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ORIGINAL ARTICLE

Emedastine Difumarate Inhibits Histamine-Induced Collagen Synthesis in Dermal Fibroblasts

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Abstract

Background: Mast cell-derived histamine is known to act on dermal fibroblasts and contribute to formation of an intractable chronic allergic dermatitis. Although this fibrotic event may also occur in other organs such as the nasal mucosa, no direct evidence has been reported as to whether responsiveness to histamine by fibroblasts derived from different organs is of the same intensity. Furthermore, while type 1 histamine receptor (H1R) blockers have been shown to be effective for alleviation of the symptoms of allergic diseases, their ability to affect histamine-induced tissue remodeling has not yet been clarified.
Objective: Our aim was to study the effect of H1R-blockers on histamine-induced tissue remodeling.
Methods: A macroarray assay was used for a comprehensive analysis of histamine–induced gene expression by normal human fibroblasts. Fibroblasts derived from skin or nasal mucosa were cultured in the presence of various concentrations of histamine, and the synthesis of type 1 collagen was measured by means of semi-quantitative reverse-transcriptase polymerase chain reaction and enzyme-linked immunosorbent assay. To determine the effect of H1R blockers, diphenhydramine hydrochloride and emedastine difumarate were investigated in this assay.
Results: Histamine induced expression of various kinds of fibrogenic molecules in fibroblasts. Increased type 1 collagen expression was observed in fibroblasts treated with high-dose (0.1 mM to 1 μM) and low-dose (1 pM) histamine. This histamine-induced type 1 collagen synthesis was effectively diminished by emedastine difumarate. While organ specificity seems to be involved, emedastine difumarate is considered to be an effective drug for reversal of such histamine-induced remodeling in the skin.
Conclusions: We found that the expression of fibroblast-derived genes is differentially regulated by different concentrations of histamine and that the robustness of the inhibitory action of H1R blockers is different for skin-derived and nasal mucosa-derived fibroblasts. We believe that our findings may contribute to a better understanding of the mechanisms of histamine-induced tissue remodeling and provide information useful for the management of refractory allergic dermatitis.

Key words: Histamine. Fibroblasts. Collagen. Antihistamines. Emedastine difumarate. Tissue remodeling. Atopic dermatitis.

Resumen

Antecedentes: Se ha constatado que la histamina derivada de los mastocitos actúa sobre los fibroblastos dérmicos y contribuye al desarrollo de dermatitis alérgica crónica resistente al tratamiento. Aunque este episodio fibrótico puede darse también en otros órganos como la mucosa nasal, no disponemos de ninguna evidencia directa de que la reactividad de los fibroblastos a la histamina derivada de distintos organos sea de la misma intensidad. Además, mientras que se ha demostrado que los antagonistas de los receptores de la histamina de tipo 1 (RH1) resultan efectivos para el alivio de los síntomas de las enfermedades alérgicas, aun no se ha esclarecido su capacidad de influencia en los procesos de remodelación de los tejidos, inducidos por la histamina.
Objetivo: Nuestro objetivo fue estudiar el efecto de los antagonistas-RH1 en los procesos de remodelación del tejido inducidos por la histamina.

Métodos: Se utilizó una técnica de macroarray para analizar exhaustivamente la expresión génica inducida por la histamina de los fibroblastos humanos normales. Los fibroblastos derivados de la piel o de la mucosa nasal se cultivaron en presencia de diversas concentraciones de histamina y se calculó la síntesis del colágeno de tipo 1 mediante la reacción en cadena de la polimerasa retrotranscriptasa semicuantitativa y enzimoinmunoanálisis de adsorción. Para determinar el efecto de los antagonistas de los RH1, en este análisis se estudiaron el hidroclorato de difenhidramina y el difumarato de emedastina.
Resultados: La histamina indujo la expresión de diversos tipos de moléculas fibrogénicas en los fibroblastos. Se observó un aumento de la expresión del colágeno de tipo 1 en los fibroblastos tratados con dosis elevadas (1 µM hasta 0,1 mM) y dosis bajas (1 pM) de histamina. El difumarato de emedastina disminuyó de modo efectivo la síntesis de colágeno de tipo 1 inducida por la histamina. Mientras que la especificidad orgánica parece estar implicada, el difumarato de emedastina se considera un fármaco capaz de revertir dicho proceso de remodelación inducido por la histamina en la piel.
Conclusiones: Encontramos que la expresión de los genes derivados del fibroblasto se regula diferencialmente mediante diferentes concentraciones de histamina y que la potencia de la acción inhibidora de los antagonistas de los RH1 no es igual para los fibroblastos derivados de la piel que para los que derivan de la mucosa nasal. Creemos que nuestras observaciones pueden contribuir a una mejor comprensión de los mecanismos de los procesos de remodelado de los tejidos inducidos por la histamina y pueden proporcionar una información interesante para el tratamiento de la dermatitis alérgica resistente al tratamiento.

Palabras clave: Histamina. Fibroblastos. Colágeno. Antihistamínico. Difumarato de emedastina. Procesos de remodelación de los tejidos. Dermatitis atópica.

Introduction

The process known as tissue remodeling and repair is thought to be an underlying cause of refractory allergic diseases such as asthmatic diseases and atopic dermatitis (AD). Based on a generally accepted definition by the Global Initiative for Asthma, it has been proposed that tissue remodeling contributes to (1) reconstitution and repair of inflammatory tissue injuries, (2) irreversibility or intractability of the process, and (3) persistence of allergic inflammation [1]. In view of these considerations, tissue remodeling appears to participate in the prolongation of chronic allergic reactions rather than in repair of tissue damaged by allergic inflammation.

AD has been demonstrated to be a feature of the tissue remodeling process during the progression from acute to subacute and chronic inflammation. In the epidermis of lesional skin, inflammatory cell infiltration, spongiosis, and acanthosis are commonly observed [2], while marked characteristics of tissue remodeling have been identified in AD-associated skin lesions, especially in the dermis. Edematous changes and perivascular infiltration of lymphocytes, eosinophils, neutrophils, and basophils occur in the early stage of AD [3]. Prolonged inflammation leads to an increase in the number of dermal fibroblasts, mast cells, and collagen bundles, and a greater number of mast cells has been found in AD skin lesions than in nonlesional skin [4]. The mechanisms underlying these tissue reactions can be partly explained by the activating or proliferating effect of mast cell-derived chemical mediators such as histamine on fibroblasts.

Histamine has distinct effects on dermal fibroblasts, effects which are characterized by increased synthesis of type 1 collagen [5] and glycosaminoglycans [6], and augmentation of fibrogenic cytokine-induced fibroblast proliferation [7]. However, there has been little or no direct evidence as to whether the effects of histamine on fibroblasts derived from different tissues are expressed at the same level.

In this study, we analyzed histamine-induced gene expression in fibroblasts. We used reverse-transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) to investigate the effects of various concentrations of histamine on the synthesis of type 1 collagen. We also examined the effect of inhibitors of the histamine H1 receptor (H1R) on histamine-induced gene expression.

Materials and Methods

Cell Culture

Normal human dermal fibroblasts (NHDFs) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 5% CO_2.

Primary Culture of Fibroblasts

For isolation of dermal fibroblasts, minced adult skin samples obtained with informed consent during surgical operations were treated with 0.2% collagenase (Sigma, St Louis, Missouri, USA) at 37°C for 1 hour. The isolated dermal fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum at 37°C in 5% CO_2. For isolation of nasal mucosa-derived fibroblasts, extirpated nasal polyps were used and cells were prepared in the same manner as for dermal fibroblasts.

Treatment With H1R Blockers

Emedastine difumarate and diphenhydramine hydrochloride (gifts from Kowa Pharmaceutical Company, Tokyo, Japan) were dissolved in DMEM. The emedastine difumarate dosages were 0.1, 1, 10 µg/mL and the diphenhydramine hydrochloride dosage was 1 µg/mL. After 24 hours of culture with these compounds, conditioned medium and total RNA were harvested for ELISA and RT-PCR.

Determination of the Number of Mast Cells

Paraffin sections from cases with various kinds of skin disease (atopic dermatitis, n=13; psoriasis vulgaris, n=9; prurigo nodularis, n=14; contact dermatitis, n=5; drug eruption, n=4) and from healthy control subjects (n = 6) were stained with Giemsa. Mast cells in the upper dermis were counted as the number of cells per unit area with a depth of 450 μm from the basement membrane and a width of 1 mm. Data were expressed as the mean (SD).

Immunohistochemical Staining for Tryptase-Positive Mast Cells

A 4 μm paraffin section from an atopic dermatitis skin lesion was deparaffinized and heated for antigen retrieval. The primary antibodies recognized tryptase (1:50; DAKO, Santa Fe, California, USA). Staining was done with the streptavidin-biotin amplification LSAB2 system (DAKO).

