—Original—

Inhibitory Effects of S-carboxymethylcystein on Goblet Cell Proliferation in Cultured Epithelium

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Background: The influence of S-carboxymethylcystein (S-CMC) on the proliferation ability of goblet cells in nasal polyp epithelium in response to inflammatory stimulation was examined.

Methods: The subjects were patients with chronic paranasal sinusitis. An epithelial cell culture system was established using nasal polyp mucosa excised during endoscopic paranasal sinus surgery. The samples were divided into 4 groups (group a: control group, group b: 10 ng/mL tumor necrosis factor-α (TNF-α) treatment group, group c: 10^{-7} M S-CMC and 10 ng/mL TNF-α treatment group, group d: 10^{-5} M S-CMC and 10 ng/mL TNF-α treatment group). The total number of epithelial cells and number of goblet cells were measured under a microscope, and the ratio of goblet cells to the total number of epithelial cells was calculated.

Results: In group b, 10 ng/mL of TNF-α significantly increased the number of goblet cells compared with group a, suggesting involvement of TNF-α in goblet cell proliferation. In addition, the number of goblet cells significantly decreased in group d compared with that in group b, and it also decreased in group c compared with that in group b, although the difference was not significant, and the decrease was smaller than that in group d, suggesting that S-CMC inhibited goblet cell proliferation in a concentration-dependent manner.

Conclusion: TNF-α promoted goblet cell proliferation in nasal polyps, suggesting its influence on nasal polyp formation. As S-CMC inhibited inflammatory stimulation-induced goblet cell proliferation in nasal polyp epithelium, it may be useful for the treatment of sinusitis.
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Key words: S-carboxymethylcystein, TNF-α, chronic sinusitis, goblet cell, nasal polyp

Introduction

Several factors have been reported to be involved in the transition of paranasal sinusitis to a chronic state such as physical ventilatory and excretory disorders of the paranasal sinus due to infection, allergy, and nasal polyp formation in the paranasal sinus mucosa, as well as functional disorders of the mucociliary system[1]. Goblet cells in nasal polyp epithelium secrete mucus and are considered to influence mucociliary transport through mucin secretion in chronic sinusitis[2]. In addition, a potent ability to proliferate has been demonstrated by goblet cells in nasal polyp epithelium, suggesting their involvement in nasal polyp enlargement[3]. An inflammatory cytokine, tumor necrosis factor-α (TNF-α), is detected in nasal discharge and the paranasal sinus mucosa in chronic sinusitis, indicating its involvement in the pathology of chronic sinusitis[4,5]; however, to our knowledge, its influence on goblet cell proliferation has not been reported.

In Japan, chronic sinusitis is conventionally treated with S-carboxymethylcystein (S-CMC) combined with macrolide therapy as conservative treatment. The pharmacological effects of S-CMC have been reported to be a

Table 1 Subjects

Case	Age	Sex	Chief complaint	JESREC Score
1	46	M	Nasal obstruction	8
2	74	M	Postnasal drip	5
3	23	M	Postnasal drip	9
4	66	M	Nasal obstruction	10

All the subjects were male, aged 23–74 years. Their main complaints were nasal congestion and postnasal drip (2 cases each). JESREC scores were 5–10.
※JESREC Score: ≥11=eosinophilic sinusitis
(Tokunaga T. et al: Novel scoring system and algorithm for classifying chronic rhinosinusitis: the JESREC study. Allergy 2015: 70: 995–1003)

normalization of the mucus properties and improvement in mucociliary transport function[6], but the effects on goblet cell proliferation have not been clarified.

In this study, using a culture system of nasal polyp epithelial cells collected from patients with surgically treated chronic sinusitis, the influence of TNF-α on goblet cell proliferation and the effects of S-CMC were examined.

Materials and Methods

Subjects

Four patients with chronic sinusitis underwent endoscopic paranasal sinus surgery between April 2012 and March 2013 at the Department of Otolaryngology, Nippon Medical School Musashi Kosugi Hospital. Nasal polyp epithelium excised during surgery was used. Patients with concomitant allergic rhinitis and bronchial asthma were excluded. All the patients were male, aged 23–74 years. Their chief complaints were nasal obstruction and postnasal drip (2 cases each). None of the patients was diagnosed with eosinophilic chronic rhinosinusitis (ECRS), with JESREC scores[7] of 5–10 (**Table 1**).

