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Modulation of Ciliary Activity by Tumor Necrosis Factor-alpha in Cultured Sinus Epithelial Cells. Possible Roles of Nitric Oxide.

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ABSTRACT

The primary function of well-differentiated ciliated epithelium in the paranasal sinus is to eliminate harmful agents through the beating action of cilia. Respiratory epithelium also contributes to local inflammatory processes through the release of various proinflammatory cytokines. Recently, considerable attention has been focused on the intimate relationship between the cytokine-dependent regulation of the ciliary beat frequency (CBF) and intra-cellular production of nitric oxide (NO) in ciliated epithelial cells. The aims of this study are to examine the effect of tumor necrosis factor-alpha (TNF-α), one of the major proinflammatory cytokines, on the ciliary activity of human sinus epithelial cells and to assess the hypothesis that NO is involved in this regulatory mechanism. Human maxillary or ethmoidal sinus mucosa (n=23) were cultured by the explant-outgrowth method. CBF of the outgrowth ciliated cells was measured by the photoelectrical method before and after being treated with TNF-α (0.1, 1 and 10 ng/ml) or dexamethasone (10^{-6}M and 10^{-7}M). We also investigated the expression of nitric oxide synthase (NOS) isoforms, enzymes responsible for NO synthesis, by fluorescent immunohistochemistry. TNF-α increased CBF at relatively low concentrations (0.1 and 1 ng/ml) and decreased CBF at a high concentration (10 ng/ml). Dexamethasone decreased CBF at a concentration of 10^{-6}M. Fluorescent immunohistochemistry demonstrated that the expression of inducible NOS was augmented by TNF-α and attenuated by dexamethasone, whereas that of endothelial NOS remained unchanged. We conclude that human sinus epithelial cells potentially contribute to the inflammatory process by regulating their ciliary motility through an NO-dependent pathway. Proinflammatory cytokines and steroids are able to modulate this mechanism by the induction or inhibition of expression of different NOS isoforms.

Key words: *Tumor necrosis factor alpha, Nitric oxide, Ciliary beat frequency, Laser scanning confocal microscopy*

It is well appreciated that the nasal and paranasal epithelia play an important role as a physical barrier in the protection of underlying tissue as well as maintaining the mucociliary clearance system against the harmful environment. In addition, recent evidence suggests that the epithelium contributes to airway inflammation through the release of cytokines such as interleukin-1 beta (IL-1β), IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-alpha (TNF-α)[1]. Among them, TNF-α is one of the most important cytokines for its multi-functions produced by various inflammatory cells including macrophages, lymphocytes, neutrophils and mast cells[21]. In airway inflammation, TNF-α induces the influx of inflammatory cells into the airway mucosa by increasing the expression of adhesion molecules in vascular endothelial cells. The inflammatory cells infiltrated in the airway mucosa can be also activated by the local presence of TNF-α and release a variety of other cytokines such as IL-1, platelet activating factor[3], and TNF-α itself[21]. In addition, surface epithelial cells are influenced by TNF-α and modulate airway functions. Cromwell et al showed that TNF-α stimulated cytokines/chemokines releasing such as IL-8, IL-6, and GM-CSF, from bronchial epithelial cells[4]. The effect of TNF-α on the ciliary activity of bovine tracheal epithelial cells has also been reported. The effect appears dependent on the release of nitric oxide (NO) inside the ciliated cell[11]. In this sense, the significance of NO as a second messenger for intracellular signal transduction as well as the induction of NO synthase (NOS) should be emphasized.

Recently, considerable attention has been

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focused on the intimate relationship between NO and airway epithelial cells[2,6–13,17,18]. For example, NOS isoforms were identified in human lung[2] and human nasal epithelial cells[7]. In addition, Lundberg et al reported that the surface epithelium in human paranasal sinuses was the major site of NO production[13]. NO has diverse effects on various human organ systems. In the airway events, NO plays a pivotal role in regulating pulmonary vascular tone, surveying immune responses and modulating mucociliary clearance through the effect on ciliary beat frequency (CBF)[20]. The purpose of this study is, therefore, to investigate the effect of TNF-α on CBF in human sinus epithelial cells and to assess the hypothesis that NO may be involved in this regulatory mechanism. For this purpose, we have developed a primary cell culture system by means of explant-outgrowth methods. CBF measurement of a single ciliated cell in the outgrowth sheet was performed by the photo-electrical method. We also examined the effect of dexamethasone, since glucocorticoids reduce NO production by inhibiting the expression of inducible nitric oxide synthase (iNOS) in various mammalian cells[17,18]. A laser scanning confocal microscope (LSCM) was employed to detect the immunoreactivity of fluorescence-labeled antibodies against different NOS isoforms.