Macroarray Assay

NHDFs were cultured on 10-cm culture dishes. At the subconfluent stage, they were incubated with or without 1 μM histamine (Sigma) for 6 hours. Total RNA was isolated with the RNeasy kit (QIAGEN GmbH, Hilden, Germany). The PANORAMA human cytokine gene arrays (Sigma Genosys) assay was performed according to the manufacturer's instructions. ^{33}P-labeled complementary DNA (cDNA) was prepared using the oligo(dT) primers provided. After purification of labeled cDNAs using spin columns, the cDNAs were hybridized to the PANORAMA gene array. Quantitation of gene expression signals was performed with a BAS5000 image analyzer (Fujifilm, Tokyo, Japan).

RT-PCR

Total RNA was extracted with the RNeasy Mini kit (QIAGEN Gmb) according to the protocol supplied by the manufacturer. First-strand cDNA was synthesized with an RT-PCR kit (Stratagene, La Jolla, California, USA) using oligo-dT primers, followed by amplification of the cDNA for 25 cycles. The following oligonucleotide primers were used for RT-PCR: procollagen α1 (I), 5'-TAC AGC ACG CTT GTG GAT G-3' (sense) and 5'-TTG AGT TTG GGT TGT TGG TC-3' (antisense); glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (antisense). Relative gene expression levels were expressed as the ratio of procollagen α1 (I) to GAPDH (internal standard). Gene expression levels were calculated using ImageJ software (NIH, Bethesda, Maryland, USA).

ELISA

The production of type I collagen was determined by ELISA, for which microtiter wells were coated with the samples dissolved in 50 mM carbonate buffer (pH 9.0) overnight at 4°C. The wells were then washed 3 times with 300 μL of 0.05% Tween 20 in phosphate-buffered saline (PBS) and nonspecific binding sites were blocked with 1% bovine serum albumin in PBS for 1 hour. After washing, anti-human type I collagen antibody (Sigma), diluted 1:1000 in PBS containing 0.05% Tween 20, was added to the wells and incubated for 2 hours. This was followed by another wash and the addition of horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark), diluted 1:1000 in PBS containing 0.05% Tween 20, to the wells and incubation for 1 hour. After washing, the reaction was developed with K-Blue Aqueous substrate (Neogen, Lexington, Kentucky, USA) for 20 minutes. After the reaction was terminated with 50 μL of 1N HCl, absorbance was read at 450 nm. Next, a standard curve was constructed using purified human skin type I collagen (Calbiochem, Darmstadt, Germany) diluted in PBS ranging in concentration from 1 ng/mL to 1 μg/mL.

Statistical Analysis

Statistical analysis was performed with Prism4 software (GraphPad Software Inc, San Diego, California, USA). Multivariable comparisons of means were performed by Kruskal-Wallis nonparametric test. Subsequently, the comparisons of means between pairs of groups were performed by Dunn multiple comparison test. $P < .05$ was considered statistically significant.

Results

Increase in Mast-Cell Number in Atopic Dermatitis Lesions

A comparison of the number of mast cells in various kinds of inflammatory skin disease is shown in Figure 1A. Skin lesions from atopic dermatitis, psoriasis vulgaris, prurigo nodularis, and drug eruption contained a larger number of mast cells than did skin sections from healthy controls. It is noteworthy that the number of mast cells was significantly increased in atopic dermatitis lesions compared with prurigo nodularis, contact dermatitis, and healthy controls ($P < .05$), while contact dermatitis lesions contained even fewer mast cells than seen in healthy controls. In atopic dermatitis lesions, tryptase-positive cells and degranulated mast cells were frequently observed (Figure 1B, C). Since skin fibrosis is often observed in the chronic phase of atopic dermatitis, and it has been reported that excessive tissue remodeling contributes to the pathogenesis of atopic dermatitis, psoriasis vulgaris, and prurigo nodularis [8-10], our findings suggest that mast cell degranulation is actively involved in generating dermal fibrosis in these dermatoses.

Comprehensive Analysis of Gene Expression in Histamine-Treated NHDF

To further analyze the role of mast-cell degranulation, we generated gene expression profiles in histamine-treated NHDF. The macroarray assay showed that various genes linked to tissue remodeling, such as FGF18, VEGFB, INHBA, BMP7, TGFB1, and TGFBR3, are transactivated in histamine-treated NHDF (Figure 2). Fibroblast growth factor (FGF) 18 and vascular endothelial growth factor (VEGF) B are known to act on endothelial cells and cause migratory activity and neoangiogenesis, respectively [11,12]. Activin A (encoded by the INHBA gene), transforming growth factor (TGF) ß1,

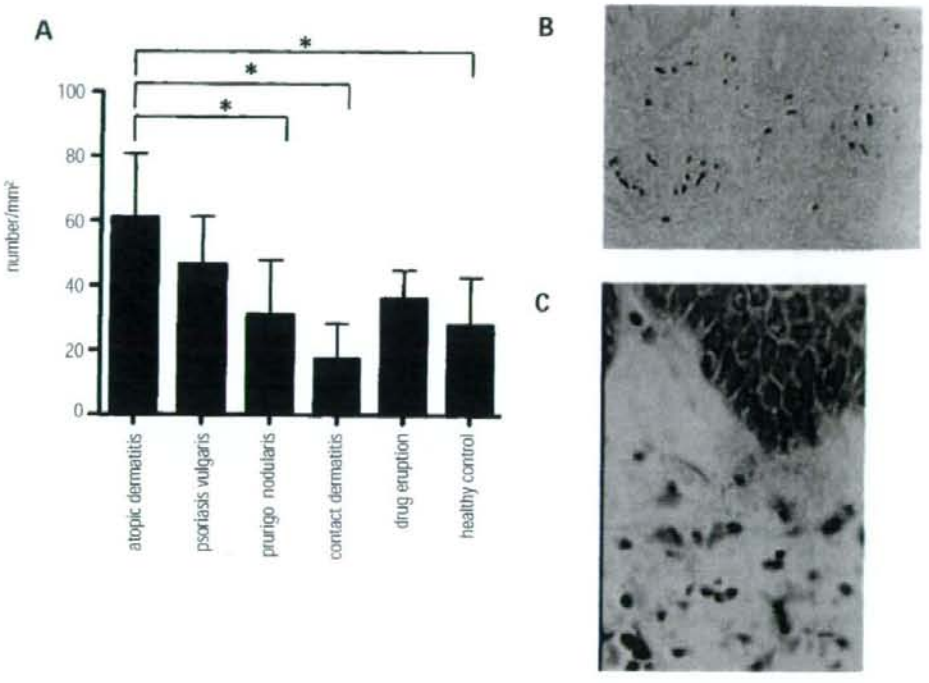

Figure 1. Accumulation of mast cells in atopic dermatitis skin lesions. A, Bars show mean values for the number of dermal mast cells in various skin diseases; whiskers show SD. Asterisks (*) indicate $P < .05$. B, Immunohistochemistry for tryptase in atopic dermatitis skin lesion (original magnification, ×200). C, Giemsa stain showing accumulation of mast cells with dark blue granules in the upper dermis of a skin lesion of atopic dermatitis (original magnification, ×400).

		Control	HT
FGF-Family	FGF18		
Angiogenic Factor	VEGF-B		
TGF-β Superfamily	Activin A		
	BMP-7		
	$TGF\text{-}\beta_1$		
	TGF-βRIII		
House Keeping	β actin		

Figure 2. Expression of genes associated with tissue remodeling in histamine-treated normal human dermal fibroblasts. The results of macroarray analysis are shown. Each gene was arrayed in duplicate. BMP indicates bone morphogenetic protein; FGF, fibroblast growth factor; HT, histamine treated; TGF, transforming growth factor; TGF-βRIII, TGF-β receptor III; VEGF, vascular endothelial cell growth factor.

both members of the TGFß superfamily, and TGFß receptor III have long been recognized as major players in tissue repair, fibrosis, and inflammation [13,14], while bone morphogenetic protein (BMP) 7, also a member of the TGF-ß superfamily, was originally identified as an inducer of cartilage and bone formation [15]. In recent years, however, additional functions of BMP-7 have been discovered, including that of an inhibitor of hair follicle formation [16]. Thus, the expression of these genes in histamine-treated NHDF indicates that histamine may perform a variety of functions in the tissue remodeling process via activation of dermal fibroblasts.

