Epithelial differentiation is a determinant in the production of eotaxin-2 and -3 by bronchial epithelial cells in response to IL-4 and IL-13

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Received 28 March 2006; accepted 16 April 2006
Available online 5 June 2006

Abstract

The composition of the airway epithelium is dynamic and epithelial differentiation is regulated by endogenous mediators as well as inhaled substances. In atopic asthma the differentiation of the epithelium is altered. Various studies have addressed the ability of cultured airway epithelial cells to release the eosinophil-attractant chemokines eotaxin, eotaxin-2 and eotaxin-3 using epithelial cell lines or poorly differentiated primary cells. Since little is known about the role of the epithelial differentiation state in the response of epithelial cells to stimuli that increase production of mediators such as the eotaxins, we analyzed the effect of differentiation state on the production of the eotaxins. In particular, we investigated the effects of the Th2 cytokines IL-4 and IL-13 on eotaxin-2 and -3 production by primary human bronchial epithelial cells and examined whether their production is affected by epithelial cell differentiation using both submerged and air–liquid interface (ALI) cultures. The results show that both IL-4 and IL-13 increase eotaxin-2 and -3 mRNA expression and protein release in submerged- and ALI-cultures. Moreover, epithelial differentiation in ALI-cultures appeared an important determinant in the regulation of eotaxin-2 and -3. Mucociliary differentiation of the epithelial cells was induced by culture in the presence of a high concentration of retinoic acid (RA), whereas low concentrations of RA resulted in a flattened squamous epithelial phenotype. Mucociliary differentiated ALI-cultures expressed and released more eotaxin-3 upon stimulation with IL-4/IL-13, whereas eotaxin-2 production was predominantly found in squamous differentiated ALI-cultures. TNFα reduced IL-4-induced eotaxin-2 release in submerged cultures but not in ALI-cultures; no effects on eotaxin-3 synthesis were observed. The results indicate that epithelial differentiation is an important determinant in Th2 cytokine-induced eotaxin-2 and -3 release by airway epithelial cells. These findings may provide new insights into the role of airway epithelial differentiation and Th2 cytokines in the pathogenesis of inflammatory lung disorders such as asthma.

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Keywords: Chemokines; Eotaxins; Th2 cytokines; Epithelial differentiation; Air–liquid interface cultures

1. Introduction

Asthma is a chronic condition characterized by variable airflow obstruction, airway hyperresponsiveness and chronic inflammation. This inflammation is particularly dominated by the infiltration of eosinophils and T lymphocytes into the airways (Bousquet et al., 2000). Increased numbers of eosinophils are present in bronchial lining fluid, mucosa and peripheral blood and are indicated to correlate with disease severity and airway hyperresponsiveness (Bousquet et al., 1990). These findings implicate eosinophils as the critical effector cells in the pathogenesis of asthma. Currently a wide range of molecules involved in eosinophil recruitment, survival and activation have been described, including the family of eotaxins. In humans, the family of eotaxins consists of three members, i.e. eotaxin (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26). They all act via the CCR3 receptor, which is expressed by various cell types including eosinophils (Forssmann et al., 1997; Kitaura et al., 1999), and have comparable functional properties. Eotaxin mRNA and protein levels are increased in both tissue and bronchoalveolar lavage fluid of patients with asthma after allergen challenge (Brown et al., 1998). The levels of eotaxin were found to correlate with eosinophilia and impaired lung function in asthmatics (Lilly et al., 2001; Nakamura et al., 1999). In addition to eotaxin, also increased expression of eotaxin-2 and -3 by epithelial cells in asthmatics as compared to healthy controls has been reported (Berkman et al., 2001; Komiya et al., 2003). A limited number of
studies suggest that the three eotaxins differentially contribute to the recruitment of eosinophils. Mice studies demonstrated that neutralization or knocking out the eotaxin gene fails to result in a strong attenuation of eosinophilia indicating that an additional mechanism is involved (Rothenberg et al., 1997). Studies in humans showed increased levels of eotaxin and eotaxin-2 in stable asthmatics when compared to healthy controls, whereas eotaxin-3 was only significantly increased after allergen challenge (Berkman et al., 2001) suggesting a role for eotaxin-3 in the allergen-induced eosinophil recruitment. Results from our own laboratory suggested that both eotaxin-2 and -3 are involved in eosinophil recruitment following allergen exposure in asthmatics (Ravensberg et al., 2005). Finally, injection of allergen into the skin of atopic subjects revealed that eotaxin-2 expression correlated with the late-phase influx of eosinophils (Ying et al., 1999). Whereas these observations point to a role of eotaxins in asthma, data about the regulation of eotaxins is limited and predominantly based on mRNA levels. Studies using airway epithelial cell lines have shown the induction of eotaxin expression upon stimulation with TNFα and IL-1β (Lilly et al., 1997). In addition, synergistic effects of TNFα and IL-4 on eotaxin release have been reported (Atasoy et al., 2003; Matsukura et al., 1999). Studies using epithelial cells have shown that IL-4 and IL-13 also induce eotaxin-2 and eotaxin-3 mRNA and protein release in primary bronchial epithelial cells (Komiya et al., 2003), and in the human A549 alveolar type II epithelial-like cells (Abonyo et al., 2005).

