

Cetirizine, an H1-receptor antagonist, suppresses the expression of macrophage migration inhibitory factor: its potential anti-inflammatory action

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Summary

Background H1-receptor antagonists are often effective in the treatment of allergic disorders such as atopic dermatitis. Cetirizine, a putative H1-receptor antagonist, has recently been shown to have anti-inflammatory properties through the inhibition of leucocyte recruitment and activation, and by the reduction of ICAM-1 expression on keratinocytes.

Objective To further elucidate the anti-inflammatory properties of cetirizine, we first examined its effects on antigen-induced eosinophilia and neutrophilia *in vivo*. We then examined the anti-inflammatory effects of cetirizine on a human keratinocyte A431 cell line.

Methods Mice were sensitized subcutaneously with ragweed pollen and were challenged intraperitoneally with the allergen. Cetirizine diluted in sterile water (0–20 mg/kg) or only sterile water was administered orally. Peritoneal cells were obtained at 8 and 24 h after challenge. The eosinophilia and neutrophilia induced by ragweed pollen extract were quantitated. Macrophage migration inhibitory factor (MIF), macrophage inflammatory protein 2 (MIP-2) and eotaxin contents of peritoneal fluid were also measured by mouse ELISA. The effects of cetirizine on MIF-induced IL-8 production in A431 cells were examined by ELISA. The effects of cetirizine on MIF expression and production in A431 cells were examined by human MIF ELISA and Northern blot analysis.

Results Eosinophilia and neutrophilia induced by ragweed pollen extract were found to be significantly reduced in cetirizine-treated mice (20 mg/kg). MIF, a pleuripotent cytokine, was significantly decreased at 8 and 24 h in the peritoneal fluid by cetirizine treatment. MIP-2 and eotaxin were also decreased 8 and 24 h, respectively, after challenge in the peritoneal fluid with cetirizine treatment. MIF stimulates IL-8 production in A431 cells. We found that MIF production in A431 cells was inhibited by 10 µM cetirizine. Consistent with this, cetirizine significantly inhibited MIF-induced IL-8 production.

Conclusion These results suggest that cetirizine exerts its anti-inflammatory effects by inhibiting MIF as well as IL-8 production, such as those involved in inflammatory allergic skin disease, suggesting a broad spectrum of action beyond its mere H1-receptor-antagonistic function.

Keywords cetirizine, chemokine, eosinophil, interleukin 8, macrophage migration inhibitory factor

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Introduction

Cetirizine, a carboxylated metabolite of hydroxyzine, belongs to the new generation of H1-receptor antagonists, which inhibit the cutaneous early-phase reaction, and is effective as an oral treatment for patients with a number of allergic skin disorders, including atopic dermatitis (AD) [1, 2]. Cetirizine also exerts an array of anti-inflammatory activities, in addition to its anti-allergic activity. In particular, it has important effects on eosinophils that inhibit chemotaxis both *in vitro* and *in vivo* [3–5]. Expression of membrane ICAM-1 and soluble ICAM-1 in epithelial cells is significantly reduced

in the presence of cetirizine [6]. High concentrations of cetirizine also markedly inhibit the IFN- γ -induced expression of membrane ICAM-1 in cultured human keratinocytes [6].

Macrophage migration inhibitory factor (MIF) was the first lymphokine reported to prevent the random migration of macrophages [7]. It plays an important role in delayed-type hypersensitivity [8]. We have recently reported the excessive expression of MIF mRNA and MIF protein in inflammatory skin lesions in AD [9, 10]. MIF originates from multiple cellular sources such as activated T lymphocytes, monocytes, eosinophils, and keratinocytes in AD [10–12]. The secretion of MIF is up-regulated *in vitro* by TNF- α and IFN- γ [13]. MIF in turn augments the secretion of TNF- α and IL-8 [14]. This inflammatory loop characterizes MIF as a pro-inflammatory cytokine. Keratinocytes are capable of producing a variety of cytokines, and are considered to be a principal

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source of cytokines in the epidermis. Cytokines are not only involved in the mediation of local inflammatory reactions within the epidermis, but also exert systemic effects by entering into the circulation.