Synthesis of Type 1 Collagen in Histamine-Treated Dermal Fibroblasts

As mentioned, type 1 collagen is synthesized by fibroblasts and is thought to play an important role in tissue remodeling and fibrosis. Histamine may induce type 1 collagen expression in fibroblasts via a direct or indirect pathway, including production of fibrogenic cytokines, as shown in Figure 2. Next, we addressed whether histamine generates the same response in fibroblasts derived from different tissues. To this end, we used RT-PCR to analyze transcripts of procollagen α1 (I) mRNA in dermal fibroblasts or nasal mucosa-derived fibroblasts following treatment with various concentrations (1 pM to 0.1 µM) of histamine (Figure 3A, B). The response of type 1 collagen synthesis to histamine in nasal mucosa-derived fibroblasts was stronger than that observed in dermal fibroblasts but, interestingly, histamine induced an inverted bell-shaped dose-response curve for expression of procollagen α1 (I) nRNA in both dermal fibroblasts and nasal mucosa-derived fibroblasts. Furthermore, ELISA confirmed the reproducibility of the outcomes of RT-PCR (Figure 3C). These findings indicate that, although histamine-induced responsiveness seems to be different for fibroblasts derived from different tissues, the response of type 1 collagen synthesis in both dermal and nasal mucosa-derived fibroblasts is similar for the same dose of histamine treatment.

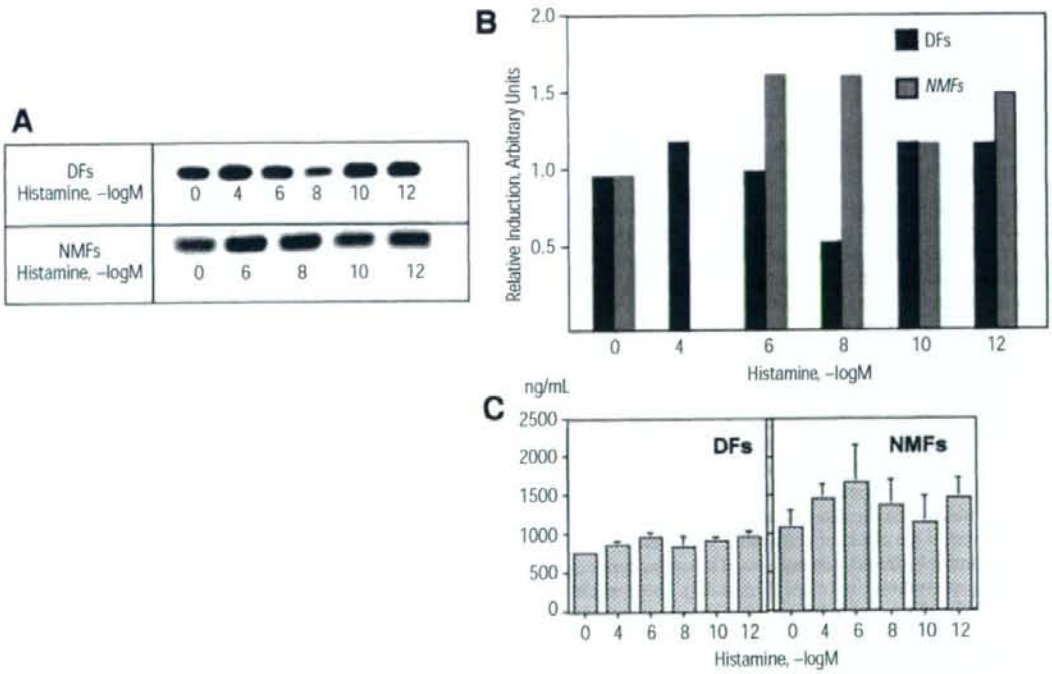

Figure 3. In both dermal fibroblasts and nasal mucosa-derived fibroblasts, histamine induces type1 collagen synthesis following an inverted bell-shaped dose-response curve. A, Results of reverse-transcriptase polymerase chain reaction for pro-collagen α1 (I) mRNA at various concentrations of histamine. B, Comparison of pro-collagen α1 (I) mRNA densities as assessed by densitometry. Treatment with 0.1µM histamine was not performed in nasal mucosa-derived fibroblasts. Bars indicate the mean of 3 cases for dermal fibroblasts and 2 cases for nasal mucosa-derived fibroblasts. C, Comparison of type 1 collagen production in 3 cases each of histamine-treated dermal fibroblasts and nasal mucosa-derived fibroblasts. Bars show mean values and whiskers SD. DF indicates dermal fibroblasts; NMF, nasal mucosa-derived fibroblasts.

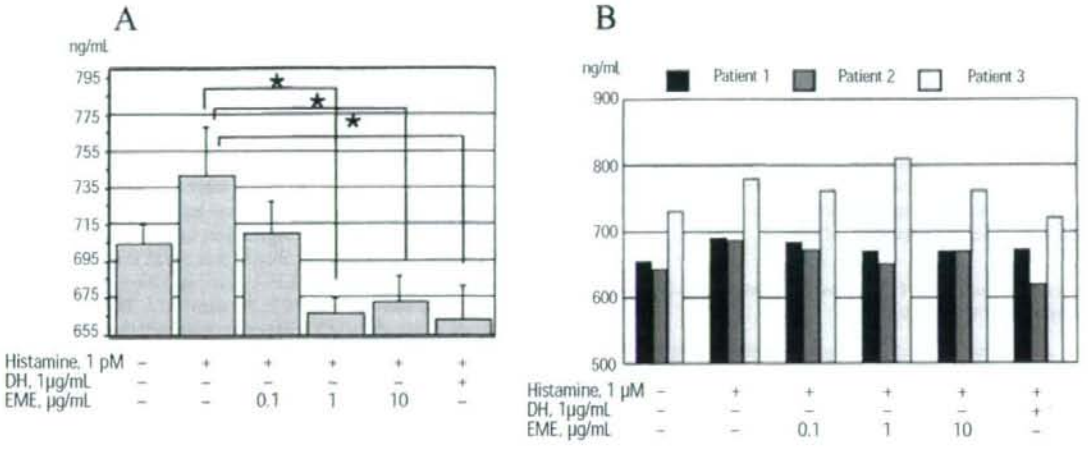

Figure 4. Emedastine difumarate inhibits histamine-induced type 1 collagen synthesis in dermal fibroblasts. A, Comparison of type 1 collagen production in dermal fibroblasts treated with 1 pM histamine with or without histamine H1 receptor blockers. Experiments were performed on dermal fibroblasts obtained from 3 individuals. Bars shown mean values and whiskers indicate SD; asterisks (*), $P < .05$. B, Comparison of type 1 collagen production in nasal mucosa-derived fibroblasts treated with 1 μM histamine with or without histamine H1 receptor blockers. Experiments were performed on nasal mucosa-derived fibroblasts obtained from 3 individuals. Values represent the concentrations of type 1 collagen from 3 different supernatants. DH indicates diphenhydramine hydrochloride; EME, emedastine difumarate.

Emedastine Difumarate Inhibits Histamine-Induced Synthesis of Type 1 Collagen in Dermal Fibroblasts

To investigate the effect of H1R inhibitors on histamine-induced type 1 collagen synthesis in dermal fibroblasts and nasal mucosa-derived fibroblasts, histamine-treated cells were cultured with first-generation (diphenhydramine hydrochloride) or second-generation (emedastine difumarate) H1R inhibitors. In the case of dermal fibroblasts, ELISA for detection of type 1 collagen in conditioned medium showed that 0.1 μg/mL emedastine difumarate inhibited histamine-induced collagen synthesis to the same extent as observed in vehicle-treated cells. Addition of diphenhydramine hydrochloride or high concentrations of emedastine difumarate to the culture medium both led to statistically significant inhibition of type 1 collagen production (Figure 4A). In the case of nasal mucosa-derived fibroblasts, on the other hand, individual differences were observed in the effect of emedastine difumarate, while diphenhydramine hydrochloride suppressed histamine-induced type 1 collagen synthesis in 3 cases (Figure 4B). These results indicate that the effect of emedastine difumarate may be tissue specific, but not that of diphenhydramine hydrochloride.

Discussion

The results of this study may provide insights into novel functional aspects of histamine in chronic allergic diseases. Histamine has been demonstrated to have multiple roles such as that of a chemical mediator in the immune response and a neurotransmitter in gastric acid production [17] and in the maintenance of the blood-brain barrier, along with hormonal functions and roles in sleep, food intake, thermoregulation, and locomotor activity [18]. These findings indicate that histamine is essential for maintaining homeostasis in living organisms.