MATERIALS AND METHODS

Preparation and culture of sinus epithelial cells

Maxillary or ethmoidal sinus mucosa were obtained from 23 patients (10 males and 13 females, mean age 48 years old) at the time of endoscopic sinus surgery for sinus diseases. All patients gave their informed consent and allowed their participation in.

The specimens obtained were immediately immersed in ice-cold physiological salt solution and transported to the laboratory. Sinus cells were cultured by using the explant-outgrowth culture technique according to the method described by Devalia et al[5]. Briefly, every specimen was rinsed three times in Hanks' balanced salt solution (HBSS) to remove blood and debris, and was then dissected into smaller sections of approximately 2 × 2 mm in size. The sectioned mucosa were harvested as explants onto sterile 35-mm plastic culture dishes precoated with poly-L-lysine (Iwaki, Scitech DIV, Japan). A small amount of 500 µl culture medium was added to enable cell attachment. The culture medium consisted of Dulbecco's modified Eagle's medium (D-MEM, GIBCO BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Sigma, St Louis, MO), L-glutamine (1 μg/ml), and antibiotics. The culture medium was replaced every 2 days. The specimens were examined under the phase-contrast microscope and were screened for bacterial or fungal contaminations. They were incubated at 37°C in humidified 5% CO_2 in air atmosphere until used for experiments. In order to standardize for both the period and size of the cultured cells, 7 to 14 day-old cell cultures from each subject were used for the following experiments.

CBF measurement and analysis of cytokine effects

The motion of the actively beating ciliated cells was quantified by measuring CBF using inverted phase-contrast microscopy (Nikon ELWD 0.3, Tokyo, Japan) equipped with a NIKON photometer P101. The specimens were placed on the heated stage maintained at 37 ± 0.5°C and allowed to equilibrate for 15 mins. Single ciliated cells in the outgrowth sheets were randomly chosen and the beating cilia were viewed at a magnification of ×400. The cilia were oriented to interrupt the passage of light through a 0.2 mm slit in the diaphragm of the attached photometer. The fluctuating light produced by the ciliary beats was processed into an electrical analog signal. The signal was amplified, filtered through 5 Hz and 30 Hz filters, digitized at 200 Hz, and displayed on the screen of an oscilloscope (VC-11, Nihon-Kohden, Tokyo, Japan). The CBF was calculated from the measurement of the signal wavelength and was expressed as beats per minute.

Conditioned D-MEM as described above, was used as the substrate medium in the preparation of all experimental solutions. Before experiments, the cultured cells were refilled with 1.2 ml of fresh medium and the baseline CBF was determined from each specimen. In this study, we selected ten to twenty outgrowth ciliated cells with actively beating cilia from one sinus explant. After initial measurement, the cells were further incubated for 24 hrs in the presence of either human recombinant TNF-α (0.1, 1 and 10 ng/ml, Life Tech) or dexamethasone (10^{-6}M, 10^{-7}M, Sigma). The control experiment was performed by adding the same volume of PBS to the medium. Following the drug stimulation, the same site on the specimen was relocated and the CBF measurement was made in the same manner.

NOS immunohistochemistry

After successive measurements of CBF, parts of specimens in each group were prepared for immunohistochemistry for NOS isoforms. Cultured cells were fixed with 4% paraformaldehyde for 15 mins, and then rinsed several times with phosphate buffered saline (PBS). They were treated with PBS containing 2% bovine serum albumin (BSA) and 0.3% Triton-X for 30 mins in order to block nonspecific binding and to enhance permeability. The primary antibodies used here were polyclonal rabbit anti-iNOS antibody (PA3–030, Affinity Bio Regents, Golden, NJ) and anti-eNOS antibody (SA-201, BIOMOL Res Lab,

Plymouth Meeting, PA). They were applied to the specimens overnight at 4°C at a dilution of ×500. After washing with PBS, the specimens were then incubated with fluorescein-isocyanate (FITC) conjugated goat immunoglobulins (Tago Immunologicals, Camarillo, CA) for 1 hr. Control sections were prepared by incubation with PBS instead of the primary antibodies. These sections were used to detect the background fluorescence level.