Methods

(1) Collection and culture of nasal polyp epithelial cells

Using nasal polyp mucosa excised from 4 patients with surgically treated chronic sinusitis, the epithelial cell culture system was prepared as follows: The collected nasal polyp was washed with phosphate buffer at pH 7.0, placed in 0.1% protease (Nacalai Tesque, Japan)-containing transfer medium [100 mL of DMEM medium (Nacalai Tesque, Japan) + 100 mL of F12 medium (GIBCO, New York) + 2 mL of penicillin-streptomycin mixed solution (Nacalai Tesque, Japan)], and kept standing in a 37℃, 5% CO_2 incubator for 1.5 h. The sample was taken out of the incubator and epithelial cells were detached by pipetting in the dish. Only the supernatant was collected and centrifuged at 980 rpm for 3 min. Only the supernatant was collected, washed with wash medium [100 mL of DMEM (Nacalai Tesque, Japan) + 100 mL of F12 medium (GIBCO, New York) + 2 mL of penicillin-streptomycin mixed solution (Nacalai Tesque, Japan) + 2 mL of fetal bovine serum (GIBCO, New York)], and centrifuged again at 980 rpm for 3 min. The supernatant was collected and kept standing in a 37℃, 5% CO_2 incubator for 1 h. As fibroblasts adhered to the dish bottom, the supernatant containing floating epithelial cells was collected. The cells were washed with subculture medium [500 mL of BEBM (LONZA, Switzerland) + 1 BEGM Single Quots kit (LONZA, Switzerland) + 300 mg of bovine serum albumin (Sigma, Missouri) + 12.5 μg of epidermal growth factor] and centrifuged at 980 rpm for 3 min. The supernatant was collected, combined with subculture medium, mixed, and cultured in a 37℃, 5% CO_2 incubator. Subculture medium was replaced every 2 days, and after confirming an epithelial cell monolayer was being cultured on the plate, the experiment was performed following the protocol below:

(2) Protocol for drug administration experiment (**Fig. 1**)

1) Cultured nasal polyp epithelium was divided into 4 groups (group a: control group, group b: TNF-α treatment group, group c: S-CMC 10^{-7} M and TNF-α treatment group, group d: S-CMC 10^{-5} M and TNF-α treatment group). After 4-day culture, groups a and b were left untreated, and S-CMC was administered to the nasal polyp epithelium at S-CMC 10^{-7} M in group c and S-CMC 10^{-5} M in group d.

2) After 48 hours, 10 ng/mL of TNF-α was administered to groups b, c, and d. The dose of TNF-α was decided based on the level previously measured in fluid retained in the paranasal sinus (unpublished data).
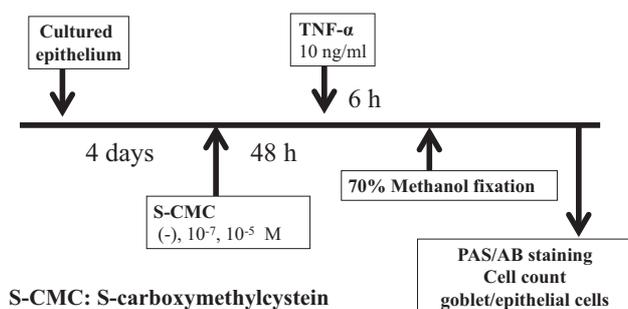
Fig. 1   Study methods
The nasal polyps collected were divided into 4 groups (group a: control group, group b: tumor necrosis factor-α (TNF-α) treatment group, group c: S-carboxymethylcystein (S-CMC) 10^{-7} M and TNF-α treatment group, group d: S-CMC 10^{-5} M and TNF-α treatment group). Only nasal polyp epithelial cells were extracted and cultured for 4 days. Groups a and b were not treated, while groups c and d were treated with S-CMC 10^{-7} M and 10^{-5} M, respectively. After 48 hours, 10 ng/mL TNF-α was added to groups b, c, and d. After 6 hours, the total number of epithelial cells and number of goblet cells were measured, and the ratio of the number of goblet cells to that of epithelial cells was compared among the 4 groups.