However, an excessive response to histamine has been shown to play an important role in the pathogenesis of chronic allergic diseases, including atopic dermatitis. Moreover, fibroblasts may be candidates for histamine-responsive cells and contribute to the development of chronic dermatitis. This possibility is supported by the observation that production of inflammatory chemokines such as eotaxin, a potent eosinophil-specific chemotactic factor, was found to be induced in fibroblasts in a dose-dependent fashion [19]. Furthermore, one study found that histamine enhanced fibroblast proliferation in a dose-dependent manner, with an optimum effect at a physiological concentration of 0.1 μM histamine [20]. In our study, dermal fibroblasts and nasal mucosa-derived fibroblasts were also stimulated at several concentrations (from 1 pM to 0.1 mM) of histamine. Unexpectedly, type 1 collagen was synthesized with a reverse bell-shaped dependence on histamine stimulus, showing a peak response at 0.1 mM, 1 μM, or 1 pM. ELISA with conditioned medium revealed a dose-dependent increase in the concentration of eotaxin in response to histamine treatment (data not shown). This dose-dependent action of histamine indicates that high concentrations of histamine may cause both inflammation and tissue remodeling, while lower concentrations may cause only tissue remodeling.

Our comprehensive study of gene expression in histamine-

treated NHDF revealed that histamine can induce the expression of various kinds of genes associated with the tissue remodeling process. Histamine is believed to play an important role in the wound-healing process, and indeed, disruption of histamine in histidine decarboxylase gene knockout mice resulted in delayed cutaneous wound healing, and the phenotype was rescued by exogenous histamine administration [21]. In that study, the mechanism underlying delayed wound healing was explained in terms of the impaired expression of histamine-activated basic fibroblast growth factor, which leads to angiogenesis and macrophage recruitment in the wound-healing process. The results of our study showed that genes encoding angiogenic factors such as VEGF-B and FGF18 were expressed by histamine-treated NHDF. Both VEGF-A and VEGF-B are known to be expressed in dermal fibroblasts and keratinocytes [22-24], and although fibroblasts treated with tumor necrosis factor α or TGFB, or irradiated with UV-A were found to be capable of releasing VEGF-A [22,23], these stimuli did not affect the expression of VEGF-B [24]. Taken together with the results of our study, these findings indicate that histamine could be a novel candidate for the previously unidentified factor inducing VEGF-B expression in fibroblasts. As VEGF is also known to function as a chemotactic factor for mast cells as well as endothelial cells [25], histamine-induced VEGF expression may be a contributing factor in allergic inflammation. On the other hand, little is known about the function of FGF18 in the skin, although a recent study found that FGF18 was strongly expressed during the anagen phase in the inner root sheath and during telogen in the hair follicles, and that subcutaneous injection of exogenous FGF18 resulted in vigorous hair growth [26]. In contrast, as mentioned earlier, BMP-7, an inhibitor of hair follicle formation, was also released from histamine-treated NHDF [16]. Taking all these findings into account, we predict that histamine may be important for the regulation of hair growth and maintenance of the skin. Moreover, we speculate that, if the balance between FGF18 and BMP-7 is altered, phenotypes such as hirsutism or hair loss (eg, the Hertoghe sign in atopic dermatitis) may appear in chronic allergic diseases. Further studies will be necessary to confirm this hypothesis.

Histamine-induced expression of type 1 collagen in dermal fibroblasts was dramatically inhibited by emedastine difumarate. The robustness of this inhibitory effect identifies it as the strongest drug among 5 different second-generation H1R-blockers (data not shown). For this reason, emedastine difumarate should be considered the most useful second-generation H1R-blocker for treating the scleroderma that is frequently observed in atopic dermatitis. Unexpectedly, the effect of emedastine difumarate was found to be different for dermal fibroblasts and nasal mucosa-derived fibroblasts. To the best of our knowledge, no reports have been published that discuss heterogeneity in the effect of H1R-blockers in these cell types. As nasal mucosa-derived fibroblasts are obtained from nasal polyps, it can be assumed that there are phenotypic differences between those cells and normal nasal mucosa-derived fibroblasts. At present, we have no explanation for this difference but further examination can be expected to yield some valuable information for tailor-made therapeutic strategies to treat allergic diseases.

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ORIGINAL ARTICLE

New aspect of anti-in ammatory action of lipo-prostaglandinE1 in the management of collagen diseases-related skin ulcer

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Abstract It is considered that the mechanism in intractable cutaneous ulcer is deeply associated with prolongation at the inflammatory phase. Having evaluated the effects of Lipo-prostaglandin E1 (Lipo-PGE1) with indicators such as the reduction ratio of the ulcer area and the values of the inflammatory markers after dividing them into two groups of collagen diseases and non-collagen diseases and giving them Lipo-PGE1, we managed to obtain the result that Lipo-PGE1 administration could influence various inflammatory markers such as C-reactive protein (CRP), IL-6, and VEGF in addition to reduction of the ulcer region. It also suggested that Lipo-PGE1 has the effect of maintaining an appropriate balance of induction of inflammation and angiogenesis. Additionally, it revealed that Lipo-PGE1 controls the production of cytokines, which are associated with the growth of collagen diseases. From these results, it can be expected that Lipo-PGE1 will act favorably on intractable collagen diseases.

Keywords Lipo-prostaglandin E1 · Skin ulcer · IL-6 · VEGF · sICAM-1 · C-Reactive protein

Introduction

Intractable cutaneous ulcer is one of the relatively common diseases, which can be seen in daily clinical practice at dermatology departments. Local manifestation associated with ulcers and the effect on general conditions combined with such symptoms may significantly compromise a patient's quality of life. Despite the fact that it is considered to be a major cutaneous symptom among dermatological disorders, different opinions have been expressed for the intractable mechanism and treatment method. For this reason, there can often be seen cases where insufficient treatment would be a cause of prolongation and refractory changes.

The wound healing process is divided mainly into three phases of the inflammatory phase, proliferation phase and remodeling phase. In normal wound healing, various types of cells for tissue repair accumulate in the ulcer region at the inflammatory phase and gather in a short period to move into the proliferation phase [1]. On the other hand, it is considered that the inflammatory phase for intractable cutaneous ulcers tend to be prolonged which may prevent the cells from growing at the local sites. It is also assumed that this phenomenon is somehow associated with the intractable mechanism. Some study reports explain that various types of inflammatory cytokines are released at the inflammatory phase; a cytokine such as VEGF, which can be seen particularly in ulcer lesions, and IL-6 [2–4]. It is also considered that such cytokine secretion at the inflammatory phase can be attributed to macrophage [2, 5]. However, there are few reports explaining how these factors affect the severity of cutaneous ulcers before and after the treatment. Therefore, it is not yet clear to what extent such factors are associated with the clinical condition.

Although PGE1 has often been used for the treatment of cutaneous ulcer with high effectiveness, lipid microspheres containing PGE1 (Lipo-PGE1) is developed to avoid the disadvantage of PGE1, such as rapid inactivation and requiring large dose of drug, using the drug delivery system. In the previous report about evaluation of clinical efficacy of Lipo-PGE1 in the patients with peripheral

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vascular diseases, it was demonstrate that 3 g of Lipo-PGE1 treated group showed significant improvement compared with 40–60 g (usual dose for Japanese) of PGE1-cyclodextrin clathrated treated group [21]. Since then, dose of 5–10 g/day of Lipo-PGE1 is recommended from manufacturer (Taisho Toyama Pharmaceutical, Japan), and is widely used for the treatment of intractable cutaneous ulcers due to chronic arterial occlusive diseases and diabetes in eastern Asia. It is also known for its anti-inflammatory effects in addition to its vasodilating action and platelet aggregation inhibitory action when it is introduced into vascular endothelial cells and macrophage after being accumulated in the ulcer lesion.

We assumed that the intractable mechanism in reflactory cutaneous ulcers is deeply associated with prolongation at the inflammatory phase. For this reason, we focused on the anti-inflammatory effects of Lipo-PGE1, which can be effective when it is taken up into macrophage, a central role at the inflammatory phase. Then we classified the patients with intractable cutaneous ulcer into two groups of collagen diseases and non-collagen diseases and gave them Lipo-PGE1 to evaluate the effects with the indicators such as the ulcer reduction ratio and the values of inflammatory markers.

