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Effect of topical anti-inflammatory drugs on epithelial cell-induced eosinophil survival and GM-CSF secretion

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ABSTRACT: Topical anti-inflammatory drugs decrease eosinophil infiltration. This action may be due to an effect on the release of epithelial cell products responsible for promoting eosinophil survival. We investigated the effect of fluticasone propionate, budesonide, beclomethasone dipropionate and nedocromil sodium on the release of granulocyte/macrophage colony-stimulating factor (GM-CSF) and on eosinophil survival induced by secretions from cultured nasal epithelial cells.

Human epithelial cell-conditioned media (HECM) were generated by cultured epithelial cells obtained from healthy subjects undergoing corrective nasal surgery. Normodense eosinophils isolated from peripheral blood were incubated with HECM generated with and without the drugs.

All of the drugs tested inhibited eosinophil survival, and response was dose-dependent. Fluticasone propionate had the highest inhibitory potency (25% inhibitory concentration (IC_{25}) 1×10^{-9} M), followed by budesonide (IC_{25} 3.3×10^{-8} M), beclomethasone dipropionate (IC_{25} 1.5×10^{-6} M), and nedocromil sodium (IC_{25} 5×10^{-6} M). Likewise, fluticasone was the strongest steroid in inhibiting release of GM-CSF (IC_{25} 8.4×10^{-11} M), followed by budesonide (IC_{25} 2×10^{-9} M), beclomethasone dipropionate (IC_{25} 1.3×10^{-8} M), and nedocromil sodium (IC_{25} $>10^{-5}$ M). A significant correlation was found between both inhibitory effects ($r=0.955$; $p<0.05$).

Topical anti-inflammatory drugs may decrease eosinophil survival by abrogating the promoting effect of epithelial cells. These drugs may exert part of their therapeutic effect by modulating GM-CSF release. The following rank of potency was observed: fluticasone propionate > budesonide > beclomethasone dipropionate > nedocromil sodium. The study of the interaction between epithelial cells and eosinophils may be a useful method for investigating and comparing the potency of topical drugs.

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Eosinophilic infiltration of the respiratory mucosa is a characteristic histological feature in rhinitis and bronchial asthma, both allergic and nonallergic [1, 2]. It has been shown that glucocorticoids, the most effective anti-inflammatory drugs used in the treatment of asthma and rhinitis, decrease eosinophilic infiltration of the respiratory mucosa [3]. The mechanism of action of glucocorticoids in reducing eosinophil numbers remains to be clarified: it is not yet clear which cells are the target for these drugs. In addition to the direct effect of steroids on eosinophil survival by inducing their apoptosis [4], other cells, such as T-lymphocytes, may also be involved in the anti-inflammatory effect of glucocorticoids in asthma [5]. It is also possible, however, that the anti-inflammatory effect of glucocorticoids may result, at least in part, from an inhibition of the release of epithelial cell-derived cytokines, such as granulocyte/macrophage colony-stimulating factor (GM-CSF), with the capacity for promoting eosinophil recruitment, survival and activation.

Nedocromil sodium, a topical antiallergic drug, has been widely used in the treatment of bronchial asthma [6]. In clinical studies, it has been found to be effective in inhibiting early and late phase allergy-induced asthmatic responses [7, 8]. It has also been shown that nedocromil sodium inhibits *in vitro* migration and activation of various cell types, such as mast cells, neutrophils and eosinophils, involved in the asthmatic inflammatory reaction [9–14]. Moreover, recent studies have demonstrated that nedocromil sodium is capable of inhibiting interleukin-1 (IL-1)-induced release of interleukin-8 (IL-8) and GM-CSF from human epithelial cells [15, 16].

Epithelial cells are capable of playing a role in the inflammatory response through the release of mediators that may either exert a direct effect on the airways or influence the activity of other cells, such as eosinophils. Supernatants from epithelial cell culture promote eosinophil survival, this effect being abrogated by previous incubation of eosinophils with glucocorticoids [17–19]. This finding suggests that, in asthma and rhinitis, eosinophil

infiltration may be downregulated by a direct effect of drugs on eosinophils. Because epithelial cells are probably the main target both for glucocorticoids and nedocromil sodium, it could also be anticipated that at least part of the anti-inflammatory effect of these drugs may be due to their capacity to reduce eosinophil infiltration by modulating the release of proinflammatory substances from the epithelium. Previous studies have provided evidence to suggest that GM-CSF is the most significant cytokine secreted by epithelial cells as far as eosinophil survival is concerned [17]. The ability to attenuate enhanced eosinophil survival with blocking antibodies for GM-CSF, and to a lesser extent IL-8, suggests that GM-CSF is the main contributor to increased eosinophil survival resulting from incubation of eosinophils with human epithelial cell-conditioned media (HECM) [17].