3) The cultured nasal polyp epithelial cells were fixed with 70% methanol 6 hours after TNF-α administration and subjected to Alcian Blue-PAS staining. In addition, the dynamics of the cultured cells were video-recorded under a phase-contrast microscope. The total number of epithelial cells and number of goblet cells were counted, and the ratio of goblet cells in the total number of epithelial cells was calculated. Cells were counted by a pathologist blinded to the study background.

(3) Statistical analysis

Student's t-tests were used for statistical analysis.

As this was a study using surgically collected human samples, it was performed after approval by the Ethics Committee of Nippon Medical School Musashi Kosugi Hospital (Permission number: 219-24-19) and obtaining consent from the patients.

Results

Establishment of an epithelial cell culture system with ciliary movement was confirmed under a phase-contrast microscope. Microscopic images are presented in **Fig. 2**. The number of epithelial cells (N-EC), the number of goblet cells (N-GC), and the ratios of the number of goblet cells to the total number of epithelial cells (number of goblet cells/number of epithelial cells: N-GC/N-EC) were 680.5, 4.70, and 7.12 in group a, 604.9, 5.90, and 10.24 in group b, 876.8, 7.50, and 8.66 in group c, and

476.8, 3.30, and 6.81 in group d. The N-GC/N-EC ratios were compared among the four groups (**Fig. 3**). The ratio was significantly higher in group b than a (p<0.05), suggesting involvement of TNF-α in goblet cell proliferation. The mean ratio decreased in the order of group b, c, and d, and it was significantly decreased in group d compared with that in group b (p<0.05), suggesting that S-CMC inhibited goblet cell proliferation in a concentration-dependent manner.

Discussion

In Japan, macrolides are the first-choice for drug therapy for chronic sinusitis. In general, expectorants, such as S-CMC, steroids, and anti-allergic drugs, are combined with macrolide therapy. Several factors have been reported to be involved in the transition of paranasal sinusitis to a chronic state: Bacterial and viral infections, and allergic reactions inducing inflammatory reactions in the paranasal sinus mucosa, reduction in the fluid retained in the paranasal sinus due to mucociliary transport by the nasal mucosa affected by local anatomical factors and secretions, and activation of inflammatory agents such as bacteria-derived substances, immune complexes, and cytokines[2,8–10]. Nasal polyps are formed in many cases, and possible causes are edema induced by promoted vascular permeability[11], epithelial rupture[12], and action of matrix metalloproteinase, but no cause has been clarified. Nasal polyp mucosa consists of epithelial tissue and interstitium. Epithelial tissue is pseudostratified epithelium containing goblet cells, similar to other respiratory epithelium, and the number of goblet cells varies depending on the nasal polyp development site[13]. In the interstitium, proliferation of gland tissue, vascular endothelial cells, fibroblasts, and collagen are observed in addition to infiltrating inflammatory cells. The association of nasal polyp proliferation with several cytokines and growth factors has been reported, and production of different cytokines and chemokines by fibroblasts isolated from nasal polyps has been demonstrated, suggesting their involvement in nasal polyp proliferation[14]. Goblet cells, abundantly present in nasal polyp epithelial tissue, are mucus-secreting cells, and they have been reported to be involved in mucin secretion in chronic sinusitis[2]. Goblet cells in nasal polyp epithelium have high proliferative ability, and proliferation of secretory goblet cells, in addition to epithelial cells, may be involved in epithelial hyperplasia during inflammation[3]. On the other hand, abundant TNF-α, an inflammatory cytokine, in nasal discharge and paranasal sinus mucosa in chronic sinusitis

2-1

| low-power | low-power | low-power | low-power |

| high-power | high-power | high-power | high-power |

| TNF-α (-) Group a | TNF-α (+) Group b | TNF-α (+) + S-CMC 10^{-7} M Group c | TNF-α (+) + S-CMC 10^{-5} M Group d |