Materials and methods

Patients

Thirty-five inpatients with intractable cutaneous ulcer in our hospital were divided into two groups of 20 patients with collagen diseases (Table 1) and 15 patients with non-collagen diseases (Table 2), and 16 patients with collagen diseases and 12 patients with non-collagen diseases were medicated with Lipo-PGE1. Clinical evaluation was made immediately after, and 2 weeks after the administration. Thirty-five subjects included 10 males and 25 females with a male-female ratio of 2:5 and age range of 28–82 years-old (average 58.332 years-old). The collagen disease group included seven systemic sclerosis cutaneous (SSC), five rheumatoid arthritis (RA), two systemic lupus erythematodes (SLE), two behcet disease (BD), two polyperiarteritis nodusa (PN), one anti-phospholipid antibody syndrome (APS) and one wegener glanuromatosis (WG) cases, while the non-collagen group included four livedo vasculitis (LV), three venous malfomation (VM), two arteriosclerosis obliterans (ASO), one blue toe syndrome (BTS), one stasis ulcer (SU), one calciphylaxis (CPL), one werner syndrome (WS), one radiation injury (RI) and one hyper gamma-globulinemic syndrome (HGS) (See Table 1). The subjects with wound infections were excluded from this study.

Methods and evaluation

10 g/2 ml of Lipo-PGE1 (Palxus®, Taisho Toyama Pharmaceutical, Japan), which is incorporated PGE1 in lipid microspheres, was given daily via intravenous bolus injection for 2 weeks. All cases had continued to take concomitant drugs while in this study. It was also confirmed that no drug was changed and no improvement of skin ulcer was found within 1 month before start of this study. As for an indicator of clinical evaluation, the clinical assessment of ulcers (ulcer area and reduction ratio of ulcer area, ulcer area long axis of ulcer × short axis of ulcer) were performed, and serum CRP concentration were measured. The non-treatment intervention group was given beraprost sodium, a stable PGI2 analog, orally. As for the local treatment, either silicone gauze (TOREX®, FUJI System Co., Japan) or petrolatum gauze (ADAPTIC®, Johnson and Johnson) were used to protect the area during the period.

Enzyme-linked immunosorbent assay (ELISA)

IL-6, sICAM-1, VEGF and HGF in serum were measured by using ELISA method. ELISA kit purchased from R & D systems was used for IL-6, sICAM-1 and VEGF, while IMMUNIS EIA kit (Institute of Immunology Co., Ltd.) was used for HGF. Each kit was used following its recommended protocol to measure each value with Model 680 Microplate Reader (BIO-RAD).

Statistic analysis

Prism4 software (Graph pad software, CA, USA) was used for the statistic analysis.

Results

The reduction ratio of the ulcer area 2 weeks after administration of Lipo-PGE1 showed tendency for improvement in both groups of collagen (mean 34.84%, SD ± 38.76) and non-collagen diseases (mean 31.45%, SD ± 33.68) compared with the non-intervention group (mean 14.04%, SD ± 34.59) (Fig. 1a). Notably, Lipo-PGE1 treatment had enhanced the generation of well-vascularized granulation tissue, in parallel with the increased temperature of lesional skin, while non-interventional group have not (Fig. 1b). CRP, an inflammatory indicator in this study, had significantly decreased in both the collagen disease group (Fig. 2c) and non-collagen disease group (Fig. 2d) ($p = 0.0276$, and $p = 0.0135$, respectively, Mann–Whitney test), while such a decrease was not observed in group with the non-treatment intervention group (Fig. 2b). Since CRP

Table 1 General data of patients with collagen disease

No	Diagnosis	Sex	Age	Location of ulcer	Reduction rate of area (%)	CRP (mg/dl)		IL-6 (pg/ml)		VEGF (pg/ml)		sICAM-1 (pg/ml)		Concomitant treatment	LipoPGE1 intervention
						Pre	Post	Pre	Post	Pre	Post	Pre	Post		
1	SSC	F	67	Toe	73	2.8	0.1	21.72	12.44	464.71	265.80	18.60	21.17	Limaprost alfadex 30 g/day	YES
2		M	56	Finger	8	0.2	0.16	31.22	21.97	NT	NT	NT	NT	Sarpogrelate hydrochloride 300 mg/day	YES
3		F	68	Foot	2	4.0	2.9	107.59	68.02	710.67	121.30	13.04	16.96	Limaprost alfadex 30 g/day	YES
4		F	48	Sole	5	0.8	0.05	8.09	7.61	NT	NT	NT	NT	Sarpogrelate hydrochloride 300 mg/day	YES
5		F	66	Finger	67	1.2	0.2	80.73	10.18	1,154.47	930.97	21.50	24.02	Prednisolone 5 mg/day, Sarpogrelate 300 mg/day, cilostazol 100 mg/day	YES
6		F	52	Finger	8	0.2	0.2	NT	NT	NT	NT	NT	NT	Sarpogrelate hydrochloride 300 mg/day	YES
7		F	59	Finger	3	0.2	0.2	NT	NT	NT	NT	NT	NT	Sarpogrelate hydrochloride 300 mg/day	YES
8	RA	F	56	Lower leg	56.4	7.4	0.8	42.62	7.84	936.31	713.88	48.56	22.44	Methylprednisolone 4 mg/day, mizoribine 150 mg/day	YES
9		F	57	Lower eg	−21.3	1.6	11.9	9.38	104.50	716.67	184.52	20.57	32.02	Methylprednisolone 4 mg/day, beraprost sodium 60 g/day	NO
10		F	77	Buttock	0	4.4	4.7	4.74	10.33	NT	NT	22.00	19.58	Prednisolone 35 mg/day, methotrexate 6 mg/week beraprost sodium 60 g/day	NO
11		F	53	Foot	33	2.7	0.1	191.43	3.38	256.17	108.60	15.35	14.09	Prednisolone 10 mg/day, aspirin 100 mg/day, PGI2 40 g/day	YES
12		F	70	Lower leg	10	0.6	0.1	2.25	ND	396.27	502.13	10.98	8.97	Prednisolone 8 mg/day, mizoribine 150 mg/day	YES
13	SLE	F	77	Lower leg	5	1.3	0.2	20.82	12.44	892.47	501.07	20.53	19.67	Aspirin 100 mg/day, cyclosporine A 100 mg/day	YES
14		F	79	Lower leg	0	4.6	8.9	NT	NT	397.34	476.47	16.84	16.46	Aspirin 100 mg/day	YES
15	Bechet	M	63	Lower leg	100	1.9	0.2	NT	NT	NT	NT	NT	NT	None	YES
16		F	24	Lower leg	87	0.1	0.1	1.72	ND	NT	NT	NT	NT	Prednisolone 20 mg/day, colchicines 1 mg/day	YES
17	PN	M	28	Lower leg	100	0.2	0.6	0.59	2.10	485.03	344.93	11.06	11.52	Aspirin 100 mg/day, Sarpogrelate hydrochloride 300 mg/day	YES
18		M	67	Lower leg	7	7.5	6.8	221.47	187.12	NT	NT	NT	NT	Prednisolone 10 mg/day, cyclophosphamide 25 mg/day, beraprost sodium 60 g/day	NO
19	APS	F	29	Lower leg	27	0.1	0.1	ND	ND	NT	NT	NT	NT	Aspirin 100 mg/day, beraprost sodium 60 g/day	NO
20	Wegener	F	58	Glabellar area	0	0.1	0.1	1.42	ND	NT	NT	NT	NT	Sarpogrelate hydrochloride 300 mg/day	YES

Pre pre-treatment, *Post* post-treatment, *NT* not tested, *ND* not detected

Table 2 General data of patients with non-collagen disease

No	Diagnosis	Sex	Age	Location of ulcer	Reduction rate of area (%)	CRP (mg/dl)		IL-6 (pg/ml)		VEGF (pg/ml)		sICAM1 (ng/ml)		Concomitant treatment	LipoPGE1 intervention
						Pre	Post	Pre	Post	Pre	Post	Pre	Post		
1	LV	F	29	Lower leg	2	0.1	0.1	ND	1.120	535.29	346.00	10.60	9.95	Aspirin 81 mg/day	YES
2		M	42	Lower leg	5	0.1	0.1	0.06	ND	NT	NT	NT	NT	Aspirin 100 mg/day, Sarpogrelate hydrochloride 300 mg/day	YES
3		F	39	Lower leg	0	0.1	0.4	0.59	4.89	528.87	627.26	14.73	11.67	Beraprost sodium 60 g/day	NO
4		F	40	Lower leg	0	0.7	1.6	48.06	120.20	NT	NT	10.78	10.10	Beraprost sodium 60 g/day	NO
5	VM	F	56	Lower leg	100	0.2	0.1	NT	NT	96.83	166.35	9.21	8.23	Aspirin 100 mg/day	YES
6		F	57	Foot	18.7	1.3	1.0	ND	3.69	7.00	135.33	7.84	7.63	Aspirin 100 mg/day	YES
7		F	68	Lower leg	85.6	NT	NT	NY	NT	NT	NT	NT	NT	Beraprost sodium 60 g/day	NO
8	BTS	F	74	Toe	30.6	0.4	0.1	163.89	ND	147.10	295.74	2.17	2.23	Sarpogrelate hydrochloride 300 mg/day	YES
9	ASO	M	76	Toe	10.1	8.3	0.1	22.25	ND	NT	NT	NT	NT	Aspirin 100 mg/day, warfarin potassium 5.5 mg/day	YES
10		M	78	Toe	17.6	0.3	0.1	37.72	9.19	481.82	450.81	15.94	19.52	Aspirin 100 mg/day	YES
11	SU	F	68	Lower leg	91	0.1	0.1	2.48	3.08	129.99	177.04	11.50	18.75	Cilostazol 200 mg/day	YES
12	CPL	M	53	Lower leg	52	1.4	0.1	135.29	2.10	198.43	171.69	24.09	19.58	Aspirin 10 mg/day, warfarin potassium 2.5 mg/day	YES
13	WS	M	41	Ankle	0	1.6	0.6	15.23	10.78	201.64	232.65	7.84	6.87	Sarpogrelate hydrochloride 300 mg/day	YES
14	RI	F	82	Sole	38.7	0.2	0.1	0.67	ND	92.56	62.61	NT	NT	None	YES
15	HGS	M	53	Lower leg	11.7	6.5	0.2	21.80	ND	NT	NT	NT	NT	Prednisolone 20 mg/day	YES