The FITC-labeled specimens were observed by laser scanning confocal microscopy (LSCM, Leica TCS-NT, Heidelberg, Germany) equipped with an Argon-Krypton laser at wavelengths of 488 nm for excitation. The appropriate filter combinations for the simultaneous visualization of FITC (bandpass filter 500–550 nm) and differential interference contrast (DIC) microscopy were used. The fluorescence image was obtained as a 1024 × 1024 pixel frame with 24-bit color. All other settings including scanning speed, pinhole diameter and voltage gain remained the same for all experiments.

Statistical analysis

In the analysis of CBF measurement, all data were expressed as the mean ± standard deviation (S.D.). Statistical significance was determined by ANOVA. If the analysis gave a significant result ($p<0.05$), further comparison of individual data was done by Fisher's LSD test.

RESULTS

CBF modulation by TNF-α and dexamethasone

Morphological evaluation of the cultured sinus mucosa using phase contrast microscopy revealed the presence of a well-preserved outgrowth sheet of epithelial cells with typical polygonal structure (Fig. 1A). Ciliated cells were easily recognized by their characteristic beating cilia on the cell surface. They tended to exist in abundant number in the vicinity of the explant. We found that the culture period required for the sinus mucosa to develop confluent outgrowth sheets did not vary by more than 2 to 3 days.

In this experiment, we measured CBF from a total of 949 ciliated cells. The histogram in Fig. 1B demonstrates the distribution of absolute CBF values of all ciliated cells before drug administration. The mean value of CBF is 605.6 ± 151.2 (n=949) and the distribution shows a typical standardized shape. Significant changes to the distribution of CBF values occurred 24 hrs after treatment with various concentrations of TNF-α. Histograms in Fig. 2 show representative examples of the changes of CBF distribution when cells were treated with TNF-α 1 ng/ml (A) or 10 ng/ml (B). Treatment with a relatively low dose of TNF-α enhances ciliary activity by approximately 18% and the CBF distribution shifts toward higher frequencies, maintaining the standardized curves in similar patterns. On the other hand, treatment