Airway epithelium is increasingly recognized as an important regulator of inflammatory processes in the lung through its ability to produce a range of cytokines, chemokines, and other inflammatory mediators. The pseudostratified bronchial epithelium is composed of various cell types including basal cells, mucus producing goblet cells, ciliated cells and Clara cells (Corrin, 2000; Danel, 1996). Both in vivo and in vitro studies have demonstrated that the composition of the airway epithelium is dynamic and airway epithelial differentiation is regulated by cytokines, growth factors, and other endogenous mediators as well as inhaled substances (e.g. cigarette smoke and microbial products). Most in vitro studies examining the regulation of chemokines and cytokine release by airway epithelial cells are performed either using epithelial cell lines or submerged cultures of primary bronchial epithelial cells. Submerged cultured cells are limited in their ability to develop into fully differentiated pseudostratified epithelial layers. This is at least in part explained by the fact that: (i) they are cultured on plastic surfaces; (ii) not at an air–liquid interface. Several groups, including our own, have now developed an air–liquid interface culture system consisting of polarized cells growing on an extracellular matrix with a pseudostratified mucociliary histology that mimics many of the properties of bronchial epithelium in vivo (de Jong et al., 1993; Gray et al., 1996). However, so far little attention has been paid to the functional consequences of epithelial differentiation regarding chemokine and cytokine release.

In the present study we investigated the effect of IL-4 and IL-13 on the regulation of eotaxin, eotaxin-2 and eotaxin-3 expression by primary bronchial epithelial cells in both poorly (submerged) and well-differentiated air–liquid interface airway epithelial cell cultures. For the air–liquid interface cultures, both well-differentiated mucociliary cultures and squamous differentiated cultures were used.

2. Materials and methods

2.1. Culture of human airway epithelial cells

Primary bronchial epithelial cells (PBEC) were obtained from resected lung tissue by enzymatic digestion as described previously (van Wetering et al., 2000). Cells were subcultured in a 1:1 mixture of DMEM (Gibco, Grand Island, NY) and bronchial epithelial growth medium (BEGM; Clonetics, San Diego, CA) supplemented with 0.4% (w/v) bovine pituitary extract (BPE), 0.5 ng/ml epidermal growth factor (EGF), 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 10 μg/ml transferrin, 1 μM hydrocortisone, 6.5 ng/ml T3, 0.5 μg/ml epinephrine (all from Clonetics), 1.5 μg/ml bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO), 1 mM HEPES (Gibco), 20 U/ml penicillin and 20 μg/ml streptomycin (Bio Whittaker: Walkersville, MD), hereafter referred to as growth medium.