LV Livedo vasculitis, *VM* venous malfomation, *BTS* blue toe syndrome, *ASO* arteriosclerosis obliterans, *SU* stasis ulcer, *CPL* calciphylaxis, *WS* Werner syndrome, *RI* radiation injury, *HGS* hyper gamma-globulinemic syndrome, *Pre* pre-treatment, *Post* post-treatment, *NT* not tested, *ND* not detected

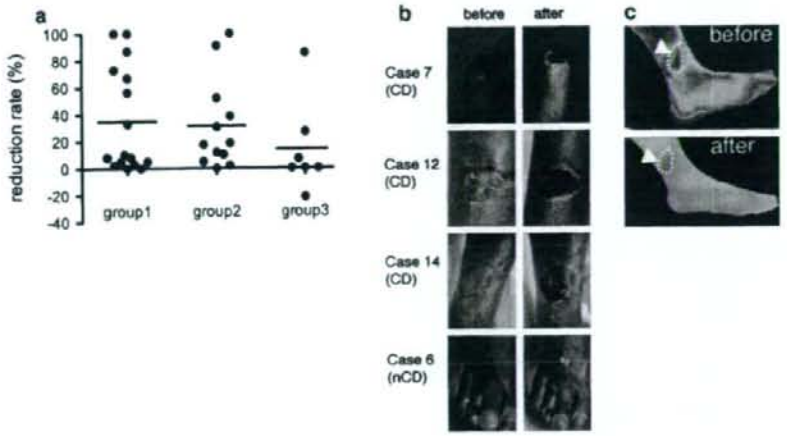

Fig. 1 **a** Reduction ratio of ulcer area. Ulcer areas before and 2 weeks after Lipo-PGE1 administrations were calculated and compared. *Group* 1 Group with diseases related to the collagen disease and treatment intervention. *Group* 2 Group with non-collagen disease and treatment intervention. *Group* 3 Group with non-treatment intervention. **b** Clinical features of some cases from intervention group. Case number is referred to Table 1 and 2. *Before* pre-treatment, *After* post-treatment, *CD* collagen disease group, *nCD* non-collagen disease group. **c** Evaluation of the lesional skin surface temperature of case number 14 from collagen disease group using thermography. Center temperature of before (pre-treatment) and after (post-treatment) is 24° and 27°, respectively. The lowest temperature area is presented as *blue color*, while the highest temperature is presented as *red color*. Before treatment, the temperature of lesional skin is lower than that of the surrounding healthy skin. After treatment, the temperature of lesional skin is recovered to be nealy identical to that of surrounding healthy skin. *Arrow* indicate lesional skin

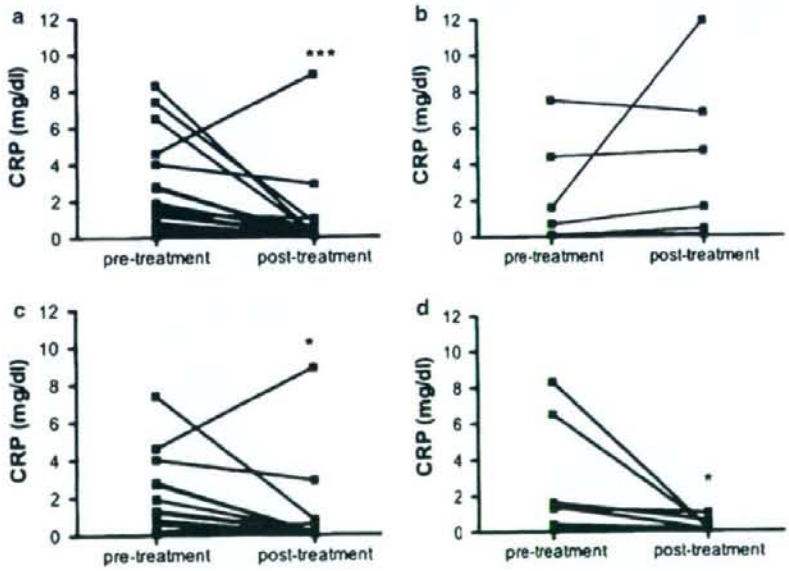

Fig. 2 Changes in the serum CRP level before and after the treatment. **a** Group with treatment intervention. Significant decrease in the CRP level was observed after the treatment ($p = 0.001$, Mann–Whitney test). **b** Group with non-treatment intervention. No obvious decrease in the CRP level was observed. **c** Only diseases related to the collagen disease were extracted from the group with treatment intervention. **d** Only diseases related to the non-collagen disease were extracted. Significant decreases in the CRP levels were observed in both collagen and non-collagen disease groups. Statistical value: $^{***}p < 0.005$, $^{*}p < 0.05$

is known to be induced by IL-6 [6], serum IL-6 levels were measured in each group. Compared with the normal control level, both the collagen disease and non-collagen disease groups showed high serum IL-6 levels ($p = 0.0028$ and $p = 0.0089$, respectively, Mann–Whitney test) (Fig. 3a, b, d, e). Such serum IL-6 levels were significantly decreased by administration of Lipo-PGE1 in overall data including collagen disease and non-collagen disease groups ($p = 0.0282$, paired t test) (Fig. 3b). The non-intervention group showed no decreased IL-6 (Fig. 3c).

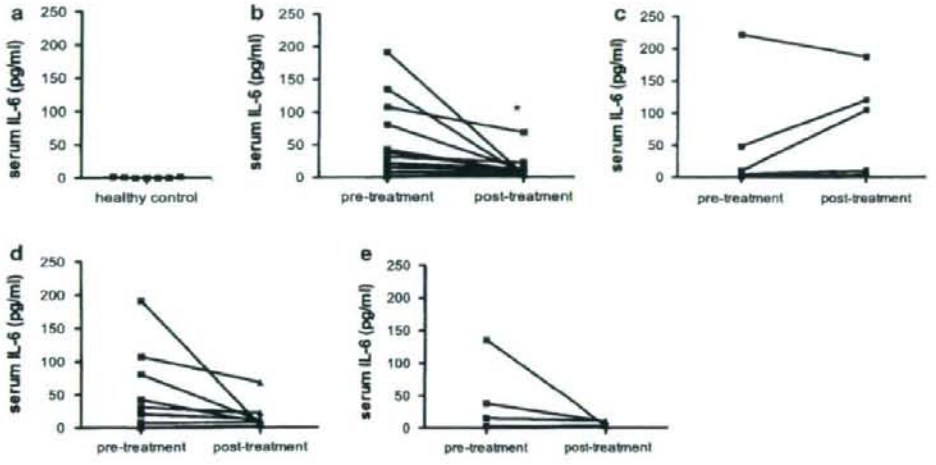

Fig. 3 Changes in the serum IL-6 level before and after the treatment. **a** IL-6 values in healthy controls. **b** All data for group with treatment intervention. The serum IL-6 level decreased significantly. Statistical value: $^{*}p < 0.05$. **c** Group with non-treatment intervention. They are only six subjects. No obvious tendencies can be confirmed. **d** No significant difference was observed in serum IL-6 changes for diseases related to the collagen disease in group with treatment intervention ($p = 0.0824$, paired t test), however, the serum IL-6 level is on a decreasing trend. **e** The same tendency was observed also in the group with non-collagen disease ($p = 0.2772$, paired t test. Pre-treatment: mean 47.68, SD ± 60.2, post-treatment: mean 6.289, SD ± 4.337)

Next, the level of VEGF was measured by the ELISA method. Before Lipo-PGE1 administration, the collagen disease group showed significantly increased levels of VEGF in serum (mean 630.6 pg/ml, SD ± 306.2) compared with that of healthy individuals ($p = 0.0056$, Mann–Whitney test) (Fig. 4a, b), as well as the non-collagen disease group (mean 210.1 pg/ml, SD ± 179.6, $p = 0.004$, Mann–Whitney test) (Fig. 4c). Notably, significant difference was observed before and after the Lipo-PGE1 administration in collagen disease group ($p = 0.0303$, paired t test).