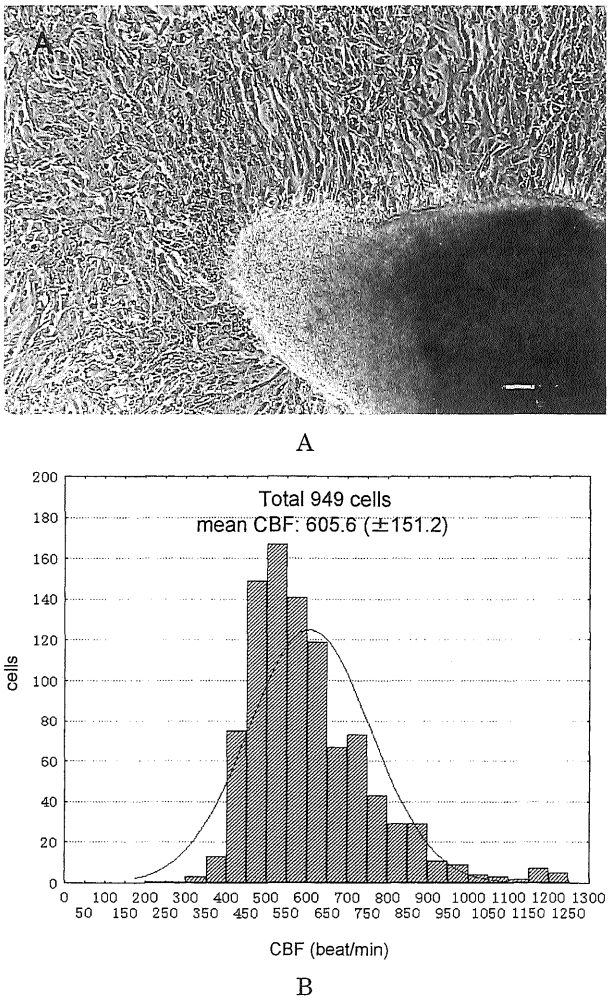


Fig. 1.
A: Phase contrast micrograph of epithelial cell outgrowth from sinus explants after one week in culture. A large number of outspreading cells with a polygonal structure is present. Bar = 10 μm.
B: The distribution of the absolute CBF values measured from the all ciliated cells (n=949) used in this study before drug exposure. The mean CBF value is 605.6 ± 151.2 beat/min. The curved line indicates estimated standardized distribution.

with a high dose of TNF-α markedly inhibited ciliary activity, the decrease in the mean values was about 25% and the CBF value of more than half of the ciliated cells was decreased. The error-bar graphs in Fig. 3 summarize the rate of CBF changes in each group 24 hrs after incubation. The CBFs are expressed as percentage changes from the baseline values before drug administration. TNF-α 0.1 and 1 ng/ml significantly increased CBF to 118.8 % and 117.7%, respectively. On the other hand, TNF-α 10 ng/ml significantly decreased CBF to 76.6%. The CBF change by dexamethasone 10^{-6}M was 91.4%. These changes are all statistically significant. No significant changes were observed in the control group or in the group treated with dexamethasone 10^{-7}M.