It has recently been reported that cetirizine reduces the release of IL-8 from epithelial cells [15]. In this context, cetirizine might not only possess anti-inflammatory activity, but also reduce the chemotactic activity of effector cells [4, 5, 16]. We investigated here the effects of cetirizine on the production of IL-8 and MIF in human epidermal keratinocytes cell line A431. Furthermore, we examined the effects of cetirizine on antigen-induced eosinophilia and neutrophilia *in vivo* in response to oral administration of this drug.

Materials and methods

Materials

Male 8-week-old BALB/c mice (25–30 g) were obtained from SLC Inc. (Shizuoka, Japan). Human A431 cells were purchased from the Health Science Research Resources Bank (Osaka, Japan). Cetirizine was kindly provided by UCB Laboratories (Braine l'Alleud, Belgium). The following materials were obtained from commercial sources: ragweed pollen extract (1 : 100, wt/vol) in aqueous solution from Torii Pharmaceutical Co. (Tokyo, Japan); human IL-8-specific ELISA kit and mouse eotaxin-specific ELISA kit from genzyme TECHNE (Cambridge, MA, USA); mouse macrophage inflammatory protein 2 (MIP-2) ELISA kit from R&D Systems (Minneapolis, MN, USA); the Isogen RNA extraction kit from Nippon Gene (Tokyo, Japan); M-MLV reverse transcriptase from GIBCO (Grand Island, NY, USA); Taq DNA polymerase from Perkin-Elmer (Norwalk, CO, USA); horseradish peroxidase-conjugated goat anti-rabbit antibody from Pierce (Rockford, IL, USA); Vector ABC Kit from Vector Laboratories (Burlingame, CA, USA); nylon membranes from Schleicher & Schuell (Keene, NH); concanavalin A (Con A) and polymyxin B from Sigma (St Louis, MO, USA); Ficoll-Plaque Plus and Protein A Sepharose from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

Immunization and challenges

The BALB/c mice were immunized and challenged with ragweed pollen according to the method of Kaneko et al. [17]. Briefly, mice were immunized by a series of five injections of a 1 : 1000 dilution of the ragweed pollen extract. On days 0 and 1, 0.1 mL was injected subcutaneously, and 0.2 mL was injected subcutaneously on days 6, 8, and 14. The mice were challenged on day 20 by an intraperitoneal injection of 0.2 mL of the dilution of the ragweed pollen extract. Experimental groups ($n = 5$) were treated with orally administered cetirizine diluted in sterile water (2 or 20 mg/kg) on days 18, 19, and 20. The control group was orally administered sterile water on days 18, 19, and 20. The naïve group was only challenged with no sensitization. Eight or 24 h after the challenge (day 21), mice were killed by incision of the carotid artery. Two millilitres of phosphate-buffered saline containing 1.0% fetal calf serum (FCS) and 5 U/mL heparin was injected intraper-

itoneally, and the abdomen was massaged. The peritoneal infusion was collected with a Pasteur pipette after the peritoneum had been opened. Differential leucocyte counts were made under a microscope after fixation and staining of the peritoneal cells with May-Grünwald-Giemsa stain. The peritoneal fluid contents were assayed for MIF, MIP-2, and eotaxin using mouse MIF, MIP-2, and eotaxin ELISA.

Cell culture

A431 cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and grown on plastic Petri dishes in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Eighty percent confluent cells were used for the following experiments. Twenty-four hours prior to the experiments, the culture medium was changed with serum-free medium.

ELISA

ELISA was used to measure the content of MIF secreted from cultured cells, as described in [10]. For this assay, we used recombinant human MIF to obtain the standard curve, with good linearity demonstrated between MIF contents (1–200 ng/mL) and absorbency. Human IL-8 release was determined with an IL-8-specific ELISA kit. Mouse eotaxin release was determined using an eotaxin-specific ELISA kit. MIF ELISA was also performed using mouse peritoneal fluid as described in [18]. MIP-2 and eotaxin contents in mouse peritoneal fluid were also measured using mouse MIP-2 and eotaxin ELISA kits, respectively.