Since sICAM-1 is also known as a marker to reflect other inflammatory disease's progress [7] in vascular diseases, we evaluated sICAM-1 level in both groups before

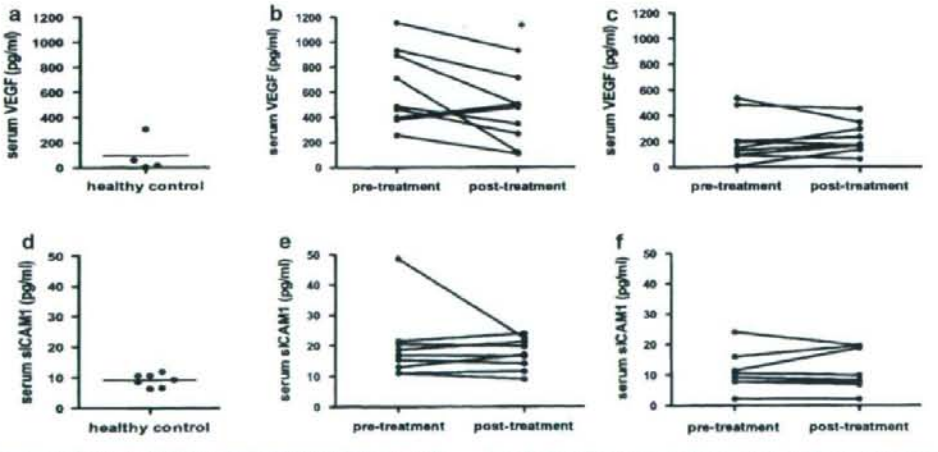

Fig. 4 Changes in the serum VEGF and sICAM1 level before and after the treatment. **a** VEGF values in healthy controls. **b** Changes in the serum VEGF level before and after the treatment in diseases related to the collagen disease with treatment intervention. Statistical value: $^{*}p < 0.05$. **c** Non-collagen disease group with treatment intervention. **d–f** Changes in the serum sICAM1 level before and after the treatment. **d** The results of healthy controls. **e** The results of diseases related to the collagen disease. **f** The results of the non-collagen disease group

and after Lipo-PGE1 administration. Before Lipo-PGE1 administration, the collagen disease group (Fig. 4e) showed a significantly increased level of sICAM-1 in serum compared with that of healthy individuals (Fig. 4d) ($p = 0.0007$, Mann–Whitney test), unlike the non-collagen disease group (Fig. 4f) ($p = 0.7282$, Mann–Whitney test). In the collagen disease group, no significant difference but a decrease in sICAM-1 level was observed before and after Lipo-PGE1 administration (mean 19.6 ng/ml, SD ± 11.51, Mean: 17.26 ng/ml, SD ± 5.077, respectively) (Fig. 4e). At the same time, no difference was observed before and after the administration in non-collagen disease group (mean 11.15 ng/ml, SD ± 6.513, Mean: 11.59 ng/ml, SD ± 6.733, respectively) (Fig. 4f).

Discussion

In addition to the benefit of DDS, Lipo-PGE1 has an anti-inflammatory effect reflected by effective uptake of PGE1 by damaged parts of the vascular. In this study, we focused on such a working mechanism of Lipo-PGE1 to evaluate the effects on intractable cutaneous ulcer. As a result, the followings are the suggestions.

Reduced ulcer size area and CRP level after Lipo-PGE1 administration

As mentioned earlier, it is considered that the prolongation at inflammatory phase may be a cause of the intractable mechanism in refractory cutaneous ulcers [1, 8]. However, there are very few reports on comparative studies on the severity of cutaneous ulcers and inflammatory markers. It is reported that the increasing serum CRP level had reflected the disease severity in pyoderma gangrenosum [9], one form of intractable cutaneous ulcer. In such situations, the present study discussed a further investigation of the correlation between the disease severity in skin ulcers and the serum inflammatory markers. This study evaluated the CRP levels in various cutaneous ulcers and confirmed that they increased in most cases regardless of the background of collagen diseases. Considering the facts that the patients with apparent wound infections were excluded and the general condition did not deteriorate in the collagen disease group, it can be assumed that inflammatory symptoms of local ulcers may have induced the elevated CRP level. In addition, these CRP levels significantly decreased in 2 weeks by Lipo-PGE1 administration. This suggested that Lipo-PGE1 might have an effect in controlling serum CRP levels increases due to inflammation in the site of ulcers other than primary vasodilating action and platelet aggregation inhibitory action. In this study, oral administration of stable PGI2 analog was applied to Lipo-PGE1 non-intervention group. It has been reported that oral administration of PGI2 analog reduced the development of ulcer and accelerated healing at least 4 weeks continuation of treatment [22]. Thus, it was assumed that the prominent effect of PGI2 analog did not appear at the 2 week evaluation.

A positive correlation between IL-6 level and CRP value and changes in serum VEGF levels due to Lipo-PGE1 administration

Among various cells recruited to ulcer areas at the inflammatory phase, microphage is known as a source of inflammatory cytokines such as IL-6 and VEGF [1, 2, 4, 5]. We assumed that the previously described reduction of CRP level due to Lipo-PGE1 administration might be to control cytokine secretory capacity from active macrophage accumulated in ulcer areas; therefore, we measured serum IL-6 and serum VEGF levels at each phase. Serum IL-6 levels in both the collagen disease and non-collagen disease groups after Lipo-PGE1 administration corresponded with our estimated values, which significantly decreased. Additionally, it was considered that the decreased serum IL-6 levels due to Lipo-PGE1 could be a cause of decreased CRP values and anti-inflammatory effects, as a positive correlation was confirmed between IL-6 level and CRP value ($r = 0.3478$).

The level of serum VEGF in the collagen disease group before treatment intervention with Lipo-PGE1 showed a significant increase compared with the healthy group, as well as the non-collagen disease group. It has been discussed that VEGF may be associated with the clinical severity of collagen disease since it is reported to be significantly increased as a cytokine associated with neoangiogenesis in RA, SLE, SSC, and PM/DM-affected individuals [10–12]. From this viewpoint, it can be explained that the serum VEGF level in the collagen disease patient group might increase under the influence of their background diseases. As for cutaneous ulcers, it is considered that serum VEGF level is increased in venous ulcers [13], but not in arterial ulcers. Drinkwater et al. made a comparative study of VEGF production in ulcer areas between healed ulcer groups and non-healing ulcer groups, and reported that increased VEGF production was observed in the non-healing ulcer group [14]. There are also a few reports, which suggest that production of monocyte-derived VEGF during cutaneous ulcer treatment with GM-CSF may have positive effects on neoangiogenesis [2]. Comprehensively understanding the above, it may be concluded that the VEGF increase is a welcome phenomenon for the wound healing of cutaneous ulcers, and contrastingly, it may be an unwelcome event as it reflects disease progress for collagen diseases. Considering the fact that the Lipo-PGE1 contributed to reducing serum VEGF level in the collagen disease

group together with the decreased IL-6, it can be assumed that Lipo-PGE1 had a good effect on the disease progress of the current diseases. In the non-collagen disease group, it may be suggested that Lipo-PGE1 has caused a favorable environment in wound healing, as the VEGF level was increased while the IL-6 level was decreased. However, IL-6 and VEGF levels showed deviations before and after the treatment, for this reason, it can also be assumed that Lipo-PGE1 has additional effects other than on macrophage.

It is known that the patients with collagen diseases have a high serum sICAM-1 level. Likewise, administration of Lipo-PGE1 controlled expression of sICAM-1 in serum as it did for serum IL-6 (Fig. 4e). This suggests that IL-6 and sICAM-1 are closely associated with each other and there is a possibility that with future advanced drug development we can expect ADL improvements in these patients.

