

Original article

Regulatory mechanisms of galectin-9 and eotaxin-3 synthesis in epidermal keratinocytes: possible involvement of galectin-9 in dermal eosinophilia of Th1-polarized skin inflammation

Background: Skin eosinophilia is a common feature of allergic skin diseases, but it is unclear how epidermal and dermal eosinophil infiltration is controlled. To investigate regulation of localization of eosinophils in skin, we examined the regulatory mechanisms of expression of eosinophil-specific chemoattractants in dermal fibroblasts and epidermal keratinocytes.

Methods: We analyzed production of eotaxin, eotaxin-2, eotaxin-3 and galectin-9 by dermal fibroblasts and epidermal keratinocytes in response to several stimuli *in vitro*.

Results: Dermal fibroblasts produced eotaxin and eotaxin-3 in response to stimulation by interleukin (IL)-4 and/or tumor necrosis factor- α . Similarly, IL-4 stimulated epidermal keratinocytes to secrete eotaxin-3. However, we did not detect eotaxin mRNA expression or protein secretion by keratinocytes stimulated *in vitro*. Interferon (IFN)- γ induced galectin-9 expression on dermal fibroblasts. Conversely, expression of galectin-9 on epidermal keratinocytes was dose-dependently inhibited by IFN- γ . The immunohistochemical assays revealed that dermal fibroblasts (but not epidermal keratinocytes) in the lesional skin of psoriasis vulgaris (a Th1-polarized disease) express significant levels of galectin-9.

Conclusion: Eotaxin-3 contributes to dermal and epidermal eosinophil infiltration in Th2-polarized skin inflammation in which IL-4 is produced. In contrast, IFN- γ -dominated inflammation appears to mediate eosinophil extravasation into the dermis and eosinophil adhesion to dermal fibroblasts via galectin-9 in association with decreased chemoattractant activity of epidermal galectin-9. The present results reveal a novel mechanism of dermal eosinophilia in IFN- γ -mediated skin inflammation, and reflect concerted chemoattractant production involving dermal and/or epidermal eosinophilia during changes in the local cytokine profile.

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Eosinophils participate in allergic inflammation in which Th2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13 are produced. IL-4, IL-13 and/or tumor necrosis factor (TNF)- α stimulate fibroblasts to generate the eosinophil-specific chemokines eotaxin and eotaxin-3 (1–5), and enhance production of eotaxin and eotaxin-3 by the bronchial epithelial cell line BEAS-2B (6–9). Tissue eosinophilia seems to be a characteristic feature of Th2-polarized disease, but weak or moderate eosinophil infiltration is observed even in Th1-polarized disease (10–12). In this context, the involvement of eosinophil chemoattractants other than eotaxins has been postulated. Recent studies have revealed that interferon (IFN)- γ (a Th1 cytokine) stimulates human fetal fibroblast-1 cells to produce the potent eosinophil chemoattractant galectin-9 (13). Galectin-9 is a β -galactoside-binding lectin that belongs to an animal lectin family of galectins (14). It has

been cloned as a T-cell-derived eosinophil chemoattractant (15), and has N- and C-terminal carbohydrate recognition domains that are connected by a linker peptide (15). Endothelial galectin-9 expression is also induced by IFN- γ (16).

Localization of extravasated eosinophils in mucosal tissues is largely dependent on the chemokines produced by submucosal and epithelial cells and the expression of several cell adhesion molecules. Bronchial epithelial cell-derived eotaxin and eotaxin-3 are probably capable of stimulating submucosal eosinophils to migrate into respiratory epithelium through basement membrane. In the present study, to elucidate regulation of localization of eosinophils in allergic skin inflammation, we examined the regulatory mechanisms of expression of eosinophil-specific chemoattractants in dermal fibroblasts and epidermal keratinocytes in response to several stimuli *in vitro*.

The present results indicate that eotaxin-3 is an eosinophil-specific chemoattractant produced by human keratinocytes stimulated by IL-4. Eotaxin was not produced by keratinocytes under any of the present experimental stimulations. In contrast, IFN- γ stimulated galectin-9 expression in dermal fibroblasts, but significantly inhibited galectin-9 expression in epidermal keratinocytes, suggesting involvement of IFN- γ -induced galectin-9 in dermal, but not epidermal, eosinophilia.