Northern blot analysis

Total cellular RNA was isolated from cultured A431 cells using an Isogen extraction kit. In brief, total RNA was extracted from cell pellets with an Isogen total RNA extraction kit. RNA was resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Total RNA (20 µg) was denatured and electrophoresed on 1% agarose formaldehyde gel. The RNA was then transferred to nylon membranes, and cross-linked by UV irradiation. Prehybridization was carried out in 0.75 M NaCl, 0.02 M Tris-HCl (pH 7.5), 2.5 mM EDTA, 0.5 × Denhardt solution, 1% sodium dodecyl sulphate (SDS), and 50% formamide at 42 °C for 4 h. Then hybridization was performed in the same buffer containing 10% dextran sulphate, salmon sperm DNA (250 mg/mL), and a radiolabelled probe at 42 °C for 20 h. The radiolabelled probe was prepared using human MIF cDNA as a template and labelled with a random primer labelling kit using [α -³²P]dCTP. The membrane was washed twice with 2 × SSC (16.7 mM NaCl, 16.7 mM sodium citrate) at 22 °C for 5 min, twice with 0.2 × SSC containing 0.1% SDS 65 °C for 15 min, and 2 × SSC at 22 °C for 20 min prior to autoradiography. Quantitative densitometric analysis was performed using an MCID Image Analyzer (Fuji Film, Tokyo, Japan). The density of each MIF band was normalized by the intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistics

All statistical comparisons were performed with the Student's *t*-test. All *P*-values were two-sided, and summary data are expressed as means $\pm$ SEM.

Results

Effects of cetirizine on antigen-induced neutrophilia and eosinophilia

We first examined the effects of cetirizine on antigen-induced eosinophilia and neutrophilia. The neutrophilia at 8 h and eosinophilia at 24 h induced by ragweed pollen extract *per os*

were significantly reduced in the mice treated with 20 mg/kg cetirizine (Fig. 1). We found that 2 mg/kg cetirizine had no effect on eosinophilia and neutrophilia. In this protocol, eosinophil accumulation reached a peak at 24 h, and the response gradually declined thereafter. In contrast, the accumulation of neutrophils reached a peak 8 h after challenge, and then their number declined [19]. This result indicated that cetirizine possessed not only potent H1-receptor-blocking activity, but also inhibitory action with regard to neutrophils and eosinophil infiltration into allergen-induced late-phase reaction sites. The MIF content of peritoneal fluid was significantly decreased in the mice treated with cetirizine compared with the control (Table 1).