Effects of Lipo-PGE1 on serum sICAM-1

The level of serum sICAM-1 in the collagen disease group before treatment intervention with Lipo-PGE1 showed a significant increase compared with the healthy group. Furthermore, sICAM-1 levels in serum showed no significant differences, but a tendency to be decreased by Lipo-PGE1 administration (Fig. 4d–f). It is considered that ICAM-1, a cell-adhesion molecule, is involved in leukocyte extravasation at local inflammations and plays an important role in the formation of autoimmune diseases. It is reported that the cleaved cell surface ICAM-1 is released to circulation as sICAM-1 in various inflammatory diseases including collagen diseases. It is reported to be a marker, which reflects disease progression of RA [15–17], SSC [18, 19] and SLE [16]. In the collagen disease group in this study, sICAM-1 levels showed the same level as serum VEGF level. Therefore, it is suggested and interesting that LipoPGE1 may play a role in the control of disease progress of collagen diseases.

In this study, Lipo-PGE1 was administered to intractable cutaneous ulcers with collagen diseases and non-collagen diseases. These results revealed that Lipo-PGE1 has significant effects on reducing various inflammatory cytokine levels, such as IL-6, CRP, and sICAM-1 in addition to the reduction of the ulcer area in intractable cutaneous ulcer cases.

This study also revealed that Lipo-PGE1 controls cytokine production, related to the disease progress of collagen vascular diseases. Steroid and immunosuppressants therapies are major choices in the treatment of collagen diseases, and sometimes compromise the quality of life of patients. They also require continuous attention for side-effects, and complications such as opportunistic infections make it even more difficult for therapeutic intervention [20]. It is anticipated that Lipo-PGE1 can express effective actions in these cases. Therefore, further accumulations of additional cases are expected.

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Dear Editor

Does Drug-induced Hypersensitivity Syndrome Elicit Bullous Pemphigoid?

A 68-year-old Japanese woman presented to our hospital for disseminated erythema on the whole body, lymphadenitis and high fever on 10 August 2003 (Fig. 1a). The erythema developed with a sudden onset after taking minocycline hydrochloride for pharyngitis and 38-degree fever on 6 August 2003 (day 0). She had taken carbamazepine and zonisamide for one year to control trigeminal neuralgia. Histopathological examination demonstrated perivascular dermatitis, and infiltration cells mainly consisted of eosinophils and lymphocytes in the upper dermis. She was given a diagnosis of drug eruption, and all the medication was stopped (day 4). However, erythema continued to develop, and we administered 60mg/day of oral prednisolone (day 8), after which the fever and erythema gradually ameliorated. Laboratory data showed leukocytosis (20.14×10^9/L), hypereosinophilia (23%), atypical lymphocytes (4%), elevated γ-glutamyltranspeptidase (385 U/L) and elevated liver enzymes (aspartate transaminase 19U/L, alanine aminotransferase 61U/L). Thereafter, titer for human herpes virus 6 (HHV-6) IgG increased from × 10 (day 8) to ×1280 (day 25). Collectively, a diagnosis of drug-induced hypersensitivity syndrome (DIHS) was established. We could not perform patch testing, and results of drug-induced lymphocyte stimulation tests for carbamazepine, zonisamide and minocycline hydrochloride were negative. It remained unclear which drug elicited the eruption. While tapering oral prednisolone, we started cyclosporine (3mg/kg) because we found slight recurrence of erythema (day 58), and we finally reduced oral prednisolone to 20 mg/day, with slight erythema remaining (day 68). However, 9 days later (day 77), itchy edematous erythema and tense bullae developed on the trunk and extremities, and there was no relationship between the distribution of the DIHS eruptions and the new eruptions (Fig. 1b). Biopsy specimens revealed subepidermal blisters with eosinophil and lymphocyte infiltration. Direct and indirect immunofluorescence showed lesional and circulating IgG autoantibodies at the basement membrane (Fig. 2). The laboratory data demonstrated hypereosinophilia (36.6%) and high index of anti-BP180 (2780) antibody by ELISA (day 92). Anti-nuclear antibody was negative. Thus, we diagnosed bullous pemphigoid (BP). Despite of the combination therapy with oral prednisolone (50mg/day), cyclosporine (3–5mg/kg), azathio-

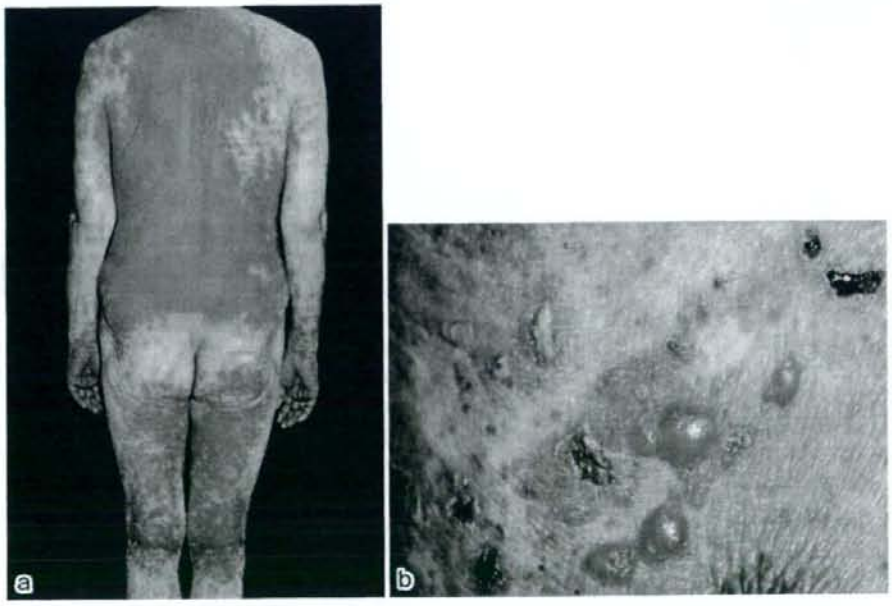

Fig. 1 Clinical appearance at the first visit (**a**) and the later occurrence of the bullous disease (**b**).

prine (100mg/day), cyclophosphamide pulse (500 mg/day), or double filtration plasmapheresis, the eruptions were recalcitrant. Finally a remission was achieved by starting mycophenolate mofetil (3g/day) and the titer of anti-BP180 antibody began to decrease markedly (Fig. 3).

To the best of our knowledge, this is the first report of BP developing consecutively after DIHS. The pathological or immunological linkage as to whether BP occurred incidentally after DIHS or was induced by DIHS remains unclear. However, Kano *et al.* reported a case of sclerodermoid graft-versus-host disease-like lesions occurring after DIHS.[1] They suggested that the autoimmune manifestations observed in patients with chronic GVHD could also be seen in patients with DIHS in view of clinical similarity between GVHD and DIHS. Although a recent report showed that a decrease in immunoglobulin levels and B-cell counts can be associated with HHV-6 reactivation and the subsequent onset of DIHS,[2] our present case suggests that this disease may involve or evolve into other immunological events including autoimmunity.

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Fig. 2 Positive deposition of IgG at the basement membrane zone on direct immunofluorescence (DIF) studies.

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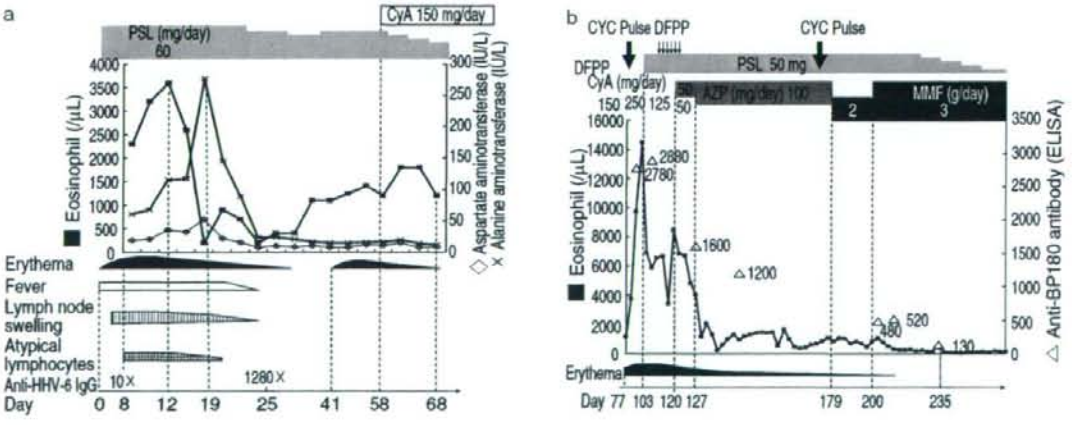

Fig. 3 Clinical and laboratory course of DIHS (**a**) and BP (**b**). HHV-6, human herpesvirus 6; PSL, prednisolone; CyA, cyclosporine; CYC, cyclophosphamide; DFPP. Double filtration plasmapheresis; AZP, azathioprine; MMF, mycophenolate mofetil.