Fluorescent immunohistochemistry expression of NOS

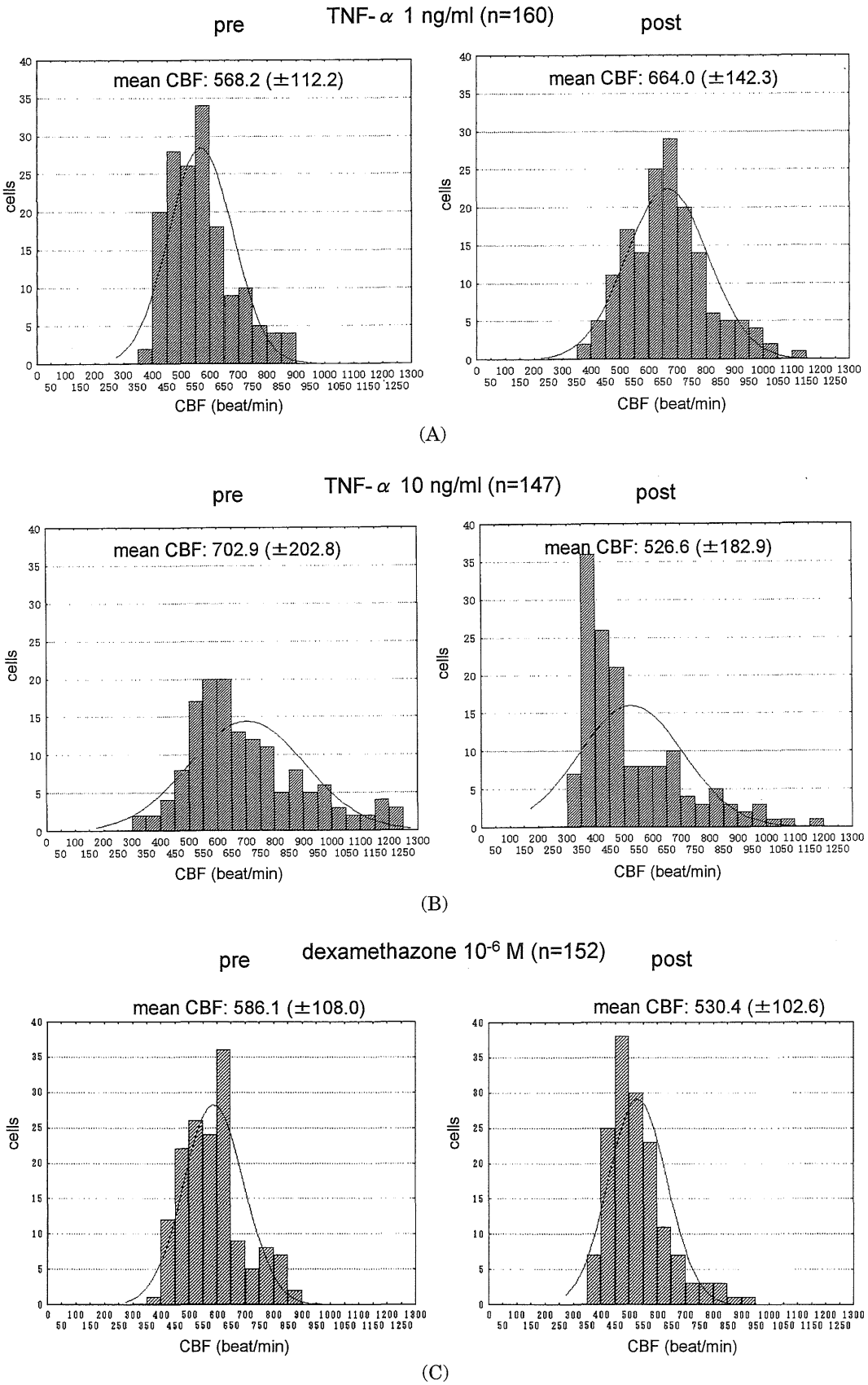


Fig. 2. Changes in the CBF distribution of cultured ciliated cells before and after treatment with (A) TNF-α 1 ng/ml (B) TNF-α 10 ng/ml and (C) Dexamethasone10^{-6} M. Note that the mean CBF values shift in different directions by adding different concentrations of TNF-α.

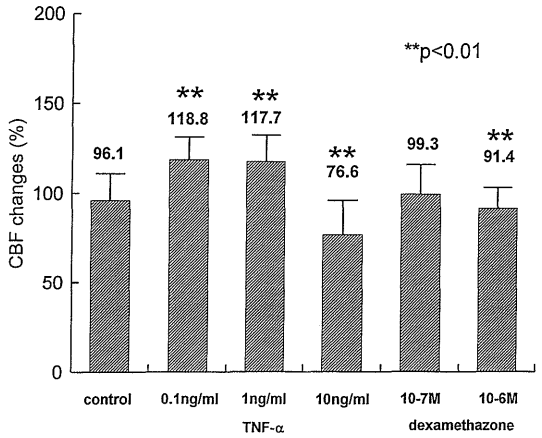
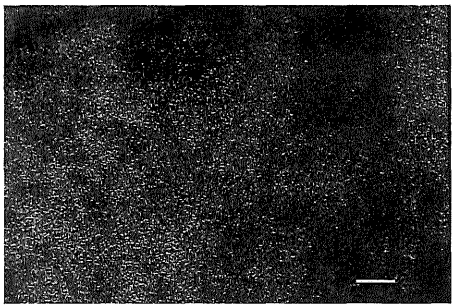
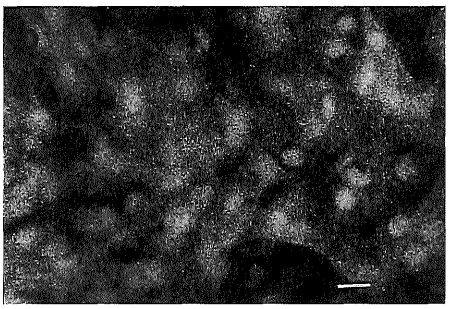


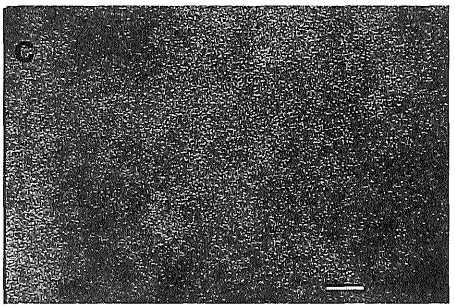
Fig. 3. The error-bar graph summarizes the change rate of each group after 24 hrs incubation. CBF is expressed as percentage changes from the baseline values before drug administration.



