed a significant abolishment of the inhibitory activity($p<0.05$)(Fig. 3). Such abolishment was not found in mouse IgG(5μg/ml), suggesting that the inhibitory activity of IFNγ was via the IFNγ receptor on these cells.

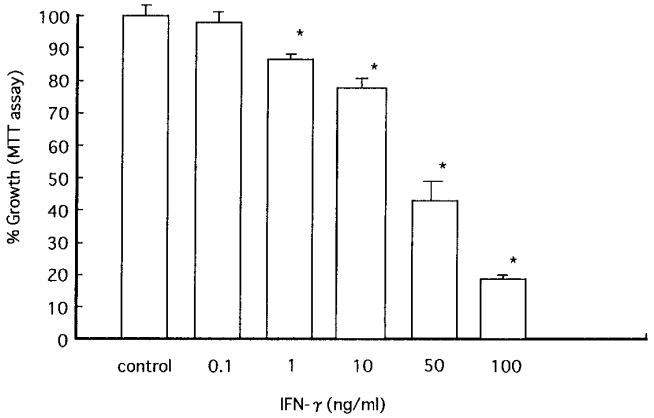

FIG. 2. Effect of IFN-γ on the growth of BEAS-2B cells assessed by colorimetric MTT test. IFN-γ showed a dose-dependent growth inhibitory effect of BEAS-2B cells in culture. Abscissa, the concentration of IFN-γ added to the cells; ordinate, percentage of growth when the control (Ham's F12 medium only) was calculated as 100%. *$p<0.05$.

Effect of TGF β, IL-1α, IL-1β, TNFα, and IL-4 on the Proliferation of Human Bronchial Epithelial Cells

We also examined the effect of other cytokines including human recombinant TGF β-1, IL-1α, IL-1β, TNFα and IL-4 on the proliferation of human bronchial epithelial cells. As previously described, BEAS-2B cells were cultured in the absence or presence of various concentrations of TGF β-1, IL-1α, IL-1β, TNFα or IL-4 for 7 days. On day 7, we examined the proliferation of human bronchial epithelial cells by using MTT assay. As shown in Fig. 4, the addition of IL-1α, IL-1β, TNFα or IL-4 showed no significant effect on the proliferation of BEAS-2B cells. In contrast, TGFβ-1 showed a dose-dependent inhibitory effect as reported previously(16).

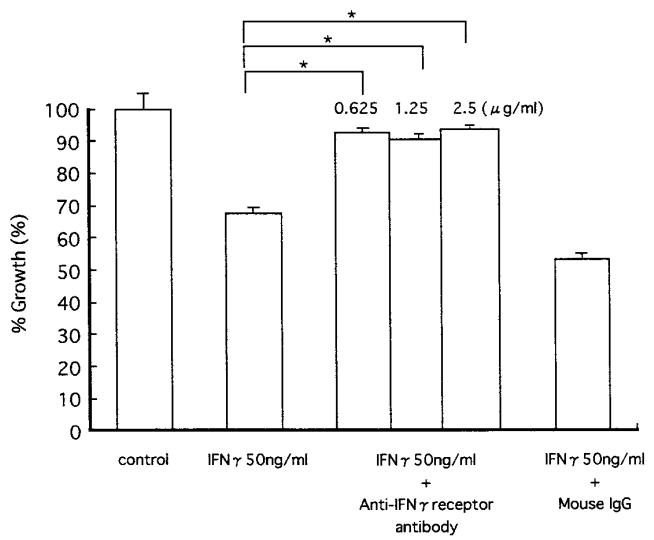

FIG. 3. Effect of preincubation with anti-IFNγ receptor antibody on inhibitory effect of IFNγ. Specific monoclonal anti-IFNγ receptor antibody abolished the activity of IFN γ, whereas control mouse IgG had no such effect. *$p<0.0001$ vs. controls.