For air–liquid interface (ALI) cultures, cells were seeded (40,000 cells per insert) on semi-permeable Transwell membranes (Transwell, 0.4 μm pore-size, 6.5 mm diameter Corning Costar; Cambridge, MA) that were precoated with a mixture of 10 μg/ml BSA, 30 μg/ml Nitrogen (30 μg/ml Celsrix Laboratories, Palo Alto, CA) and 10 μg/ml fibronectin in phosphate buffered saline (PBS). Cells were either cultured in the same medium as described above or in this medium supplemented with a higher concentration of retinoic acid (RA, 15 ng/ml; Sigma). These cultures are hereafter referred to as RA-low and RA-high cultures, respectively. Cultures were grown submerged for 4–7 days after which they were exposed to an air–liquid interface for another 2 weeks. Mucociliary differentiation was observed between days 7 and 10 after exposure to air–liquid interface. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2.

2.2. Stimulation of airway epithelial cells

To analyze the effect of Th2 cytokines on cytokine expression and release in airway epithelial cells, PBEC were cultured in 24-well plates in growth medium until confluence. Prior to the experiments, cells were cultured overnight in stimulation medium (growth medium without BPE and EGF) to obtain quiescent cell cultures. Cells were stimulated in triplicate for various time-periods with different concentrations of IL-4 and IL-13 (Peprotech, Rocky Hill, NJ). To examine a possible effect of TNFα (Peprotech) on Th2 cytokine-induced synthesis of eotaxins, cells were stimulated either with Th2 cytokines alone or in combination with TNFα (20 ng/ml).

ALI-cultures were stimulated at the basal side with IL-4 or IL-13 (20 ng/ml) alone or in combination with TNFα (20 ng/ml) in complete medium for the duration of the culture (e.g. 2 weeks). Medium with and without stimulus was refreshed every 48 h. Stimulation was started the first day cells were exposed to air.
Cell counting was performed at the end of the culture by trypan blue staining.

2.3. RNA isolation and RT-PCR

PBEC were treated as described above and total RNA was extracted using the RNeasy Minikit (Qiagen, Valencia, CA) and reverse transcribed using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) as recommended by manufacturers. Equal loading of cDNA was evaluated by assessing the expression of the housekeeping gene β-actin. The PCR primers for β-actin were AAGGAAGGCTGGAAGAGTG (forward) CTACAATGAGCTGCGTGTTGG (reverse); for eotaxin: CCCCACCGACTGGAGAGC (forward) and ACACCTGCTGCTGCTGCTGCTG (reverse); for eotaxin-2: CCCACCAGACGACTCCCTCAC (forward) and GTCGCCACAGAACTGCTG (reverse) and for eotaxin-3: GCCTGATTTGCAGCATCATGATGG (forward) and CGGATGACAATTCAGCTGAGTCAC (reverse). PCR amplification was performed in a final volume of 25 μl PCR buffer (Promega, Madison, WI) containing varying final concentrations of MgCl₂ (2 mM for β-actin and eotaxin, 2.5 mM for eotaxin-2 and 3 mM for eotaxin-3), 2 pmol of each primer, 0.2 mM dNTP (Invitrogen) and 0.04 U/μl Taq polymerase (Promega). Amplified products were loaded on a 1% agarose gel.

2.4. Measurement of chemokine release

Culture supernatants of both submerged and ALI-cultured PBECs were harvested at various time-points and assessed for eotaxin, eotaxin-2 and -3 by specific sandwich ELISAs from R&D systems (R&D systems, Abingdon, UK). The detection limit of the eotaxin and eotaxin-2 ELISA was 20 pg/ml, whereas the detection limit for eotaxin-3 was 50 pg/ml. IL-8 levels were measured in cell-free supernatants using a IL-8-specific sandwich ELISA (BioSource International, Nivelles, Belgium).

2.5. Immunohistochemistry

ALI-cultures were fixed in 10% buffered formalin overnight at 4 °C, dehydrated and embedded in paraffin. Four micrometers...
sections were stained with hematoxylin and eosin, and examined by light microscopy.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical differences were assessed using the Student t-test. Differences with a P-value < 0.05 were considered significant.