2-2

| low-power | low-power | low-power | low-power |

| high-power | high-power | high-power | high-power |

➡ **Goblet cell**

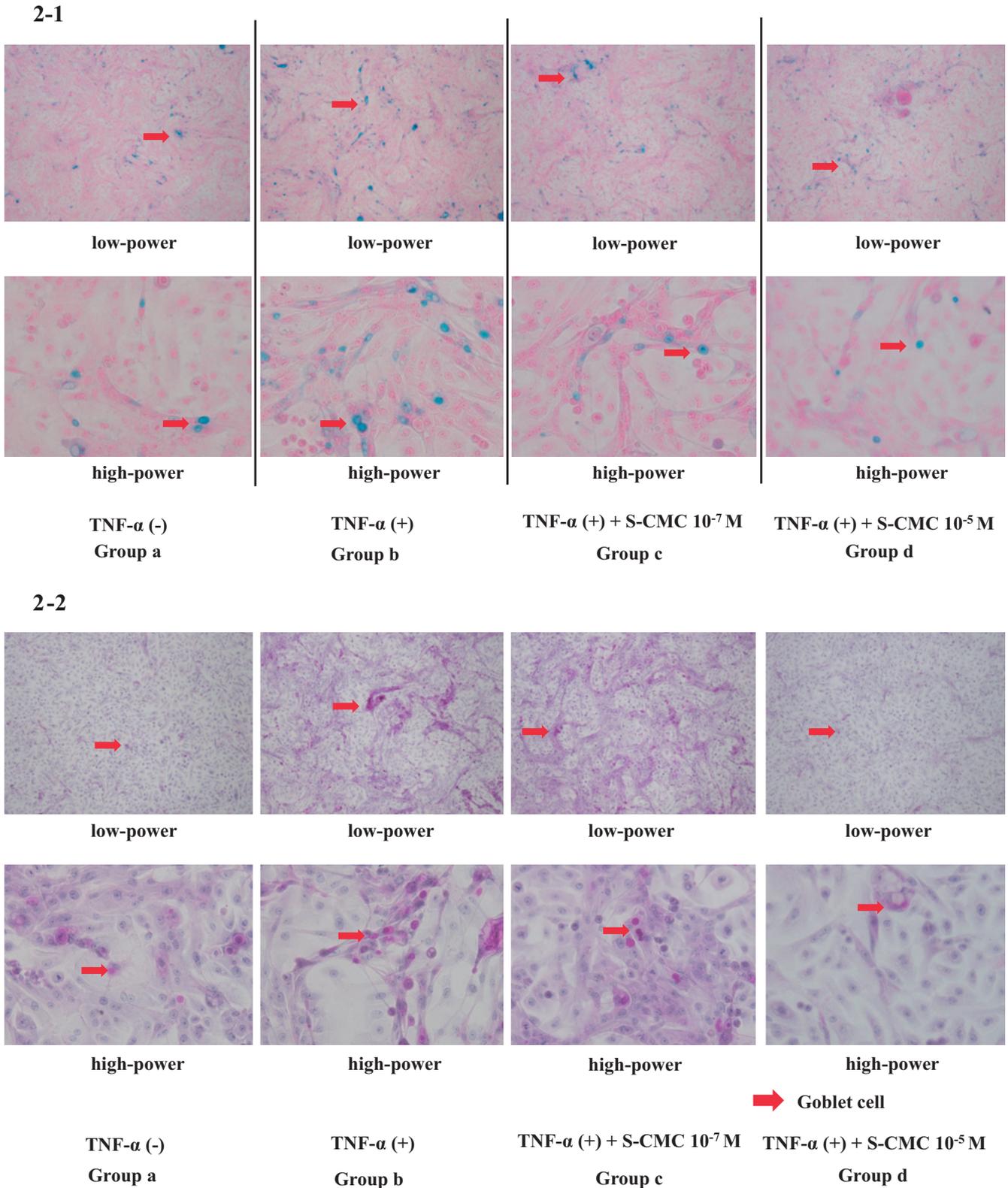| TNF-α (-) Group a | TNF-α (+) Group b | TNF-α (+) + S-CMC 10^{-7} M Group c | TNF-α (+) + S-CMC 10^{-5} M Group d |

Fig. 2　Microscopic images (Fig. 2-1: Alcian blue staining Fig. 2-2: PAS staining)

Fig. 2-1 shows a microscopic image made of Alcian blue staining, and Fig. 2-2 shows that of PAS staining. The microscope images show the low-powered field on the upper level, and the high-powered field on the lower level. Acidic mucus produced from goblet cells was increased in group b, and reduced in group c and group d.