iNOS of control

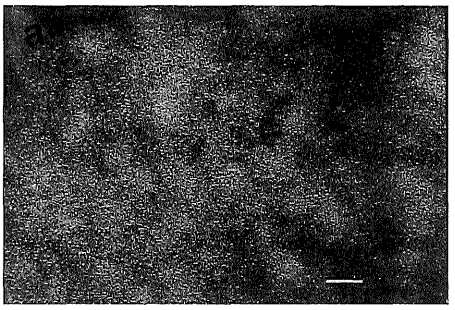


iNOS of TNF-α 10 ng/ml

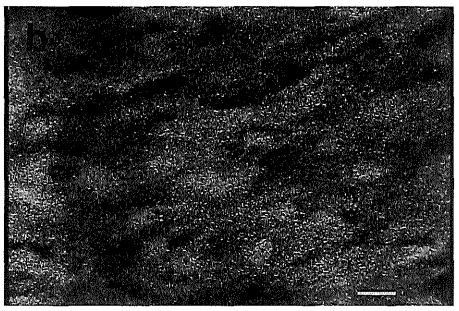


iNOS of dexamethasone 10^{-6}M

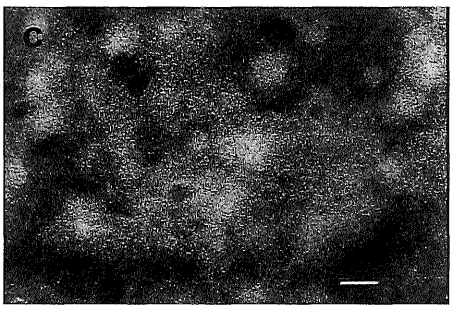
Fig. 4. Inducible NOS expression of cultured sinus epithelial cells in the control (a), TNF-α 10 ng/ml (b), and dexamethasone 10^{-6}M groups (c) as measured by LSCM. All three images were obtained from the same subject. Strong immunoreactivity was observed in the TNF-α 10 ng/ml group compared with the control group. No visible immunoreactivity was seen in the dexamethasone group.



eNOS of control



eNOS of TNF-α 10 ng/ml



eNOS of dexamethasone 10^{-6}M

Fig. 5. Endothelial NOS expression of cultured sinus epithelial cells in the control (a), TNF-α 10 ng/ml (b) and dexamethasone10^{-6}M groups (c) as measured by LSCM. All specimens reveal positive staining against anti-eNOS antibodies and no remakable changes to their immunoreactivity exist among the groups.

Fig. 4 and Fig. 5 demonstrate representative examples of iNOS and eNOS expression of the cultured epithelial cells detected by LSCM. Inducible NOS immunoreactivity remarkably increased after incubation with TNF-α (0.1, 1 and 10 ng/ml) compared with the control group. Strong fluorescence intensity of iNOS expression was observed in the TNF-α 10 ng/ml group compared with that of the control group. On the other hand, iNOS immunoreactivity was markedly decreased after incubation with dexamethasone 10^{-6}M. For eNOS immunoreactivity, the cultured epithelial cells sampled from all subjects uniformly revealed positive staining and the degree appeared the same.

DISCUSSION

The mechanism of CBF modulation is complex, because various factors can affect it. Among them, the potential role of NO in regulating ciliary activity has recently been discussed by Jain et al[10,11].

For example, CBF stimulation by substance P and bradykinin has been shown to be accompanied by a rise in intracellular Ca^{2+} and constitutive NOS (cNOS) activation[10], while TNF-α and IL-1β upregulated CBF in an NO-dependent manner mediated via iNOS induction in bovine airway epithelium[11]. However, the detailed mechanism is still not clear. In human airways, ciliated epithelial cells are considered to be not only a major site of NO production[15], but also a "target" for various active mediators such as cytokines, chemokines and reactive oxygen species including NO itself[1]. Jain et al demonstrated that the maximum increase in cytokine-induced CBF stimulation was observed in a concentration of TNF-α 1 ng/ml after 24 hrs incubation[11]. This is consistent with our results that TNF-α increased CBF at concentrations of 0.1 and 1 ng/ml. In addition, we demonstrated a novel finding that a further high concentration of 10 ng/ml of TNF-α decreased CBF in the same experiment.