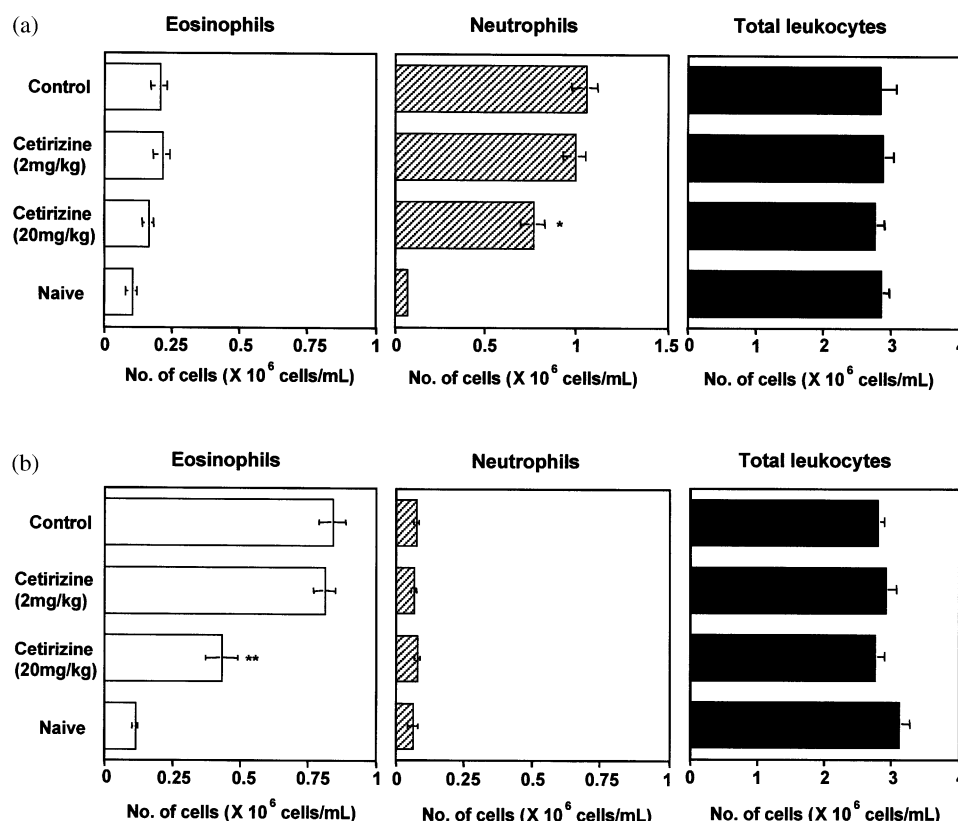


Fig. 1. Effects of cetirizine on antigen-induced neutrophilia and eosinophilia in mice. Mice were sensitized subcutaneously with an allergen on days 0, 1, 6, 8, and 14, and were challenged intraperitoneally with the same allergen on day 20. Peritoneal cells were obtained 8 h (a) or 24 h (b) after challenge. Cetirizine diluted in sterile water (2 or 20 mg/kg) or only sterile water (control group) was administered orally on days 18, 19, and 20. The naïve group was only challenged, with no sensitization. Each value represents the mean $\pm$ SEM of five animals. **P* < 0.01, ***P* < 0.005 as compared with the control group.

Table 1. MIF, MIP-2 and eotaxin contents in peritoneal fluid

Treatment†	MIF(ng/mL)‡		MIP-2(pg/mL)‡		Eotaxin(pg/mL)‡	
	8h	24h	8h	24h	8h	24h
Control	153.1 $\pm$ 15.7	169.5 $\pm$ 15.9	133.3 $\pm$ 9.4	11.0 $\pm$ 1.2	8.6 $\pm$ 1.4	44.8 $\pm$ 2.7
Cetirizine(2mg/kg)	147.6 $\pm$ 17.9	151.7 $\pm$ 16.7	125.2 $\pm$ 9.2	10.8 $\pm$ 2.3	8.2 $\pm$ 1.1	43.1 $\pm$ 3.5
Cetirizine(20mg/kg)	73.1 $\pm$ 9.9*	75.7 $\pm$ 7.4*	75.8 $\pm$ 9.1**	8.7 $\pm$ 1.1	8.1 $\pm$ 1.4	24.1 $\pm$ 2.8**
Naïve	38.4 $\pm$ 4.0	44.1 $\pm$ 6.9	13.3 $\pm$ 1.2	8.1 $\pm$ 0.5	5.2 $\pm$ 1.0	5.6 $\pm$ 0.9

†The mice were treated with orally administered cetirizine diluted in sterile water (2 or 20 mg/kg) on days 18, 19, and 20. The control group was orally administered with sterile water on days 18, 19, and 20. The naïve group was only challenged without sensitization (*n* = 5 in each group). ‡Eight or 24 hours after the challenge (day 21), MIF, MIP-2 and eotaxin contents of peritoneal fluid were assayed using mouse ELISA kits as described in Methods. MIF, migration inhibitory factor; MIP-2, macrophage inflammatory protein 2. **P* < 0.005; ***P* < 0.001 vs. control.

Furthermore, the MIP-2 content at 8 h and eotaxin content at 24 h were significantly decreased by cetirizine treatments.

Stimulation of IL-8 release from A431 cells by MIF

It has been reported that cetirizine also has anti-inflammatory properties [20]. In this context, we tested whether cetirizine exhibits any anti-inflammatory activity in relation to human keratinocytes. We carried out an experiment to determine