Materials and methods

Reagents

Recombinant human IL-4, IFN- γ and TNF- α were obtained from Genzyme (Cambridge, MA).

Generation and purification of a polyclonal Ab for human galectin-9

Anti-galectin-9 IgG was generated and purified from sera of rabbits immunized with a mixture of recombinant C-terminal carbohydrate recognition domain of human galectin-9 and complete Freund's adjuvant (13).

Cell culture

Normal human keratinocytes and fibroblasts were isolated from skin specimens obtained from plastic surgery of patients who gave written informed consent. The keratinocytes were grown in keratinocyte-SFM (Gibco-BRL, Gaithersburg, MD) supplemented with 0.2 ng/ml epidermal growth factor, 25 mg/ml bovine pituitary extract (BPE) and 1% antibiotics/antimycotics (Gibco-BRL) and the fibroblasts were grown in Dullbecco's modified Eagle's medium (Sigma, St Louis, MO) supplemented with 10% fetal calf serum (Sigma) and 1% antibiotics/antimycotics (Gibco-BRL). For each experiment, the three-passaged keratinocytes and the six-passaged dermal fibroblasts were used with about 70% confluency.

Assay for galectin-9 expression by cell-based ELISA technique

Three-passaged keratinocytes or six-passaged dermal fibroblasts were maintained in the each medium as described above (for dermal fibroblasts, serum was starved for 12 h), and incubated with recombinant human IFN- γ (Genzyme) for 24 h in the 96-well clear enzyme-linked immunosorbent assay (ELISA) plate. Cell-based ELISA for galectin-9 was performed as reported previously with some modifications (17).

Briefly, the plate was fixed with 4% formaldehyde. After washing with buffer A [phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA)], non-specific binding sites were blocked by incubation with 200 μ l of blocking buffer (PBS containing 1% BSA) for 30 min at 37°C. Then, cells were incubated with 6 μ g/ml of rabbit-anti-human galectin-9 antibody for 45 min at 37°C. After washing with buffer A, cells were incubated with goat-anti-rabbit immunoglobulins conjugated with horseradish peroxidase (1/1000; Dako, Glostrup, Denmark) for 45 min at 37°C. The plate was washed with buffer A and 100 μ l of 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate (Sigma) was added and incubated at room temperature for 5–10 min. The reaction was stopped by adding 100 μ l of 1 M H₂SO₄. The absorbance was read using

ELISA plate reader (BIO-RAD, MODEL 2550; Bio-Rad Laboratories, Hercules, CA) at 450 nm.

Measurement of eotaxin and eotaxin-3

Normal human keratinocytes or dermal fibroblasts were cultured with appropriate combinations of cytokines for 24 h and the supernatants were collected. Eotaxin and eotaxin-3 were measured with ELISA kits (range: 15.6–1000 pg/ml for eotaxin, 7.8–500 pg/ml for eotaxin-3; R&D systems, Minneapolis).

Immunohistochemical staining and confocal laser scanning analysis

Deparaffinized and rehydrated tissue sections were immersed into preheated (95–99°C) Target Retrieval Solution® (Dako) and incubated for 10 min. After cooling down at room temperature, the sections were washed three times with TBS-T (Tris-buffered saline containing 0.1% Tween20, pH 7.8) and nonspecific binding sites were blocked by incubation with Protein Block Serum-Free® (Dako Corp., Carpinteria, CA) for 15 min at room temperature. After washing with TBS-T, the sections were incubated with rabbit-anti-human galectin-9 antibody for overnight at 4°C. After three-times washing, tissue sections were reacted with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes Inc., Eugene, OR) for 45 min at 37°C. After further washing, sections were mounted with Fluoprep® (Biomérieux, Marcy l'Etoile, France). Laser scanning confocal microscopic analysis was performed using a Zeiss LSM 510 system (Carl Zeiss Microscopy, Jena, Germany) according to the manufacture's instructions.