3. Results

3.1. Effect of IL-4 and IL-13 on eotaxin, eotaxin-2 and eotaxin-3 release by submerged primary bronchial epithelial cells cultures

The effect of IL-4 and IL-13 on eotaxin, eotaxin-2 and -3 release by submerged cultures of primary bronchial epithelial cells (PBEC) was studied by stimulating cells for various time-periods with different concentrations of Th2 cytokines. Both IL-4 and IL-13-induced eotaxin-2 and -3 release in a dose- and time-dependent manner (Fig. 1A–D). In contrast, eotaxin release was not detected (not shown). The effect of IL-4 on both eotaxin-2 and -3 protein was already observed within 24 h of stimulation at concentrations starting from 10 ng/ml. Whereas eotaxin-2 protein levels continued to increase up to 72 h of stimulation (Fig. 1C), a striking increase in eotaxin-3 protein was observed after 48 h of stimulation (Fig. 1D). Similar results were obtained with IL-13, although, based on protein concentration, IL-13 was a far less potent stimulus than IL-4.

The increase in eotaxin-2 and -3 production upon stimulation with IL-4 and IL-13 was accompanied by increased mRNA levels of the corresponding genes (Fig. 1E), indicating that there was de novo synthesis of the eotaxins. Both eotaxin-2 and -3 mRNA were enhanced upon stimulation of cells with IL-4 and IL-13 at concentrations of 10 ng/ml or higher at all time-points studied.

3.2. Effect of IL-4 and IL-13 on eotaxin, eotaxin-2 and eotaxin-3 release by air–liquid interface cultures of primary bronchial epithelial cells

Air–liquid interface (ALI) cultures are increasingly used as a model to study airway epithelial cell function, since this model enables the study of a well-differentiated cell culture consisting of various epithelial cell types. It is, however, unclear whether these well-differentiated cultures respond similarly to Th2 cytokines as the poorly differentiated submerged cultures. Therefore, ALI-cultures were stimulated at the basal side with IL-4 or IL-13 during the period of culture at the ALI and the basolateral release of eotaxins was examined. In addition, we studied whether the release of eotaxins was affected by the type of differentiation (squamous or mucociliary differentiated cells). Therefore, cells were cultured either in the absence or presence of additional retinoic acid (RA), an agent known to induce mucociliary differentiation (Gray et al., 1996; Lopez-Souza et al., 2004). As shown in Fig. 2A (left upper panel), in the absence of additional RA (RA-low cultures), morphological features of a flattened squamous phenotype with signs of keratinisation are observed. In the presence of additional RA (RA-high cultures) a fully mucociliary differentiated culture is obtained (Fig. 2B, right upper panel), as demonstrated by the presence of goblet cells, ciliated cells and basal epithelial cells. Furthermore, stimulation of both RA-low and RA-high cultures with IL-4 and IL-13 resulted in an increase in the number of cells in both cultures (Fig. 2A, left lower panel and B), suggesting that both IL-4 and IL-13 induce proliferation of the epithelial cells.

In both the RA-low (squamous differentiated) and RA-high (mucociliary differentiated) cultures, IL-4/IL-13 stimulation resulted in a dose-dependent increase in eotaxin-2 and -3 protein release (Fig. 3). Stimulation of cells with a combination of IL-4 and IL-13 did not result in an additive effect (not shown). In addition, the results show that the type of epithelial cell differentiation affects the release of IL-4/IL-13-induced eotaxin-2 and -3 protein. This is illustrated by the observation that both the spontaneous and IL-4/IL-13-induced eotaxin-2 production is higher in the RA-low cultures when compared to the RA-high...
Fig. 3. Effect of IL-4 and IL-13 on eotaxin-2 and -3 protein synthesis release in air–liquid interface cultures: modulation by retinoic acid. ALI-cultures were cultured either in the absence (open bars) or presence (hatched bars) of additional retinoic acid and IL-4/IL-13 (10 ng/ml) during culture at the air–liquid interface. Basal media were harvested and analyzed for eotaxin-2 and -3 protein by ELISA; mRNA was isolated and analyzed by RT-PCR. Panels A and B) IL-4/IL-13-induced eotaxin-2 (panel A) and eotaxin-3 (panel B) protein release. Results are depicted as mean ± S.E.M. of at least six different experiments each performed in triplicate and using cells from different donors.* P<0.01 as compared to the corresponding control (RA-low or RA-high); # P<0.01 as compared to cells treated with the same stimulus in the absence of RA.

cultures (Fig. 3A), whereas the IL-4/IL-13-induced eotaxin-3 production is higher in the RA-high cultures as compared to the RA-low cultures (Fig. 3B). Finally, as observed for the submerged cultures, no eotaxin synthesis, either protein or mRNA, could be detected (not shown).