The objective of the present study was to investigate and compare the potency of topically-applied drugs in inhibiting both the promoting effect on eosinophil survival and on the secretion of GM-CSF by cultured nasal epithelial cells.

Materials and methods

Materials

Ham's F12 medium was obtained from Biochorm KG (Berlin, Germany). Trypan blue, penicillin-streptomycin, hydroxyethylpiperazine ethanesulphonic acid (Hepes) buffer, foetal calf serum (FCS), and RPMI 1640 culture medium were purchased from Flow Laboratories (Irvin, UK), and 24-well tissue culture clusters from Costar (Cambridge, MA, USA). Amphotericin B was acquired from Squibb (Esplugues de Llobregat, Spain). Hydrocortisone, human transferrin, bovine insulin, 3,3',5-triiodo-L-tyrosine sodium salt, protease type XIV, beclomethasone dipropionate and glutamine were provided by Sigma Chemical Co. (St Louis, MO, USA). Endothelial cell growth supplement, epidermal growth factor rat tail collagen type-I were supplied by Collaborative Research Inc. (Bedfort, MA, USA). Percoll® was supplied by Pharmacia LKB (Uppsala, Sweden). Budesonide was obtained from Astra (Esplugues, Spain); fluticasone propionate from Glaxo (Madrid, Spain), and nedocromil sodium from Fisons Ibérica (Zaragoza, Spain). GM-CSF enzyme-linked immunosorbent assay (ELISA) kits were supplied by Amersham Ibérica (Madrid, Spain).

Methods

Population. Nasal mucosal specimens were obtained from 17 patients (12 males and 5 females) aged 32±3 yrs (range 13–61 yrs), undergoing nasal obstruction corrective surgery for septal dismorphy, turbinate hypertrophy, or both. One patient suffered from allergic rhinitis and another from nonallergic rhinitis. Specimens were placed in Ham's F12 medium supplemented with 100 international units (IU) of penicillin, streptomycin, 100 $\mu g \cdot mL^{-1}$, and amphotericin B, 2 $\mu g \cdot mL^{-1}$, and immediately transported to the laboratory.

Epithelial cell culture. Isolation of epithelial cells from human nasal mucosa and epithelial cell cultures was carried out according to the method described previously [17]. Briefly, epithelial cells isolated by protease digestion were plated on collagen-coated wells (100,000 $cells \cdot well^{-1}$), with 2 mL of serum-free Ham's F12 medium supplemented with antibiotics, glutamine and growth factors and placed in a 5% CO_2 humidified incubator at 37°C. Culture media were changed every 2 days until culture confluence was reached after 7–10 days. Characterization of epithelial cells was made by optic microscopy (May-Grünwald Giemsa stain), and by immunocytochemistry using the monoclonal antibody to cytokeratin (CK 1).

Generation of human epithelial-conditioned media (HECM). After reaching confluence, HECM were generated as described previously [17]. Since previous studies have shown that non-stimulated epithelial cells produce low levels of GM-CSF, FCS was used to increase the production of this cytokine. In order to avoid different effects of FCS from different batches or sources, the same FCS batch from Flow Laboratories was used in all experiments. Cultured epithelial cells were incubated for 48 h with RPMI 1640 culture medium supplemented with 10% FCS, in the presence or absence of budesonide, beclomethasone dipropionate or fluticasone propionate at concentrations ranging 10^{-13} to 10^{-5} M, or nedocromil sodium (10^{-5} to 10^{-8} M). HECM were harvested, centrifuged at 400×g for 10 min at room temperature, sterilized by passing through 0.22 μm filters (Millipore), and stored at -70°C until assayed. Nedocromil sodium was diluted in culture media (RPMI), while glucocorticoids were diluted in dimethylsulphoxide (DMSO). The final concentration of DMSO (0.1%) had no effect on FCS-induced GM-CSF release (FCS alone=305±137 $pg \cdot mL^{-1}$; FCS plus DMSO=270±92 $pg \cdot mL^{-1}$; n=4).