Reverse transcriptase-polymerase chain reaction to detect human eotaxin, eotaxin-2, eotaxin-3, and galectin-9

Total cellular RNA isolated using RNeasy™ B (Tel-Test Inc., Friendswood, TX) was digested with DNase I (Takara Biomedicals, Tokyo, Japan). Twenty microliters of reverse transcription (RT) mix consisted of 8 μ l of 5x buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 50 mM DTT, 15 mM MgCl₂), 4 μ l of hexanucleotide mixture (62.5 A₂₆₀ u/ml; Boehringer Mannheim, Mannheim, Germany), 2 μ l of dNTP (2.5 mM each), 4 μ l of 20 u/ μ l of human placenta ribonuclease inhibitor (Takara Biomedicals), 2 μ l of 200 u/ μ l reverse transcriptase (Moloney murine leukemia virus; Takara Biomedicals). The RT mixture was dispensed at 20 μ l/tube with 20 μ l of 40 ng/ μ l total RNA, vortex-mixed, and then incubated at 37°C for 60 min. Reverse transcriptase was inactivated at 70°C for 10 min, then the samples were stored at –70°C.

The polymerase chain reaction (PCR) mixture contained 5 μ l reverse-transcribed RNA (100 ng total RNA), 5 μ l of 10x buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂), 4 μ l of dNTP (2.5 mM each), 2.5 μ l of 20 μ M sense primer, 2.5 μ l of 20 μ M antisense-primer, 0.5 μ l of 1 u/ μ l Perfect Match (Stratagene, La Jolla, CA), 0.4 μ l of 5 u/ μ l Taq DNA polymerase (Takara Biomedicals), and 30.1 μ l of water. The tubes were transferred to a thermal cycler (DNA amplifier PC-700; Astec, Fukuoka, Japan). The reaction was started at 94°C for 3 min, followed by repeated 1 min cycles of 94, 60 and 72°C.

The primers were: β -actin mRNA, 5'-CGCGAGAAGATG ACCCAGATC-3' and 5'-ATCACGATGCCAGTGGTACGG-3'; eotaxin, 5'-CCCAACCACCTGCTGCTTTAACCTG-3' and 5'-TGGCTTTGGAGTTGGAGATTTTGG-3'; for eotaxin-2, 5'-CCATAGTAACCAGCCTTCTGTTC-3' and 5'-GCGTCCA GGTCTTCATGTACCTC-3'; for eotaxin-3, 5'-AACTCCGA

AACAATTGTGACTCAGCTG-3' and 5'-GTAAGTCTGGG AGGAAACACCCTCTCC; for galectin-9, 5'-GATG AGAATGCTGTGGTCCG-3' and 5'-GAAGCCGCCTATG TCTGCA-3'. Aliquots from each sample were resolved by electrophoresis on 3.3% agarose in glycine buffer and stained with ethidium bromide. The reactions were stopped after various numbers of cycles to assess the region of a linear response. Experiments were repeated three times.

Real-time quantitative PCR

Quantitative RT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye (Brilliant SYBR Green QPCR Master Mix, Stratagene) with Mx3000P Real-Time PCR system (Stratagene). The primers for galectin-9 were purchased from Takara Biomedicals.

Statistical analyses

Student's *t*-test was used to determine statistical differences between mean values. *P* < 0.05 was considered as statistically significant. Results are expressed as mean $\pm$ SD.

Results

Expression of eosinophil-specific chemoattractants in human dermal fibroblasts and epidermal keratinocytes

The mechanisms that regulate mRNA of eotaxin, eotaxin-2 and eotaxin-3 in fibroblasts have been analyzed in detail (1–5), but little is known about such mechanisms in keratinocytes. To clarify differences in production of eosinophil-specific chemoattractants in response to various stimuli between dermal fibroblasts and keratinocytes, we measured mRNA levels for eotaxin, eotaxin-2, eotaxin-3, and galectin-9 under stimulation by cytokines.

Dermal fibroblasts expressed eotaxin and eotaxin-3 mRNA in response to IL-4 (Fig. 1). TNF- α synergistically enhanced IL-4-induced mRNA expression of eotaxin, but not eotaxin-3; this is consistent with previous findings (3, 4). Eotaxin-2 mRNA was not detected. Galectin-9 expression was induced by IFN- γ , and to a lesser extent by TNF- α .