Although the exact mechanism underlying this CBF downregulation is not clear, we should consider another aspect of NO, the so-called "double-edged sword". Excessive and unregulated NO synthesis has been implicated as causal or contributory to pathophysiological contradictions of many lethal and debilitating diseases in humans such as autoimmune disease, immune rejection of allografted organs, and sepsis[20]. This may be a consequence of the reaction of a large amount of NO with reactive oxygen intermediates such as superoxide to form peroxynitrite which is a major cause of tissue damage[20]. Interestingly, the representative ciliated epithelial damage observed in two chronic respiratory diseases (pertussis and asthma) has been shown to be mediated by excessive NO[6]. We can postulate a causal link between excessive NO production and the subsequent ciliated epithelial damage which results from the autotoxic capacity of NO inside the ciliated cells. A previous observation that NO inhibits a number of iron-containing enzymes involved in ATP synthesis, which is essential for ciliary activity maintenance, also supports our hypothesis[6].

NO is produced from L-arginine to L-citrulline by NO synthase (NOS) catalyzation[14]. Three distinct types of human NOS isoforms have been recognized. These are constitutive isoforms [neuronal NOS (nNOS) and endothelial NOS (eNOS)] and inducible isoform (iNOS). In this study, we showed the expression of both eNOS and iNOS isoforms in cultured human sinus epithelial cells under normal conditions. These results clearly indicate that cultured human sinus epithelial cells are able to generate and release NO through eNOS and iNOS expression.

Endothelial NOS (eNOS) produces picomolar concentrations of NO in a resting state and the activity of NO produced by this isoform is generally local and short-lived[15]. In our study, strong eNOS immunostaining was expressed uniformly in all of the specimens, and pretreatment with neither TNF-α nor dexamethasone affected their immunoreactivity. This is consistent with the report by Furukawa et al that there was no correlation between the degree of epithelial eNOS immunoreactivity and the extent of chronic inflammation[7]. These results suggest that eNOS expressed in sinus epithelial cells is mainly responsible for the regulation of paranasal homeostasis.

Inducible NOS produces a nanomolar concentration of NO once transcriptionally activated by cytokines or LPS endotoxin[15]. Unlike the eNOS isoform, iNOS expression is inhibited by corticosteroids[19]. In human airway epithelial cells, iNOS gene expression was also induced by cytokine combinations of TNF-α, IFN-γ, and IL-1β[2,17], and dexamethasone down-regulated the cytokine induced iNOS expression[17]. Nathan et al reported that iNOS lay on the high-output path and that this enzyme was often expressed in an infection or inflammation state[15]. Under such conditions, NO is often produced in excess and becomes pathogenic, as is the case in bronchial asthma. Asthmatic patients express strong iNOS immunoreactivity in the airway epithelium[9] and have elevated levels of NO in exhaled air[12]. The excessive NO may result in the detachment of pseudo-stratified ciliated columnar epithelial cells from the basal layer[9]. Our immunohistochemical staining demonstrated that cultured sinus epithelial cells stimulated by TNF-α alone showed significantly increased expression of iNOS compared with the control group. In contrast, dexamethasone 10^{-6}M inhibited its reactivity. These findings are consistent with previous reports[2,9,17]. We speculate that a low level of iNOS activity is important for airway homeostasis in the inflammatory condition by increasing ciliary activity. However, excessive NO produced by iNOS through cytokine activation would cause toxic consequences such as ciliary activity decreasing, as observed in our experiment.

In summary, our study demonstrates that cultured sinus epithelial cells have an ability to express two different NOS isoforms and their ciliary motility is affected by TNF-α, which may be NO-dependent. NO may exert cytoprotective or cytotoxic effects according to the magnitude of NOS activity. We conclude that sinus epithelial cells potentially contribute to the inflammatory process through the production of NO. In addition, the explant-outgrowth system provides a reliable tool for the study of NOS expression in the human airway.