Eosinophil isolation. Normodense eosinophils were obtained from peripheral blood of volunteers with more than 3% eosinophils using Percoll® discontinuous gradients, as described previously [17]. Cell viability (>95%) was assessed by trypan blue dye exclusion, and the percentage of eosinophils obtained (>95%) was quantified by cytocentrifuge smears stained with May-Grünwald Giemsa.

Experimental design. Eosinophils (at a concentration of approximately 2.5×10^5 $cells \cdot mL^{-1}$ per well) were incubated in 24-well culture plates with (positive control) or without (negative control) 25% HECM. This concentration of HECM was chosen because it showed the most significant effect on eosinophil survival in a previous study [17]. Eosinophil suspensions were incubated with HECM generated in the presence of topical drugs. In all the experiments, the eosinophil survival index was assessed on day 4, and was calculated as follows: (number of eosinophils recovered) × (percentage of eosinophil viability)/(number of eosinophils delivered on day 0).

The method used in this study to incubate epithelial cells with glucocorticoids and nedocromil sodium has a potential drawback. When HECM are generated in the

presence of drugs, some concentration of the drugs may remain in the HECM, and this could have a direct effect on eosinophils. To investigate this possibility, in a preliminary study, we evaluated the effect of HECM generated with dexamethasone, which was maintained or removed from the culture before adding the epithelial cell supernatant to eosinophils. HECM were generated in the presence or absence of dexamethasone at a concentration of 10^{-5} M over 48 h. HECM were then recovered, the dishes were washed with phosphate-buffered saline (PBS) 1 M, and fresh RPMI culture medium, supplemented with 10% FCS without dexamethasone, was added to the dishes for an extra incubation period of 48 h. HECM generated by both methods were added to eosinophil suspensions, and the survival index measured on day 4 (fig. 1). No differences were found between the effect of these HECM on eosinophil survival, suggesting that the inhibition of eosinophil survival is due to an effect of these anti-inflammatory drugs on epithelial cells and that the glucocorticoid remaining in the supernatant does not have a significant effect on eosinophils.

The study was approved by the Ethics Committee of our institution and informed consent was obtained from each subject.

Cytokine ELISA. The concentration of GM-CSF in HECM was measured directly by ELISA using a "sandwich" technique. The limit of detection for GM-CSF was 4 $pg \cdot mL^{-1}$.

Statistical analysis

Statistical evaluation was performed on a Power Macintosh 6100/60 (Apple Computer, Cupertino, CA, USA) using the statistical software package Statview II (Brainpower Inc., Calabasas, CA, USA). All results are expressed as mean±SEM. Parametric (Student's t-test for paired and unpaired sample analysis) and nonparametric (Wilcoxon signed rank) tests were used for statistical comparisons. A p-value less than 0.05 was considered statistically significant.

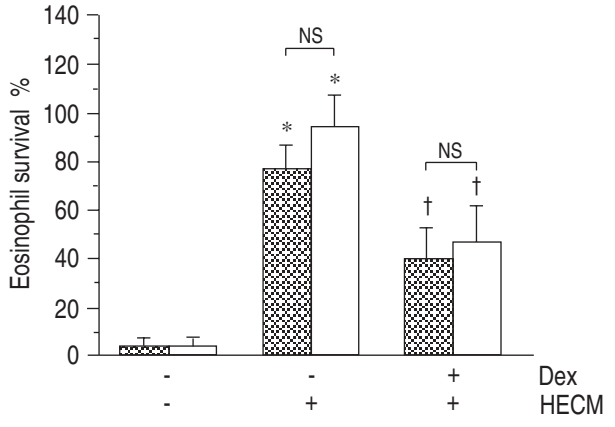

Fig. 1. – Effect of dexamethasone (Dex) on eosinophil survival induced by two different human epithelial-cell-conditioned media (HECM), that contained (▨), or did not contain (□), Dex. *: HECM 25% significantly increased eosinophil survival compared to control ($p<0.05$); †: Dex (10 μM) significantly inhibited the HECM-induced eosinophil survival ($p<0.05$); NS: no statistical difference was found between the effects of HECM generated from Dex-treated epithelial cells that contained or did not contain the steroid. Values are expressed as mean±SEM. Student's paired t-test was used for statistical analysis. For further details, see Materials and methods.