In epidermal keratinocytes, we detected minimal constitutive eotaxin-2 expression, which was not affected by the cytokines tested (Fig. 1). Eotaxin mRNA was not detected, even in the presence of IL-4, IFN- γ and/or TNF- α ; this is in striking contrast to previous findings that IFN- γ /TNF- α and IL-4/TNF- α synergistically up-regulated eotaxin production in human keratinocytes (18). Expression of eotaxin-3 mRNA in keratinocytes was up-regulated by IL-4; this is similar to the results for dermal fibroblasts. Galectin-9 was constitutively expressed in keratinocytes. Notably, unlike the results for dermal fibroblasts, IFN- γ inhibited galectin-9 mRNA expression in epidermal keratinocytes.

Eotaxin-3, but not eotaxin, was secreted by keratinocytes stimulated with IL-4

In mRNA expression analysis, eotaxin-3 transcripts were detected in keratinocytes under cytokine stimulation, but eotaxin transcripts were not (Fig. 1). Given the previous finding that eotaxin was produced by keratinocytes stimulated by TNF- α /IL-4 or TNF- α /IFN- γ (18), we assessed secretion of eotaxin and eotaxin-3 protein by keratinocytes in response to cytokine stimulation.

Consistent with the results of mRNA expression analysis, eotaxin protein was not detected in supernatant from keratinocytes under cytokine stimulation, whereas concomitant stimulation with TNF- α and IL-4

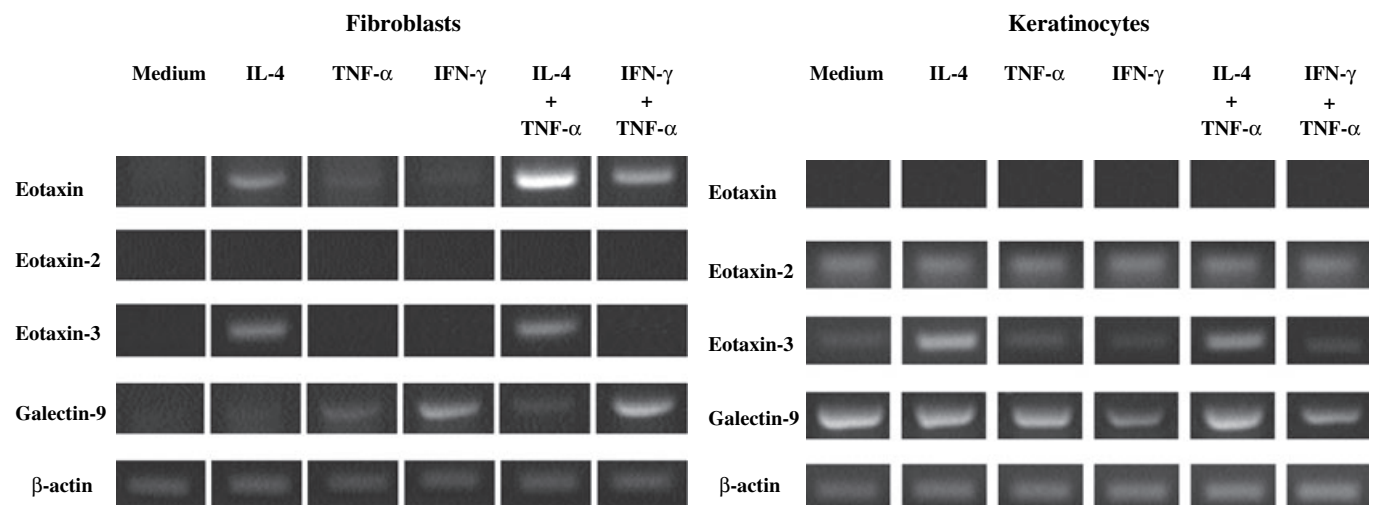


Figure 1. mRNA expression of eosinophil chemoattractants in fibroblasts and keratinocytes. Human dermal fibroblasts and epidermal keratinocytes were incubated with IL-4 (10 ng/ml), tumor necrosis factor- α (10 ng/ml), and/or interferon- γ (10 ng/ml) for 24 h. The levels of mRNA of eotaxin, eotaxin-2, eotaxin-3 and galectin-9 were analyzed. The results shown are from a single representative of three separate experiments.