3.3. Effect of TNFα on Th2 cytokine-induced eotaxin-2 and -3 release in both submerged and ALI-primary bronchial epithelial cultures

Previous studies demonstrated a synergistic effect of TNFα and IL-4 on eotaxin release by various cell types, including airway epithelial cells (Fujisawa et al., 2000; Terada et al., 2000). In the present study, we analyzed whether TNFα would also affect IL-4/IL-13-induced eotaxin-2 and -3 production in both submerged and ALI-cultures. In the submerged cultures, TNFα itself had no effect on the release of eotaxins, but significantly reduced IL-4-induced eotaxin-2 release and tended to decrease IL-13-induced eotaxin-2 protein production. In contrast, no effect was observed on eotaxin-3 protein release (Fig. 4A and B). Furthermore, TNFα did not reduce IL-4-induced eotaxin-2 mRNA expression (Fig. 4D), suggesting that the inhibitory effect of TNFα on eotaxin-2 protein release is regulated at a post-transcriptional level. To demonstrate that TNFα was biologically active, the amount of IL-8 secreted by the airway epithelial cells was analyzed. As expected, TNFα caused a marked increase in

Fig. 4. Modulation of IL-4/IL-13-induced eotaxin-2 and -3 synthesis by TNFα. Submerged PBEC cultures were stimulated with IL-4 (10 ng/ml), IL-13 (10 ng/ml) or medium alone (open bars) or in the presence of TNFα (20 ng/ml, closed bars) for 24 h. Supernatants were harvested and analyzed for eotaxin-2 (panel A), eotaxin-3 (panel B) or IL-8 (panel C) by ELISA. Results are shown as mean ± S.E.M. of at least six individual experiments performed in triplicate and each using cells of different donors. * P<0.05 as compared to corresponding control–treated cells, † P<0.05 as compared to TNFα-stimulated cells and ‡ P<0.05 as compared to corresponding sample stimulated in the presence of TNFα. (Panel D) Representative PCR showing the effect of TNFα on IL-4/IL-13-induced eotaxin-2 and -3 mRNA synthesis.
Various studies have demonstrated increased eotaxin-2 and -3 production by IL-4/IL-13 in ALI-cultures. Nevertheless, others have shown increased eotaxin release by airway epithelial cells after stimulation with TNFα (Atasoy et al., 2003; Takamura et al., 2004). This discrepancy may be attributed to the culture technique used.

In our cultures we were unable to detect eotaxin synthesis upon stimulation with IL-4 and IL-13 alone or in combination with TNFα. This is in agreement with a study by Komiy et al. (2003), which also reported a lack of basal and IL-4/IL-13-induced eotaxin expression in submerged cultures of primary bronchial epithelial cells. Nevertheless, others have shown increased eotaxin release by airway epithelial cells after stimulation with Th2 cytokines (Atasoy et al., 2003; Meyer-Hoffert et al., 2003; Takamura et al., 2004). This discrepancy may be attributed to the culture technique used.

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4. Discussion

The results of the present study demonstrate that epithelial differentiation in ALI-cultures appears to be a major determinant of eotaxin-2 and -3 production by IL-4/IL-13. Mucociliary differentiated cultures express and release more eotaxin-3 upon stimulation with IL-4/IL-13, whereas squamous differentiated cultures express and release more eotaxin-2. In addition, a difference in IL-4/IL-13-induced eotaxin-2 and -3 synthesis was observed in poorly differentiated (submerged) versus differentiated (both squamous and mucociliary) air–liquid interface (ALI) cultures of primary bronchial epithelial cells. Eotaxin production was not detected in either culture system used. Furthermore, TNFα reduced IL-4/IL-13-induced eotaxin-2 release without affecting eotaxin-3 synthesis in submerged but not in ALI-cultures. These data indicate that epithelial differentiation affects the levels of cytokine release.