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REFERENCES

1. **Albert, J.P. and David, P.** 1998. Epithelial cells as regulators of airway inflammation. J. Allergy Clin. Immunol. **102:** 714–718.
2. **Asano, K., Chee, C.B.E., Gaston, B., Lilly, C.M., Gerard, C., Drazen, J.M. and Stamler, J.S.** 1994. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. Proc. Natl. Acad. Sci. USA. **91:** 10089–10093.
3. **Bachwich, P.R., Chensue, S.W., Larrick, J.W. and Kunkel, S.L.** 1986. Tumor necrosis factor stimulates interleukin-1 and prostaglandin E2 production in resting macrophages. Biochem. Biophys. Res. Commun. **136:** 94–101.
4. **Cromwell, O., Hamid, Q., Corrigan, C.J., Barkans, J., Meng, Q., Collins, P.D. and Kay, A.B.** 1992. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1β and tumor necrosis factor-α. Immunol. **77:** 330–337.
5. **Devalia, J.L., Sapsford, R.J., Wells, C.W., Richman, P. and Davies, R.J.** 1990. Culture and comparison of human bronchial and nasal epithelial cells in vitro. Respir. Med. **84:** 303–312.
6. **Flak, T.A. and Goldman, W.E.** 1996. Autotoxicity of nitric oxide in airway disease. Am. J. Respir. Crit. Care Med. **154:** S202–S206.
7. **Furukawa, K., Harrison, D.G., Saleh, D., Shennib, H., Chagnon, F.P. and Giaid, A.** 1996. Expression of nitric oxide synthase in the human nasal mucosa. Am. J. Respir. Crit. Care Med. **153:** 847–850.
8. **Gaston, B., Drazen, J.M., Loscalzo, J. and Stamler, J.S.** 1994. The biology of nitrogen oxide in the airways. Am. J. Respir. Crit. Care Med. **149:** 538–551.
9. **Hamid, Q., Springall, D.R., Moreno, V.R., Chanez, P., Howarth, P., Redington, A., Bousquet, J., Godard, P., Holgate, S. and Polak, J.M.** 1993. Induction of nitric oxide synthase in asthma. Lancet **342:** 1510–1513.
10. **Jain, B., Rubinstein, I., Robbins, R.A., Leise, K.L. and Sisson, J.H.** 1993. Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. Biochem. Biophys. Res. Commun. **191:** 83–88.
11. **Jain, B., Rubinstein, I., Robbins, R.A. and Sisson, J.H.** 1995. TNF-α and IL-1β upregulate nitric oxide-dependent ciliary motility in bovine airway epithelium. The American Journal of Physiology **268:** L911–917.
12. **Kharitonov, S.A., Yates, D., Robbins, R.A., Sinclair, R.L., Shinebourne, E.A. and Barnes, P.J.** 1994. Increased nitric oxide in exhaled air of asthmatic patients. Lancet **343:** 133–135.
13. **Lundberg, J.O.N., Farkas-Szallasi, T., Weitzberg, E., Rinder, J., Lidholm, J., Anggard, A., Hokfelt, T., Lundberg, J.M. and Alving, K.** 1995. High nitric oxide production in human paranasal sinuses. Nature Med. **1:** 370–373.
14. **Moncada, S., Parmer, R.P.M. and Higgs, E.A.** 1991. Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol. Rev. **43:** 109–142.
15. **Nathan, C. and Xie, Q.W.** 1994. Nitric oxide synthases: roles, tolls, and controls. Cell **78:** 915–918.
16. **Nathan, C. and Xie, Q.W.** 1994. Regulation of biosynthesis of nitric oxide. J. Bio. Chem. **19:** 13725–13728.
17. **Robbins, R.A., Barnes, P.J., Springall, D.R., Warren, J.B., Kwon, O.J., Buttery, L.D.K., Wilson, A.J., Geller, D.A. and Polak, J.M.** 1994. Expression of inducible nitric oxide in human lung epithelial cells. Biochem. Biophys. Res. Commun. **203:** 209–218.
18. **Robbins, R.A., Springall, D.R., Warren, J.B., Kwon, O.J., Buttery, L.D.K., Wilson, A.J., Adcock, I.M., Riveros-Moreno, V., Moncada, S., Polak, J. and Barnes, P.J.** 1994. Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. Bio. Biophys. Res. Commun. **198:** 835–843.
19. **Radomski, M.W., Palmer, R.M.J. and Moncada, S.** 1990. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc. Natl. Acad. Sci. USA. **87:** 10043–10047.
20. **Schmidt, H.H.H.W. and Walter, U.** 1994. NO at work. Cell **78:** 919–925.
21. **Shah, A., Church, M.K. and Holgate, S.T.** 1995. Tumour necrosis factor alpha: a potential mediator of asthma. Clin. Exp. Allergy **25:** 1038–1044.