Results

Effects of glucocorticoids and nedocromil sodium on GM-CSF release

In a preliminary study, we investigated the effect of FCS on the release of GM-CSF, with respect to dose-response (0, 0.5, 1, 2.5, 5 and 10%) and time course (6, 12, 24 and 48 h). FCS increased GM-CSF release in a dose-related fashion, the effect being maximal and similar at 24 and 48 h using 10% concentration (fig. 2). Since the variability of GM-CSF release was lower at 48 h than at 24 h, 48 h was finally chosen as the optimal time course for further experiments.

The release of GM-CSF from cultured nasal epithelial cells was inhibited in a dose-dependent manner by fluticasone propionate (up to 62% at 10^{-5} M, (n=6);

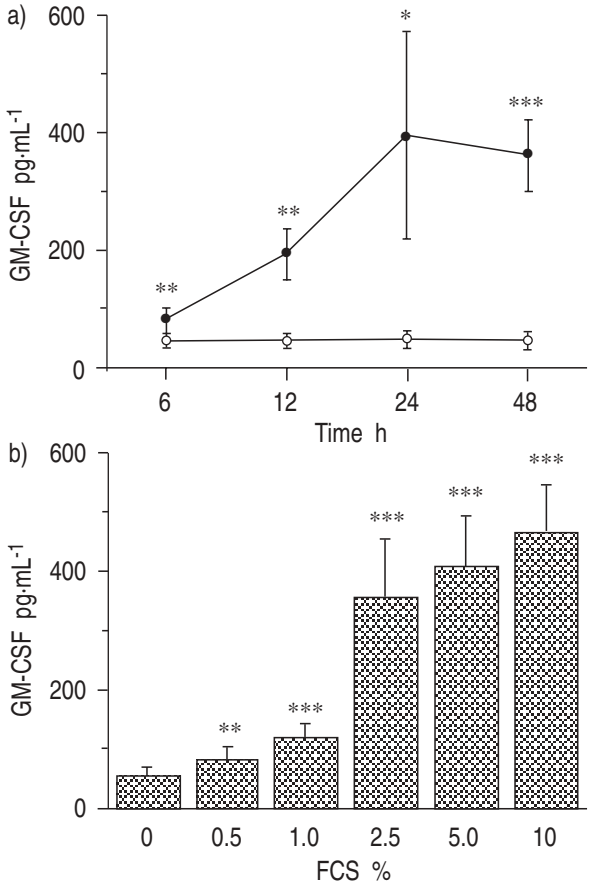

Fig. 2. – Release of granulocyte/macrophage colony-stimulating factor (GM-CSF) from cultured nasal epithelial cells in response to foetal calf serum (FCS). a) Time course of GM-CSF release ($pg \cdot mL^{-1}$) in response to 10% FCS (n=5). FCS (—●—) induced a significant release of GM-CSF compared to media-treated control cells (—○—) from 6–48 h. b) Dose-response of GM-CSF release in response to increasing concentrations of FCS (0.5–10%) during 48 h (n=6). Values are expressed as mean±SEM. Student's paired t-test was used for statistical analysis. *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$.