Various studies have demonstrated increased eotaxin-2 and -3 synthesis upon stimulation with IL-4/IL-13 in both airway epithelial cell lines and primary bronchial epithelial cells. However, most studies explored mRNA levels and protein data are limited (Komiy et al., 2003; Atasoy et al., 2003; Meyer-Hoffert et al., 2003). Our study is the first study to investigate the effect of IL-4/IL-13 on production of eotaxins by primary bronchial epithelial cells grown in an ALI-culture and its regulation by cellular differentiation. The use of the ALI-culture model enabled us to study a pseudostratified mucociliary cell culture, composed of a mixture of cells also seen in vivo. To date various other studies demonstrated that epithelial differentiation is an important determinant in controlling epithelial cell function. Kikuchi et al. (2004) showed that the ability of epithelial cells to release GM-CSF and TGFβ was abolished when cells were allowed to differentiate into a mucociliary phenotype. This effect could not be mimicked by the addition of retinoic acid to the submerged cultures. Furthermore, a differentiation-dependent increase of production of several mucins has been reported (Bernacki et al., 1999), and the resistance of a differentiated epithelial cell layer to infection by rhinovirus is much higher as compared to submerged cultures (Lopez-Souza et al., 2004). Finally, in a recent study it was shown that CINC-2β (a splice variant of CINC-2) was only expressed in differentiated rat alveolar type II epithelial cells (Nishina et al., 2005). These studies point to a role of epithelial differentiation in the secretion of inflammatory mediators by these cells. In addition, these observations emphasize that the culture technique used should be taken into account when interpreting and designing studies aimed to understand responses of the airway epithelium.

Previous studies in fibroblasts and airway epithelial cells reported a synergistic interaction between TNFα and IL-4 on eotaxin release (Atasoy et al., 2003; Matsukura et al., 1999). In our cultures we were unable to detect eotaxin synthesis upon stimulation with IL-4 and IL-13 alone or in combination with TNFα. This is in agreement with a study by Komiy et al. (2003), which also reported a lack of basal and IL-4/IL-13-induced eotaxin expression in submerged cultures of primary bronchial epithelial cells. Nevertheless, others have shown increased eotaxin release by airway epithelial cells after stimulation with Th2 cytokines (Atasoy et al., 2003; Meyer-Hoffert et al., 2003; Takamura et al., 2004). This discrepancy may be attributed to the culture technique used.

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explained by the fact that these studies were performed using airway epithelial cell lines. Another possibility is that retinoic acid present in culture media used to culture primary epithelial cells suppresses eotaxin transcription (Takamura et al., 2004). This seems, however, unlikely since the concentrations normally used in media (10^{-8} M) are lower than the inhibitory concentrations reported. Increased expression of eotaxin in airway epithelial cells has been reported in lung tissue from asthmatics as compared to healthy controls (Lilly et al., 2001; Komiya et al., 2003), indicating that airway epithelial cells are able to express eotaxin in situ. In our hands stimulation of cultured primary epithelial cells with Th2 cytokines or TNFα did not result in increased eotaxin release suggesting that other factors present in the airways may contribute to eotaxin expression in epithelial cells.