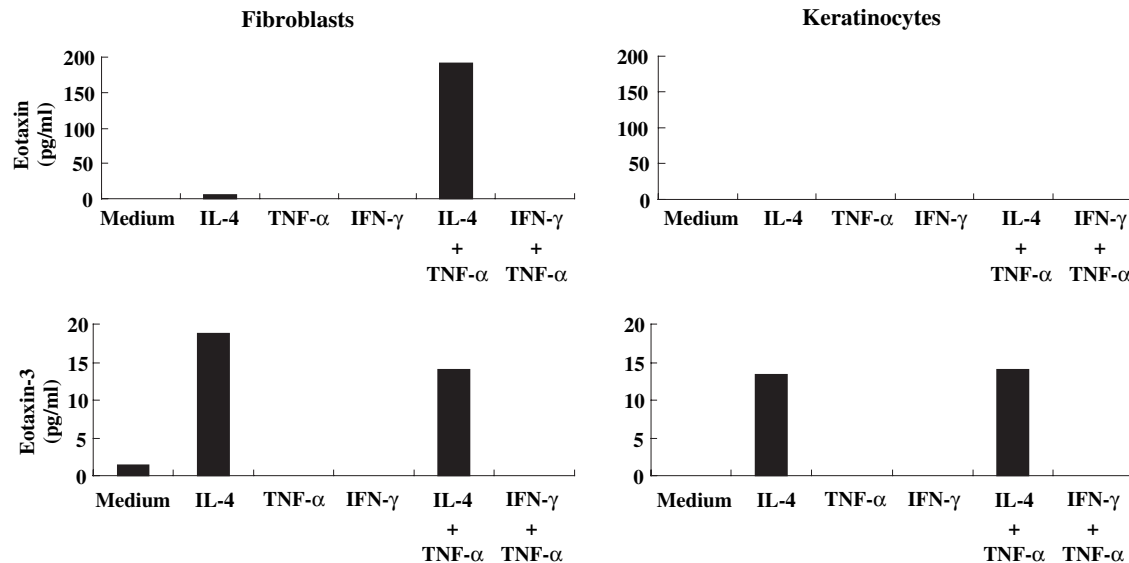


Figure 2. Eotaxin and eotaxin-3 production by keratinocytes and dermal fibroblasts. Human epidermal keratinocytes and dermal fibroblasts were cultured with interleukin (IL)-4, tumor necrosis factor- α and/or interferon- γ for 24 h. Secretion of eotaxin and eotaxin-3 into the culture supernatant was evaluated by enzyme-linked immunosorbent assay. Eotaxin-3, but not eotaxin, was produced by keratinocytes in response to IL-4. The results shown are from a single representative of two separate experiments.

significantly induced production of eotaxin by dermal fibroblasts (Fig. 2). In contrast to the results for eotaxin, IL-4 markedly enhanced production of eotaxin-3 by both keratinocytes and fibroblasts.

IFN- γ stimulated surface expression of galectin-9 on dermal fibroblasts, but inhibited its expression on epidermal keratinocytes

Interferon- γ stimulated mRNA expression of galectin-9 in dermal fibroblasts, but it suppressed mRNA expression of galectin-9 in epidermal keratinocytes (Fig. 1). This inhibitory effect of IFN- γ on keratinocytes was dose-dependent (Fig. 3A). Real-time PCR analysis further confirmed that IFN- γ weakly but significantly inhibited galectin-9 mRNA expression in keratinocytes (Fig. 3B).

We next used cell-based ELISA to assess the reciprocal effect of IFN- γ on galectin-9 synthesis in fibroblasts and keratinocytes. IFN- γ stimulated galectin-9 expression on fibroblasts, but it dose-dependently reduced surface galectin-9 expression on keratinocytes (Fig. 4).

It has been reported that IFN- γ induces apoptosis in keratinocytes (19, 20). Thus, we postulated that the present inhibition of galectin-9 production was because of IFN- γ -induced keratinocyte apoptosis. However, in the MTS assay and trypan blue dye exclusion assay, we did not detect a decrease in cell viability in IFN- γ -stimulated keratinocytes at the concentrations tested (data not shown).