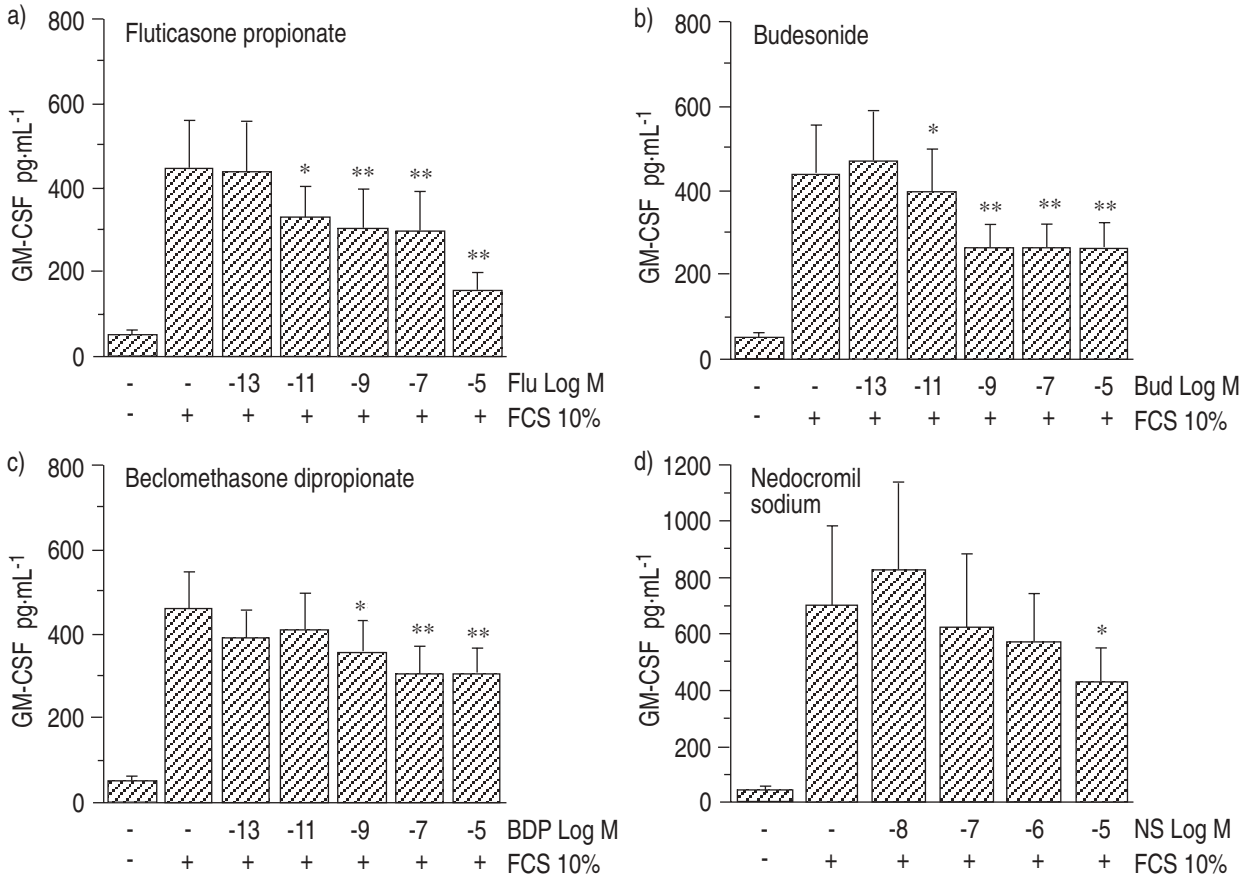

Fig. 3. – Effect of topical glucocorticoids and nedocromil sodium on production of granulocyte/macrophage colony-stimulating factor (GM-CSF). Foetal calf serum (FCS) 10% increased GM-CSF release from cultured nasal epithelial cells compared to media-treated cells. a) Fluticasone propionate (Flu; n=6); b) budesonide (Bud; n=6); c) beclomethasone dipropionate (BDP; n=7); and d) nedocromil sodium (NS; n=7) caused a dose-related inhibitory effect on FCS-induced GM-CSF release compared to FCS alone. Values are expressed as mean±SEM. Student's paired t-test was used for statistical analysis. *: $p<0.05$; **: $p<0.01$.

$p<0.001$), budesonide (up to 36% at 10^{-5} M (n=6); $p<0.01$), and beclomethasone dipropionate (up to 33% at 10^{-5} M, (n=7); $p<0.001$). The minimal inhibitory concentration was 10^{-11} M for the three glucocorticoids. In comparison to glucocorticoids, nedocromil sodium had a weaker effect on GM-CSF release (up to 18% at 10^{-5} M, (n=7); $p<0.05$) (fig. 3).

Effects of glucocorticoids and nedocromil sodium on eosinophil survival

HECM from healthy nasal mucosa, at a concentration of 25%, significantly increased eosinophil survival with respect to controls, while HECM generated in the presence of glucocorticoids decreased eosinophil survival (fig. 4). HECM-induced eosinophil survival was inhibited in a dose-related fashion by fluticasone propionate (up to 84% at 10^{-5} M (n=5); $p<0.001$), budesonide (up to 62% at 10^{-5} M (n=6); $p<0.01$), and beclomethasone dipropionate (up to 52% at 10^{-5} M (n=5); $p<0.001$). The minimal inhibitory concentration was 10^{-8} M for fluticasone propionate, 10^{-7} M for budesonide and 10^{-5} for beclomethasone dipropionate. In contrast with glucocorticoids, incubation of epithelial cells with different concentrations of nedocromil sodium caused a lower inhibition of HECM-induced eosinophil survival. A significant inhibitory effect, however, was detected with the 10^{-5} M concentration (33% inhibition (n=6); $p<0.05$).