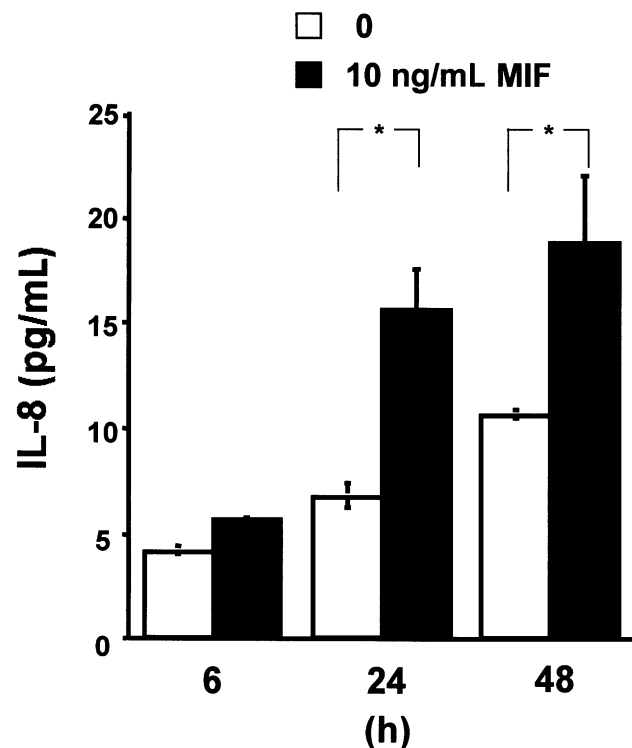


Fig. 2. IL-8 release from A431 cells stimulated by migration inhibitory factor (MIF). Human keratinocyte cell line A431 cells were incubated with various concentrations of MIF for 24 h. The IL-8 content was measured in the culture media of A431 cells with an IL-8-specific ELISA kit. Values are means $\pm$ SEM from three independent experiments. * P < 0.01.

whether MIF stimulates the production of IL-8, the multi-functional pro-inflammatory cytokine. The human keratinocyte cell line of A431 cells was incubated with various concentrations of MIF. The results indicate that MIF (10 ng/mL) enhanced IL-8 production within 24 h (Fig. 2).

Effects of cetirizine on MIF-induced IL-8 production in A431 cells

To analyse whether cetirizine has inhibitory effects on the MIF-stimulated IL-8 release from A431 cells, the cells were pre-incubated with various concentrations of cetirizine, followed by stimulation with 10 ng/mL MIF. The addition of 0.1 μ M cetirizine significantly inhibited the production of IL-8 in A431 cells at 24 h, as did that of 1 μ M cetirizine at 6 h (Fig. 3).

Effects of cetirizine on MIF expression and production in A431 cells

Next we examined whether cetirizine directly inhibits MIF production in A431 cells. Cells were pre-incubated with increasing concentrations of cetirizine, from 1 to 100 μ M. After 6 and 24 h of treatment, the release of MIF was significantly inhibited at concentrations of 10 μ M (Fig. 4a), indicating that cetirizine has the potential to inhibit not only IL-8 but also MIF production in A431 cells. To confirm the effects of cetirizine on MIF expression at the mRNA level, we carried out Northern blot analysis. The result showed that the MIF mRNA levels were significantly inhibited (0.46-fold decrease) by cetirizine after 6 h of treatment at 10 μ M concentrations (Fig. 4b).

Discussion

Cetirizine is a carboxylated analogue of hydroxyzine, that which potent and specific H1-receptor-blocking activity. Cetirizine has anti-inflammatory activities unrelated to its histamine antagonism. For example, it is also known to