Galectin-9 expression in human skin

Galectin-9 expression in human skin was evaluated by laser scanning microscopy. Epidermal cells, but not

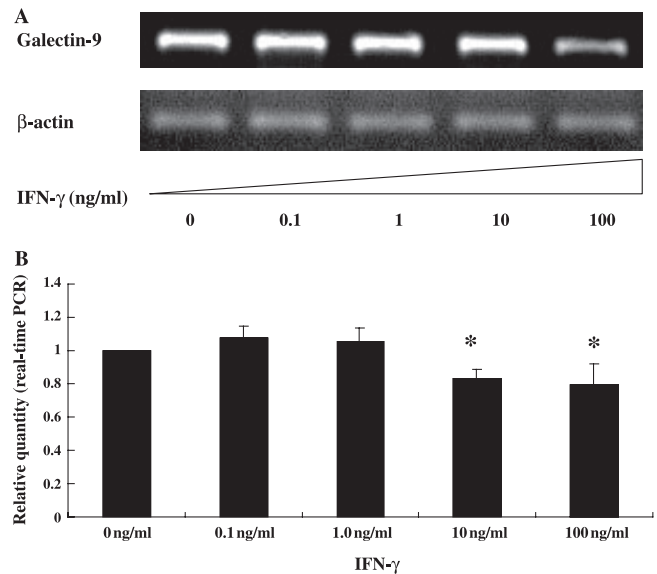


Figure 3. Inhibitory effect of interferon (IFN)- γ on galectin-9 mRNA expression of keratinocytes. Epidermal keratinocytes were stimulated with various doses of IFN- γ for 24 h. The expression of galectin-9 mRNA was dose-dependently suppressed by IFN- γ . The results shown are from a single representative of three separate experiments (A). Real-time PCR analysis of triplicate samples confirmed the inhibitory effect of IFN- γ on galectin-9 mRNA levels in keratinocytes. The value 'relative quantity' indicates the relative amount of mRNA of target genes, compared with levels of β -actin mRNA ($n = 3$). The relative quantity of genes in unstimulated cells (medium alone) was designated as 1.0.

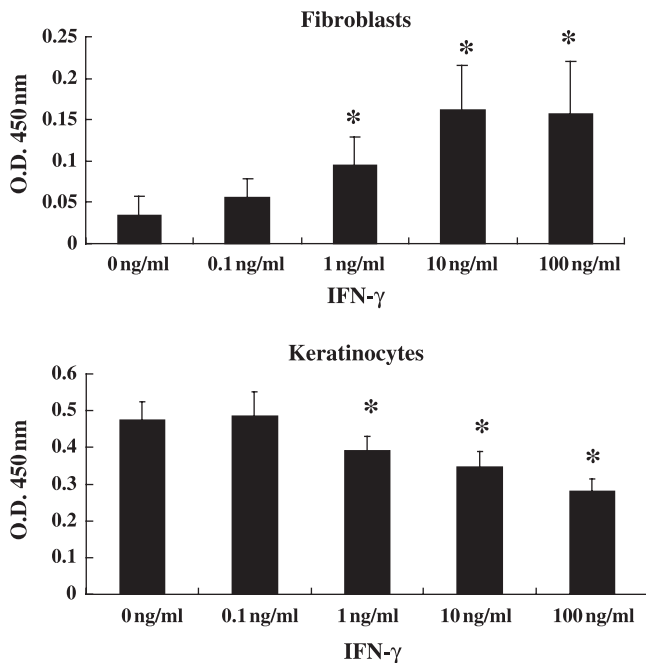


Figure 4. Effect of interferon (IFN)- γ on surface expression of galectin-9 on fibroblasts and keratinocytes. Human dermal fibroblasts and epidermal keratinocytes were incubated with IFN- γ for 24 h. The surface expression of galectin-9 was assessed by cell-based enzyme-linked immunosorbent assay. Whereas IFN- γ enhanced galectin-9 expression in dermal fibroblasts, it dose-dependently suppressed surface galectin-9 expression of epidermal keratinocytes.

dermal fibroblasts, of normal skin ($n = 3$) weakly expressed galectin-9 (Fig. 5E). Among cells obtained from lesions of psoriasis vulgaris (a Th1-polarized disease) ($n = 5$), dermal fibroblasts exhibited significant galectin-9 expression, whereas cells from the middle and upper layer of the epidermis weakly expressed galectin-9 (Fig. 5A). A small but significant number of eosinophils were attached to dermal fibroblasts in psoriasis vulgaris lesions (Fig. 5C,D). In contrast, epidermal cells (but not dermal fibroblasts) from lesions of Th2-dominated bullous pemphigoid ($n = 4$) exhibited a strongly positive droplet-like staining pattern for galectin-9 (Fig. 5B).