Comparison of glucocorticoid and nedocromil sodium inhibitory potency on eosinophil survival and GM-CSF release

Since most of the drugs did not reach 50% inhibition of FCS-induced GM-CSF release, their inhibitory potencies for both GM-CSF production and eosinophil survival were compared by using the 25% inhibitory concentration (IC_{25}). In FCS-induced GM-CSF production, fluticasone propionate showed the highest inhibitory potency ($IC_{25} = 8.4\times10^{-11}$ M), followed by budesonide ($IC_{25} = 2\times10^{-9}$ M), beclomethasone dipropionate ($IC_{25} = 1.3\times10^{-8}$ M), and nedocromil sodium ($IC_{25} > 10^{-5}$ M) (table 1). In HECM-induced eosinophil survival, fluticasone propionate also showed the highest inhibitory potency ($IC_{25} = 1\times10^{-9}$ M), followed by budesonide ($IC_{25} = 3.3\times10^{-8}$ M), beclomethasone dipropionate ($IC_{25} = 1.5\times10^{-6}$ M), and nedocromil sodium ($IC_{25} = 5\times10^{-6}$ M) (table 1).

The effects of the four topical drugs on eosinophil survival and GM-CSF release were highly and significantly correlated ($r=0.955$; $p<0.05$).

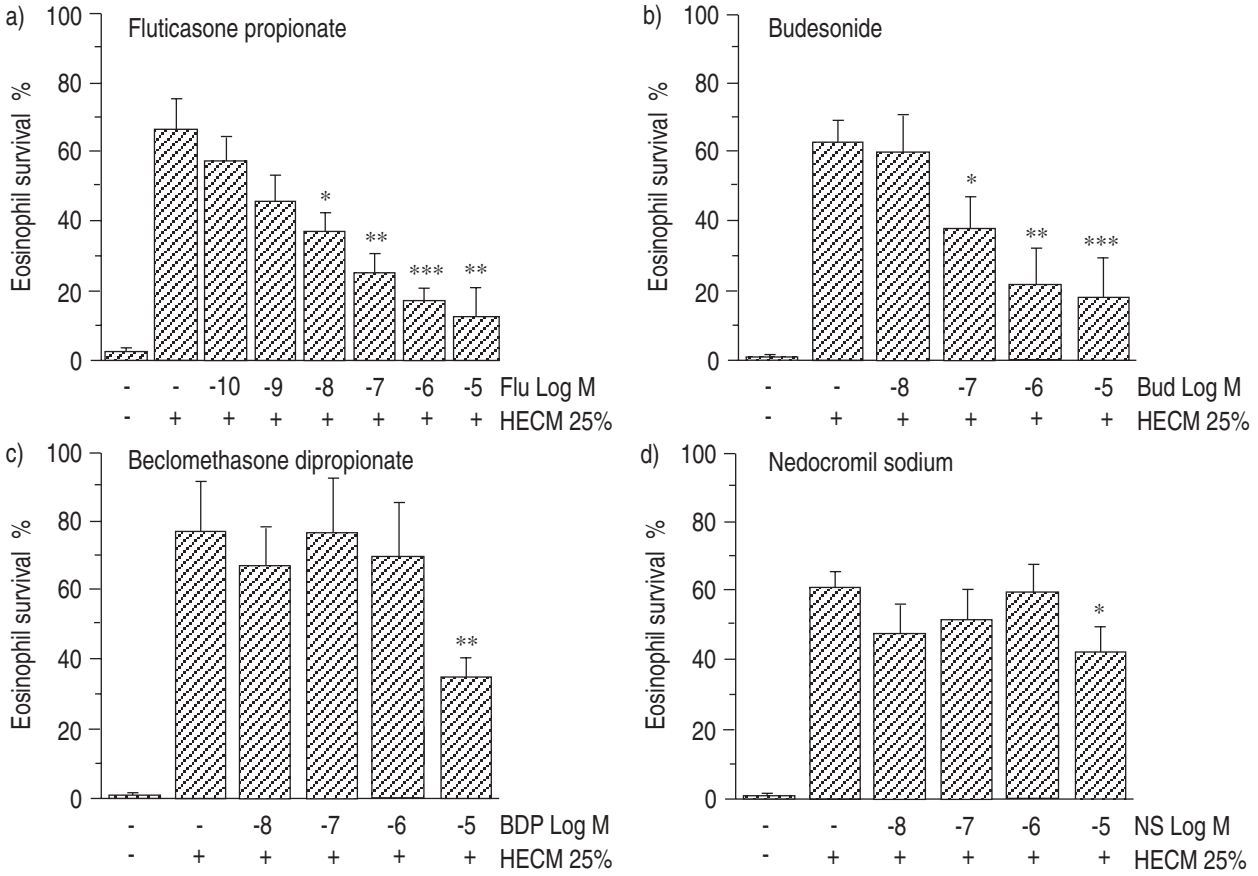

Fig. 4. – Effect of human epithelial cell-conditioned media (HECM) generated with topical glucocorticoids and nedocromil sodium on eosinophil survival. HECM generated without drugs increased eosinophil survival compared to controls. HECM generated with increasing concentrations of: a) fluticasone propionate (Flu, n=5); b) budesonide (Bud, n=6); c) beclomethasone dipropionate (BDP, n=5); and d) nedocromil sodium (NS, n=6) showed a dose-related inhibitory effect on eosinophil survival compared to HECM generated without drugs. Values are expressed as percentage of eosinophil survival index (mean±SEM). Student's paired t-test was used for statistical analysis. Asterisks represent values significantly different from the HECM value. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Table 1. – IC_{25} of topical glucocorticoids and nedocromil sodium on induced eosinophil (Eos) survival and GM-CSF release from nasal epithelial cells

Drugs	IC_{25}	
	Eos survival M	GM-CSF* M
Fluticasone propionate	1×10^{-9}	8.4×10^{-11}