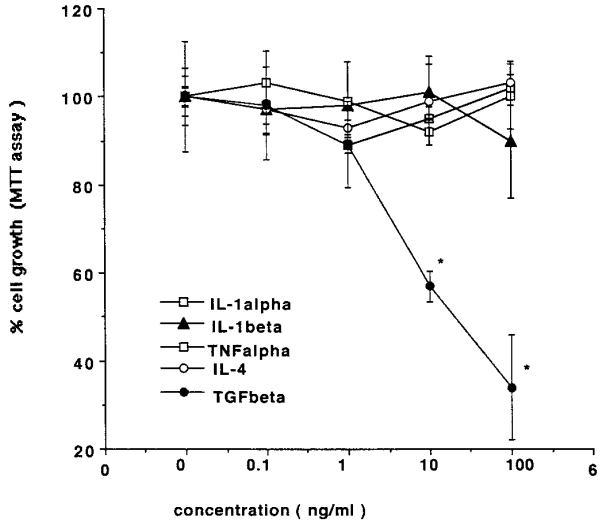

FIG. 4. Effect of a variety of cytokines and growth factors on the growth of BEAS-2B cells assessed by colorimetric MTT test. Human recombinant IL-1α, IL-1β, TNF-α or IL-4 showed no significant effect on cell growth of BEAS-2B. However, human recombinant TGF-β inhibited the cell proliferation of BEAS-2B cells in a dose-dependent manner. *$p<0.0001$ vs. controls.

In some experiments, we also evaluated the effect of combinations of growth factors. When the cells were treated with TGFβ-1 and IFNγ, the effect was only additive(data not shown). When the cells were treated with TNFβ followed by IFNγ, TNFα significantly augmented the inhibitory effect of IFNγ(Fig.5). Flow cytometric analysis (Epics V, Coulter Corp., Hialeah, FL) with anti-IFNγ receptor antibodies showed that TNFα(10ng/ml) induced a 65.5 $\pm$ 10.2% increase in the mean fluorescence intensity (n=3), whereas IL-1α or IL-1β showed no such effect. Therefore, TNFα showed a selective increasing effect on IFNγ receptor density on the epithelial cells.

Effect of Anti-TGFβ-1 Antibodies on IFNγ-Induced Growth Inhibition

To determine if IFNγ-induced growth inhibition was via the effect of endogenously secreted active form of TGFβ-1, neutralizing anti-TGFβ-1 antibodies(16) were added before the treatment of IFNγ. The results showed that anti-TGFβ-1 antibodies had no effect on the growth-inhibitory effect of IFNγ(Table 1).

Effect of NOS Inhibitor L-NMMA on IFNγ-Induced Growth Inhibition

To determine the role of NO on IFNγ-induced growth inhibition, a specific NOS inhibitor L-NMMA was

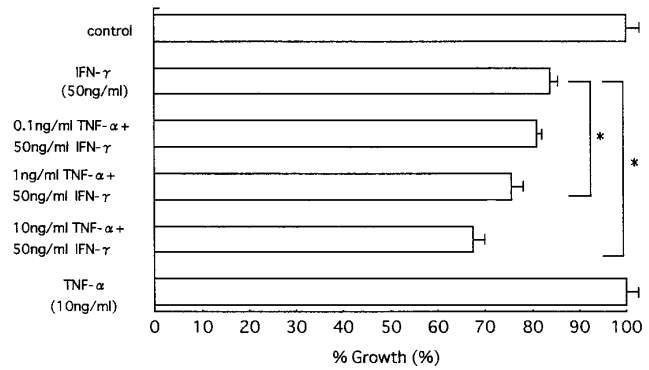

FIG. 5. Effect of combination of IFNγ and TNFα on growth of BEAS-2B cells. Pretreatment with TNFα followed by IFNγ significantly potentiated the inhibitory activity of IFNγ on the cell proliferation. *$p<0.01$ vs. 50ng/ml of IFNγ alone.

added to the epithelial cells. As shown in Table 1, the agent showed no significant effect on growth inhibition.