In the lung several cell types, including monocytes, fibroblasts, smooth muscle cells and epithelial cells, have been reported to produce eotaxins (Komiya et al., 2003; Lamkhioued et al., 1997; Teran et al., 1999; Zuyderduyn et al., 2004). The mechanisms that control the expression of the eotaxins have been partially unravelled. The transcription factor STAT-6 has been shown to be involved in the regulation of all three eotaxins (Atasoy et al., 2003; Matsuura et al., 1999; Zimmermann et al., 2000; Hock and Woietschlager, 2001). The presence of a NF-κB binding site has so far only been reported for the eotaxin promoter (Matsukura et al., 1999). In vivo studies have suggested that eotaxin expression is associated with the early phase of allergen-induced recruitment of eosinophils (Brown et al., 1998), whereas eotaxin-2 and -3 may be involved in the late-phase eosinophil recruitment (Berkman et al., 2001; Ying et al., 1999). Also our results point to differential regulation of the eotaxins. First, IL-4/IL-13-induced eotaxin-2 and -3 production without affecting eotaxin synthesis. Secondly, although we observed a significant increase in eotaxin-2 and -3 release after 24 h of incubation with IL-4 and IL-13, the time-courses of eotaxin-2 and -3 production were different (Fig. 1C and D). For eotaxin-2, the increase upon IL-4 stimulation was comparable for all time-periods. For eotaxin-3, however, the most striking increase upon IL-4/IL-13 stimulation was observed after 48 and 72 h. Furthermore, evidence for a differential regulation of eotaxin-2 and -3 expression was also obtained by our demonstration that cellular differentiation differentially affects eotaxin-2 and -3 release. Finally, TNFα reduced eotaxin-2 protein release without affecting eotaxin-3 release in the submerged cultures. This is in line with results reported by Komiya et al. using primary bronchial epithelial cells and cells of the airway epithelial cell line BEAS-2B, whereas the opposite effect was observed in A549 cells (Komiya et al., 2003). In addition, TNFα also increased IL-4-induced eotaxin-2 release in nasal epithelial cells (Meyer-Hoffert et al., 2003; Lezcano-Meza et al., 2003). Whereas these differences may be explained by differences in cellular origin, it may also suggest that regulation of eotaxins is different between various cell types. In addition, the modulating effect of TNFα on eotaxin-2 synthesis was not found in the ALI-cultures, which may be explained by the different culture technique used or by the different exposure times.

In our experiments we observed that IL-4 was much more potent in inducing eotaxin-2 and -3 release than IL-13. Both IL-4 and IL-13 exhibit overlapping, but not identical effector profiles due to the shared use of type II IL-4 receptor composed of the α-chain of the IL-4 (IL-4Rα) and IL-13 (IL-13Rα1) receptor (Shirakawa et al., 2000). The type I IL-4 receptor is composed of IL-4Rα and a common γ-chain also used by other receptors. In addition, IL-13 binds to IL-13Rα2, which negatively regulates IL-13 function by competing for IL-13 binding and promoting receptor internalization (Kawakami et al., 2001). These receptors are reported to be present on airway epithelial cells (Lordan et al., 2002) and may explain the differences between the ability of IL-4 and IL-13 to induce release of eotaxins. Further research is required to explore this possibility.

As discussed above, epithelial differentiation affects the function of epithelial cells. In our study epithelial differentiation affected the release of eotaxin-2 and -3, although the underlying processes are not known. Retinoic acid was shown to be an essential component for obtaining mucociliary differentiation in vitro. As shown in the present report and also shown by others (Gray et al., 1996; Lopez-Souza et al., 2004), cells are known to develop a squamous phenotype in the absence of retinoic acid. Previously, it was shown that retinoic acid inhibits IL-4-induced eotaxin release in the airway epithelial cell line BEAS-2B via a yet unresolved mechanism (Takamura et al., 2004). Our results cannot be explained by this inhibitory effect of retinoic acid, since addition of retinoic acid did not affect IL-4/IL-13-induced eotaxin-2 and -3 synthesis in submerged cultures (data not shown). Furthermore, in the present study we observed that TNFα-induced IL-8 release is significantly higher in well-differentiated cultures as compared to the squamous differentiated cultures. Whether this is a result of epithelial differentiation or an effect of retinoic acid is not clear. It has been shown that retinoic acid enhances TNFα-induced IL-8 release by activating NF-κB (Chang et al., 2000). Also in our submerged cultures, addition of retinoic acid resulted in a strong increase in TNFα-induced IL-8 release (data not shown).

In conclusion, the results of the present study demonstrate that epithelial differentiation is an important determinant in the regulation of eotaxin-2 and -3 release upon stimulation with the Th2 cytokines IL-4 and IL-13. This may provide new insights in the role of the airway epithelium and Th2-dependent effects in the pathogenesis of asthma.

Acknowledgements

This study was supported by a grant (# 01.27) from The Netherlands Asthma Foundation and from AstraZeneca (Lund, Sweden).

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