Budesonide	3.3×10^{-8}	2×10^{-9}
Beclomethasone dipropionate	1.5×10^{-6}	1.3×10^{-8}
Nedocromil sodium	5×10^{-6}	$>10^{-5}$

IC_{25}: 25% inhibitory concentration; GM-CSF: granulocyte/macrophage colony-stimulating factor; FCS: foetal calf serum. *: the inhibitory effect of topical glucocorticoids and nedocromil sodium on GM-CSF release induced by 10% FCS in cultured nasal mucosal epithelial cells correlated highly and significantly ($r=0.955$; $p<0.05$) with the effect on eosinophil survival induced by epithelial cell secretions.

Discussion

Epithelial cell secretions enhance eosinophil survival *in vitro*, and this effect is abrogated by previous incubation of epithelial cells with steroids. Our results suggest that topical anti-inflammatory drugs may decrease eosinophil infiltration by acting on epithelial cells. Using this *in vitro* model, we have investigated the potency of several topically-administered glucocorticoids (fluticasone propionate, budesonide and beclomethasone dipropionate) and nedocromil sodium.

Fluticasone propionate was found to be the most potent glucocorticoid, followed by budesonide and beclomethasone dipropionate. The responses were concentration-dependent and showed a rank of steroid potency profile similar to that seen in other *in vitro* assays [20, 21]. However, the differences in potency among the three glucocorticoids were more marked in this *in vitro* method than those found by the vasoconstrictor test. Fluticasone propionate, for instance, has been found to be only twice as potent as beclomethasone dipropionate in some studies [22]. However, the capacity of steroids to blanch the skin is not necessarily correlated with their anti-inflammatory properties in the airways. The vasoconstrictor test is influenced by differences in tissue penetration [21]. A high lipophilicity of the glucocorticoid will enhance penetration through the skin, but the skin and the airway tissues are very different. Glucocorticoids do not have to cross a barrier after topical application, as in the case of dermal products, and therefore additional factors such as lipophilicity and absorption have less influence on the nose and bronchi than on the skin.