DISCUSSION

In the present report, we demonstrated that IFNγ had the inhibitory effect on the proliferation of human bronchial epithelial cells under a variety of conditions *in vitro*. This effect was via its specific receptors on the cells, and was augmented by the pretreatment of TNFα, which induced an increase in the receptor density on the cells.

Several lines of evidence have suggested that IFNγ was involved in the pathogenesis of airway inflammatory disorders. It induced bronchial epithelial cells to express major histocompatibility complex (MHC) class II antigen as well as MHC class I antigen, and intercellular adhesion molecule-1 (ICAM-1)(19,20,21), suggesting a role for cell activation and adhesion. IFNγ also increased the amount of prostaglandin E2 release in human bronchial epithelial cells(22). Bruinier and co-workers(23) found that lung T cells showed an increased adherence to lung epithelial cells by IFNγ. Taken together, IFNγ plays an important role in the pathogenesis of airway disorders such as asthma.

However, it remains unclear if this lymphokine plays any role in the epithelial repair. IFNγ has been reported to suppress the growth of epithelial and carcinoma cells such as melanoma tumor cells(13), human colorectal carinoma cell lines(14), human colon carcinoma cell line(15), human keratinocytes(24), and human salivary gland epithelial cell line(25). Although the precise mechanisms of how this lymphokine acts on cell cycles remain unclear, recent reports suggested that NO might be involved in cell growth arrest(26,27). Since, NO synthase (NOS) gene expression and NO release were increased in the pulmonary epithelial cells by IFNγ(28), we studied the effect of NOS inhibitor on the effect of IFNγ. We also speculated that the effect might be via an endogenously produced TGFβ, a well documented growth inhibiting factor of epithelial cells. The results, however, showed no evidence supporting this hypothesis. Raitano et al(14) reported that IFNγ showed the growth inhibition in a human colorectal carinoma cell line and its effect was enhanced by IL-1. They showed that IL-1 upregulated the expression of IFNγ receptors on the cells. The augmenting effect of TNFα on IFNγ activity in the present report appeared to be due to the similar mechanism.

In asthmatic patients, there was an increase in the numbers of cells expressing mRNA for IL-4 and IL-5, but not IL-2 or IFNγ(29). However, increased levels of IFNγ were found in BAL fluid of asthmatic patients after segmental allergen challenge, suggesting that not only Th2 cells but also Th1 cells were activated after allergen instillation(30). Recent reports suggested that airway hyperresponsiveness was dependent on IFNγ , but not on eosinophils in vivo(31). Our present findings suggested that Th-1 T cells-derived IFNγ, but not Th-2 cytokine IL-4, might be involved in the regulation of repair processes in the airway mucosa.

Because the BEAS-2B cell line is transformed by SV-40/adenovirus-hybrids(17), the exact effect of IFNγ on respiratory epithelial cell proliferation should be further confirmed by non-virally-transformed cell lines such as HS24 or normal bronchial epithelial cells.

In conclusion, we found that human IFNγ showed an inhibitory action on the proliferation of human bronchial epithelial cells independently of the action of TGFβ and NO production, and that its efffect might be important to understanding the mechanism of mucosal repair in the airways.

TABLE 1

Effect of Anti-TGFβ Antibody and L-NMMA on IFNγ (50ng/ml)-Induced Growth Inhibition of BEAS-2B Cells

	% growth (MTT assay, IFN-treated cells = 100%)
anti-TGFβ antibody (μg/ml) (n = 3)	
0	100
1	98.1 ± 11.2$^{n.s.}$
10	105 ± 9.81$^{n.s.}$
L-NMMA (μM) (n = 3)	
0	100
1	94.6 ± 10.8$^{n.s.}$
10	99.0 ± 21.5$^{n.s.}$
100	90.9 ± 22.9$^{n.s.}$

n.s. not significant ($p > 0.05$) as compared to control (ANOVA)

ACKNOWLEDGMENTS

This work was supported in part by the Grant from Adult Diseases Memorial Foundation, from the Ministry of Education, Science and Culture, and Manabe Medical Foundation.

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