Discussion

Eosinophil infiltration into the skin is a common feature of inflammatory skin diseases such as acute eczematous reactions, prurigo reactions, urticaria, autoimmune bullous skin diseases, drug-induced skin eruptions, parasitic infections, eosinophilic pustular dermatosis, and Wells' syndrome. However, eosinophil infiltration into the epidermis is observed in a more limited range of inflammatory skin conditions, including a subtype of eczema, eosinophilic spongiosis in autoimmune bullous diseases, incontinentia pigmenti, and eosinophilic pustular derma-

tosis. The underlying mechanisms of epidermal eosinophil infiltration are not fully understood. Previous findings suggest that in the vesiculobullous stage of incontinentia pigmenti, eotaxin expressed by keratinocytes is a precipitating factor of epidermal eosinophilia (18). Immunoreactive eotaxin has been detected in the epidermis of skin lesions. In an *in vitro* study, keratinocytes produced eotaxin in response to TNF- α /IL-4 or TNF- α /IFN- γ (18). However, in the present study, neither TNF- α , IL-4 nor IFN- γ stimulated keratinocytes to produce eotaxin. This discrepancy may be due to differences in origins among the prepared keratinocytes. Whereas Jean-Baptiste et al. (18) isolated keratinocytes from neonatal foreskin, we used keratinocytes from adult skin. Alternatively, the characteristic properties of the keratinocytes in subconfluent culture in the present study may be different from those of keratinocytes in confluent culture. Further study is needed to clearly identify the signals that stimulate eotaxin secretion by keratinocytes.

In the present study, eotaxin-2 mRNA was minimally detectable in keratinocytes and undetectable in fibroblasts. Eotaxin-2 mRNA expression was not affected by any of the present cytokine stimulations. Conversely, eotaxin-3 mRNA and protein synthesis was enhanced by IL-4 in both epidermal keratinocytes and dermal fibroblasts (Figs 1 and 2). We performed immunohistochemical staining for eotaxin-3 in skin tissue specimens of bullous pemphigoid, eosinophilic pustular dermatosis, atopic dermatitis and psoriasis vulgaris. Immunoreactive eotaxin-3 was detected in epidermis, dermal fibroblasts, smooth muscle cells, and endothelial cells of both normal and disease skin. It was difficult to identify increases or decreases in eotaxin-3 levels in the disease specimens, compared with normal skin (data not shown). However, given the present *in vitro* findings (Fig. 2), we speculate that IL-4-mediated eotaxin-3 production contributes to the chemoattraction of eosinophils to the dermis and epidermis in Th2-dominated skin diseases. This supposition is partly supported by the recent finding that serum levels of eotaxin-3, but not eotaxin-2, correlate with severity of atopic dermatitis (21).

Notably, IFN- γ markedly inhibited epidermal galectin-9 production in the present study. This is in striking contrast to the present finding that IFN- γ stimulated dermal fibroblasts to produce galectin-9 (Fig. 4) (13). These opposite effects of IFN- γ on galectin-9 expression by keratinocytes and dermal fibroblasts are intriguing in the context of dermal eosinophilia in Th1-polarizing inflammation. IFN- γ -mediated galectin-9 expression by dermal fibroblasts probably induces extravasation and accumulation of eosinophils in the dermis. The inhibitory effect of IFN- γ on galectin-9 production by keratinocytes may reduce chemoattraction of eosinophils into the epidermis.

Dermal eosinophilia is not a characteristic feature of psoriasis vulgaris, which is a Th1-polarized disease (22). However, in some cases of psoriasis vulgaris, we have observed a moderate degree of eosinophil infiltration into