Ideally, the properties of drugs used topically in the treatment of rhinitis and bronchial asthma have to be investigated in *in vitro* systems, with characteristics close to the pathophysiology of allergic inflammation in the

upper airways. The present *in vitro* method is useful in the evaluation of the efficacy of old and new drugs [18]. It is also very useful for investigating the mechanism of action of glucocorticoids. Using this method, it was demonstrated that topical glucocorticoids abrogate GM-CSF release from cultured nasal epithelial cells. Since this cytokine is involved in the eosinophilic survival-promoting effect of epithelial cell supernatants [17, 19, 23], these findings suggest that the therapeutic effect of topical glucocorticoids may, at least in part, be due to their capacity to inhibit GM-CSF release. It is possible, however, that inhibition of other eosinophil survival-enhancing factors released by epithelial cells, such as IL-8 and tumour necrosis factor-α (TNF-α) [17], which were not measured in the present study, also contributes to reducing eosinophil viability. It is interesting to note that the concentrations effective in inhibiting eosinophil survival were clearly higher than those inhibiting GM-CSF release. This finding also suggests that inhibition of eosinophil-activating factors other than GM-CSF may be responsible for the steroid effect on eosinophil viability.

Nedocromil sodium was also found to inhibit epithelial cell-induced eosinophil survival, although its effect was less potent than that of topical glucocorticoids. Nedocromil sodium also showed the lowest inhibitory potency with respect to GM-CSF release. This finding is in keeping with previous studies showing that nedocromil sodium significantly prevents the upregulation of GM-CSF production by IL-1, and suggesting that it might possibly promote its anti-inflammatory action by modulating the stimulated-release of GM-CSF by other cytokines [15, 16]. Nedocromil sodium also abrogates the N-formyl-methionyl-leucyl-phenylalanine (fMLP) and zymosan-induced activation of human peripheral eosinophils [9, 10, 14], and inhibits the chemotaxis of eosinophils induced by platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) [12]. Spry *et al*. [13] demonstrated that nedocromil sodium blocks the complement-induced eosinophil cationic protein and eosinophil peroxidase release.

Although extrapolation of the present *in vitro* results to the clinical setting will require careful validation, it is interesting to note that, to some extent, these findings agree with recent clinical studies showing that fluticasone propionate is more effective than beclomethasone dipropionate [24–26] and budesonide [27]. These results are also in keeping with clinical trials demonstrating that nedocromil sodium has a moderate antiasthma effect compared to glucocorticoids [28].

The higher potency of budesonide compared to beclomethasone dipropionate that was detected in the present study, contrasts with clinical studies which reflect a similar efficacy for both drugs [29, 30]. While *in vitro* studies may show clear differences between topical drugs, demonstrating such differences clinically is less straightforward. The different results regarding potency of glucocorticoids when tested *in vitro* compared to *in vivo* assessment may be due to real differences in the experimental models. In this regard, the present results should be interpreted with caution because an *in vitro* test was used, which is very simple compared to the complexity of the inflammatory response *in vivo*. Other factors, such as lack of statistical power due to the small number of patients involved in a study, may also contribute to the failure to detect differences between treatments in some clinical trials. Only studies involving an appropriate number of patients will detect differences in the efficacy of the drugs evaluated. As an example, in a comparative study showing differences between fluticasone propionate and budesonide, 671 patients were recruited [27], whereas, in clinical trials showing a similar efficacy for beclomethasone and budesonide, only 128 patients [29], or less than 30 patients [30] were included.

Differences in the metabolism of beclomethasone and budesonide might also explain the present findings. During incubation in human lung tissue, beclomethasone dipropionate is rapidly hydrolysed to beclomethasone-17 monopropionate (17-BMP), with a much higher glucocorticoid receptor activity [31]. This observation indicates that the metabolism of BDP to 17-BMP is an important activating step, resulting in a much more potent substance. It may well be that in our *in vitro* model this transformation did not occur, or was incomplete, and that this accounted, at least in part, for the differences in potency observed between beclomethasone dipropionate and budesonide.

In conclusion, topical anti-inflammatory drugs may decrease eosinophil survival by abrogating the promoting effect of epithelial cells. These drugs may exert part of their therapeutic effect by modulating release of cytokines, such as granulocyte/macrophage colony-stimulating factor, from epithelial cells. The following rank of potency was observed: fluticasone propionate > budesonide > beclomethasone dipropionate > nedocromil sodium. Study of the interaction between epithelial cells and eosinophils may be a useful method to investigate and compare the potency of topical drugs.

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