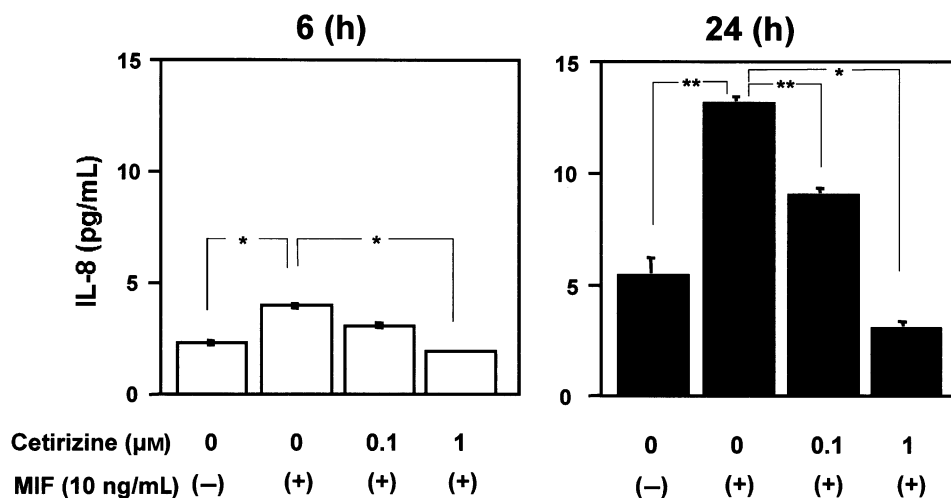


Fig. 3. Effects of cetirizine on IL-8 expression and release from A431 cells induced by migration inhibitory factor (MIF). A431 cells were pre-incubated with various concentrations of cetirizine, followed by stimulation with 10 ng/mL MIF. After incubation for 6–24 h, the IL-8 content was measured within the culture media of A431 cells with an IL-8-specific ELISA kit. Values are means $\pm$ SEM from three independent experiments. * P < 0.05, ** P < 0.01.

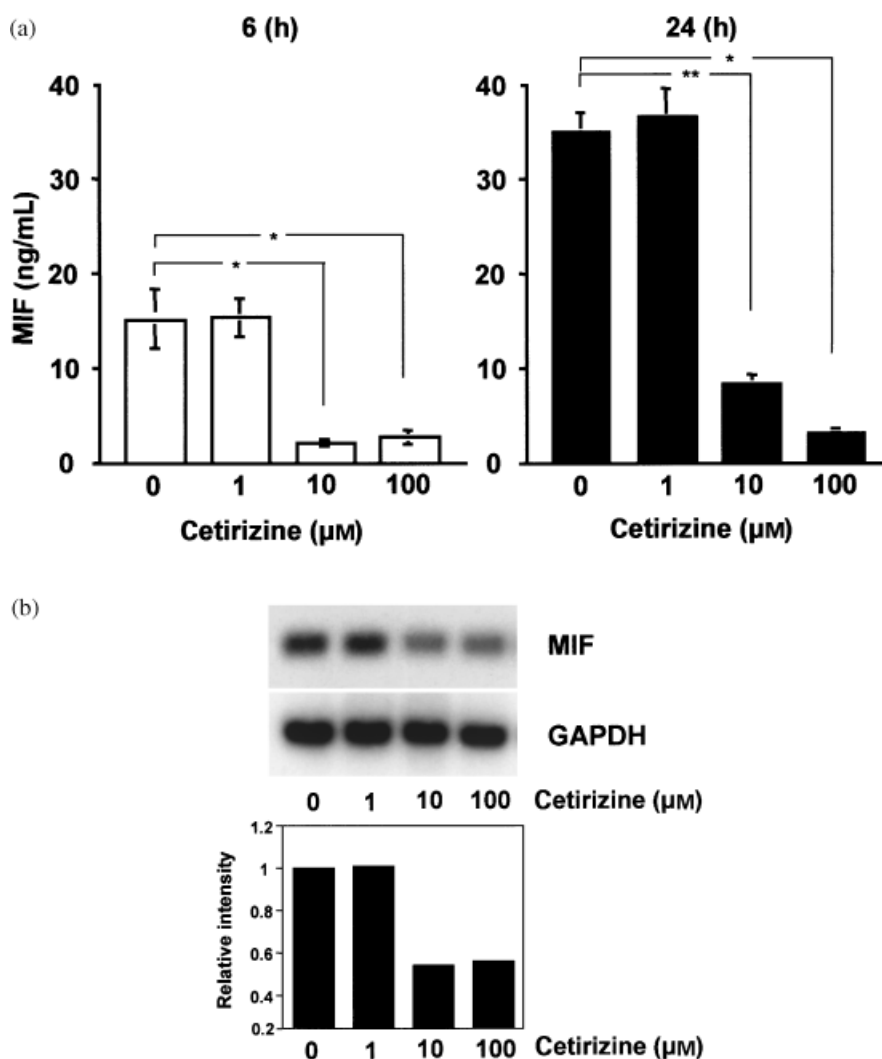


Fig. 4. Effects of cetirizine on migration inhibitory factor (MIF) mRNA expression and release from A431 cells. (a) A431 cells were pre-incubated with increasing concentrations of cetirizine from 1 to 100 µM. After 6 and 24 h of treatment, an MIF ELISA measured the release of MIF into the culture media of A431 cells. Values are means $\pm$ SEM from three independent experiments. * $P < 0.0001$, ** $P < 0.005$ vs. control (0 µg/mL cetirizine). (b) After 6 h of incubation with varying doses of cetirizine, total RNA was isolated from A431 cells. Northern blot analysis was performed as described in Materials and methods. The densities of MIF bands were normalized to the glyceraldehyde-3-phosphate dehydrogenase signals. The relative intensity was determined in comparison with that of the 0 µg/mL cetirizine.