has been demonstrated, suggesting its involvement in the pathology of chronic sinusitis[4,5]. TNF-α is generated by stimuli, such as viral and bacterial infections, to promote the release of granulocyte / macrophage colony-
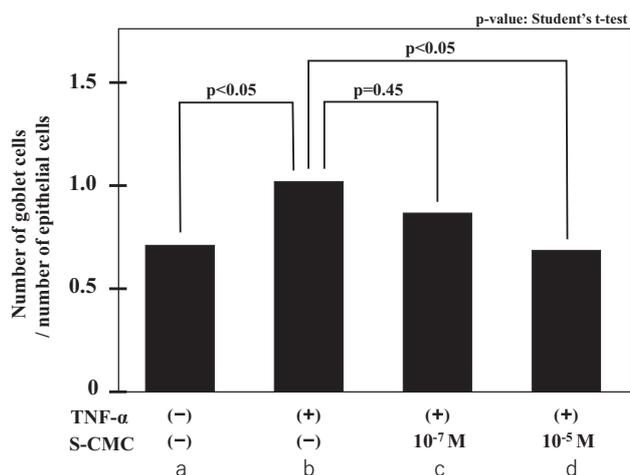
Fig. 3   Results

In the non-S-carboxymethylcystein (S-CMC) treatment groups (groups a and b), the ratio of goblet cells to the total number of epithelial cells was significantly increased by tumor necrosis factor-α (TNF-α) ($p<0.05$). In the TNF-α treatment groups (groups b, c, and d), the mean was slightly lower in the S-CMC 10^{-7} M treatment group (group c) than in the non-S-CMC treatment group (group b) ($p=0.45$), and significantly lower in the S-CMC 10^{-5} M treatment 10 group (group d) ($p<0.05$). The ratio of goblet cells to epithelial cells was lower in the S-CMC 10^{-5} M group than in the 10^{-7} M treatment group (group c), suggesting that S-CMC inhibits goblet cell proliferation in a concentration-dependent manner.

stimulating factor (GM-CSF) from the nasal mucosal epithelium and fibroblasts. GM-CSF promotes eosinophil activation[15] and enhances GM-CSF mRNA expression in nasal polyps[16]. The number of eosinophils is correlated with the number of GM-CSF-producing cells in nasal polyps[17]. Therefore, GM-CSF produced upon TNF-α stimulation may play an important role in eosinophil accumulation in nasal polyps. TNF-α also induces the expression of adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) in vascular endothelial cells and promotes eosinophil migration[18]. Thus, goblet cells in the nasal polyp epithelium may be involved in eosinophil infiltration, and TNF-α may promote eosinophil infiltration in nasal polyps and induce goblet cell proliferation. However, no report has been published on these hypotheses. In the present study, TNF-α administration to cultured nasal polyp epithelial cells significantly increased the number of goblet cells, suggesting that TNF-α is involved in goblet cell proliferation by acting on undifferentiated cells among epithelial cells.

Concerning drug therapy for chronic sinusitis, in addition to infection control in the paranasal sinus, improvement in the self-cleaning action of the paranasal sinus,

such as mucociliary transport and shrinkage of nasal polyps, is important. S-CMC, which is frequently used in combination with macrolide therapy, improves mucociliary transport[19], and improvement of subjective nasal discharge has also been reported, demonstrating its usefulness for treatment[6]. Inhibitory effects on goblet cells among nasal polyp epithelial cells were observed, but goblet cell inhibitory effects by S-CMC in normal nasal and paranasal sinus mucosae were not observed[19]. Carbocysteine does not merely decrease goblet cells, but decreases and normalizes pathologically hyperplastic goblet cells. Goblet cells significantly proliferated in chronic sinusitis with marked eosinophil infiltration, and mucus is caused by excessive secretion from goblet cells[20]. Carbocysteine suppresses mucin secretion in nasal discharge[21]. Thus, carbocysteine reduces excessive mucin secretion from goblet cells through normalization of goblet cell proliferation, thereby normalizing mucociliary transport.

Conclusion

Changes in the number of goblet cells in cultured nasal polyp epithelium induced by TNF-α and S-CMC were examined. TNF-α significantly increased the number of goblet cells, suggesting that TNF-α is involved in goblet cell proliferation and influences nasal polyp formation. S-CMC decreased the number of goblet cells in cultured nasal polyp epithelium in a concentration-dependent manner. Thus, S-CMC may normalize goblet cell proliferation in the nasal polyp epithelium and may also be involved in the normalization of mucociliary transport.

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