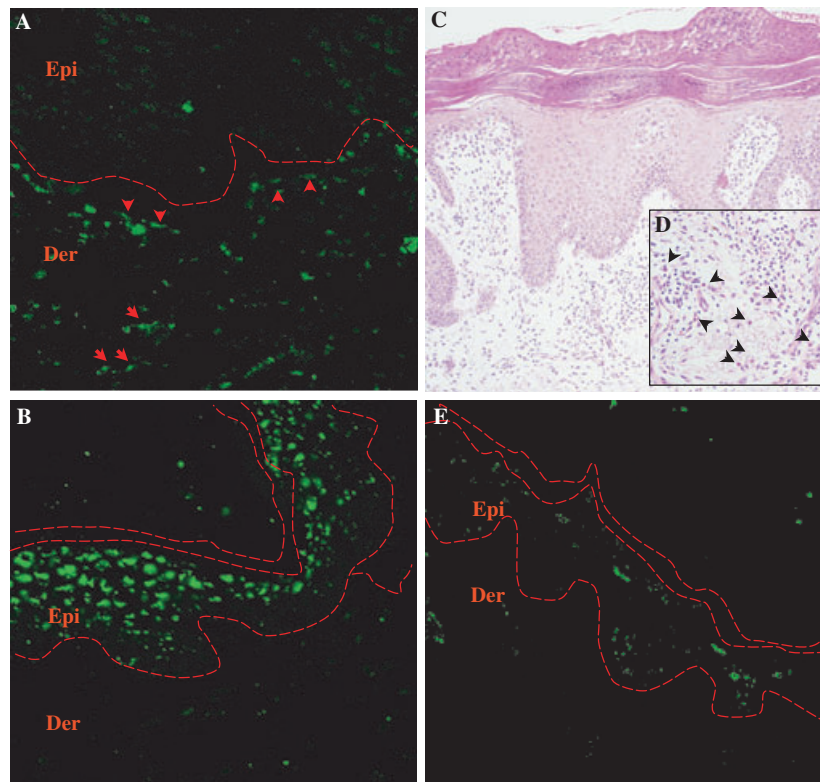


Figure 5. Galectin-9 expression in human skin. (A) Psoriasis vulgaris. Dermal fibroblasts (red arrows) exhibited markedly high expression of galectin-9. (B) Bullous pemphigoid. Galectin-9 was strongly expressed in epidermal keratinocytes, whereas it was weakly expressed in dermal fibroblasts. (C) and (D) H&E staining of tissue section A (psoriasis vulgaris). Eosinophils (arrows) were scattered throughout the dermis. (E) Normal skin.

the dermis (Fig. 5D), which is consistent with previous observations (23). Indeed, we found that galectin-9 was strongly expressed by dermal fibroblasts, but was weakly expressed by epidermal keratinocytes. RANTES, a chemoattractant for eosinophils, T cells and monocytes, has previously been detected in psoriatic skin lesions (24). A combination of IFN- γ and TNF α stimulates production of RANTES by dermal fibroblasts and keratinocytes (24–26). However, eosinophil migration toward the epidermis is not a common feature of psoriasis, unlike neutrophil migration. In previous *in vitro* studies, IFN- γ -induced galectin-9 expression on fibroblasts promoted adhesion of eosinophils, but not neutrophils, to fibroblasts (13). Thus, galectin-9 expressed on the surface of dermal fibroblasts in response to IFN- γ probably causes adhesion of eosinophils to fibroblasts and inhibition of eosinophil migration into the epidermis. However, RANTES may cooperate with galectin-9 to induce extravasation of eosinophils from blood vessels.

The roles that eosinophils play in Th-1-polarized inflammation remain unclear. IFN- γ -inducible protein-10 and monokine induced by IFN- γ (Mig) stimulate eosinophils to release eosinophilic cationic protein via CXCR3 (27). Adhesion of eosinophils to fibroblasts via galectin-9 may be important in the context of skin tissue remodeling resulting from cross-talk between these two types of cells.

However, the inhibitory effect of galectin-9 on eosinophil apoptosis is still controversial and uncertain (28). Further study is needed to ascertain the complex roles that eosinophils play in enhancement, regulation and termination of IFN- γ -associated inflammation.

In the present specimens of bullous pemphigoid, the cytoplasm of keratinocytes contained galectin-9 in a droplet-like pattern, whereas only a minimal positive reaction for galectin-9 was detected in the dermis. However, it is unclear whether increased intracytoplasmic galectin-9 in bullous pemphigoid contributes to eosinophil chemoattraction into the epidermis.

In conclusion, the present results indicate that eotaxin-3 is an important eosinophil chemoattractant produced by epidermal cells stimulated by IL4. Galectin-9 expression of dermal fibroblasts is stimulated by IFN- γ . Conversely, IFN- γ inhibits keratinocyte galectin-9 expression. The present data provide important insights into a novel regulatory mechanism of dermal and epidermal eosinophilia in skin diseases.

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