inhibit eosinophil infiltration into allergen-induced late-phase reaction sites in humans [5, 21]. There is emerging evidence that eosinophils are important effector cells in allergic skin inflammation such as those occurring in AD. Previously it was reported that subcutaneous cetirizine treatment (15–30 mg/kg) markedly reduces ovalbumin-induced eosinophil accumulations [22]. The effects of high concentrations of cetirizine (10–30 mg/kg) have been reported [22, 23]. Our present results also demonstrated that oral cetirizine administration significantly reduced the eosinophilia induced by ragweed pollen extract in mice. However, a similar H1-blocker, terfenadine, is ineffective against eosinophil accumulations when administered subcutaneously (10 or 30 mg/kg) [22]. Thus, the effects of cetirizine appear not to be related to the H1-receptor-inhibition effects of histamines. Thomson et al. [24] reported that cetirizine exhibited potent *in vitro* inhibitory effects on eosinophil transmigration through monolayers of both lung and dermal microvascular endothe-

lial cells. In a rat model, cetirizine has been found to inhibit the antigen-induced recruitment of eosinophils into the pleural cavity in animals sensitized with ovalbumin [25]. We also demonstrated that oral cetirizine administration significantly reduced neutrophilia. Cetirizine is also known to exert an anti-inflammatory effect on neutrophils [26]. Therefore, cetirizine not only exhibits potent H1-receptor-blocking activity but also inhibits eosinophil and neutrophil influx into allergen-induced late-phase reaction sites. In the present study, MIF, MIP-2, and eotaxin contents of peritoneal fluid were also significantly decreased by cetirizine treatment, indicating that the decrease in eosinophils and neutrophils caused by cetirizine is more profoundly involved in inhibiting these cytokines and chemokines. Indeed, MIF has the potential to cause eosinophils and neutrophils to accumulate [11, 12, 27]. In mice, a functional IL-8 homologue, MIP-2, induces migration of neutrophils, and eotaxin is a potent and specific eosinophil chemoattractant.

We have also found that cetirizine inhibited MIF release from A431 cells. In human allergic and inflammatory diseases, it has been reported that MIF has the potential to exacerbate asthma [12] and acute respiratory distress syndrome [14]. Therefore, it is hypothesized that cetirizine may alleviate acute inflammatory and allergic respiratory diseases through the suppression of MIF production. In the epidermis, we have demonstrated the presence of MIF in keratinocytes [28]. Following this report, we have demonstrated excessive expression of MIF mRNA as well as MIF protein in AD inflammatory skin lesions, with the serum MIF content changing in parallel with the clinical symptoms [10]. In this context, it is hypothesized that cetirizine exerts its anti-inflammatory action in allergic skin lesions by suppressing MIF production. Although a high cetirizine concentration was needed to inhibit MIF production and expression in A431 cells, this may be explained by the fact that there was no correlation between the concentration of H1-antihistamine which inhibited H1-receptor-independent anti-inflammatory effects and receptor-dependent histamine release [29]. Furthermore, MIF is known to stimulate the proliferation of fibroblasts and other cell lines [30, 31]. Recently, Cagnoni et al. [32] showed that cetirizine was able to inhibit fibroblast proliferation. These findings suggest that cetirizine may inhibit cell proliferation by the suppression of MIF bioactivity.

Furthermore, we have demonstrated in the present study that MIF stimulates the release of IL-8 in A431 cells. IL-8 may also play a role in inflammatory skin disorders such as AD, as it has been reported to be produced by keratinocytes [33]. IL-8 stimulates neutrophil chemotaxis and also affects other leucocyte cell types, including lymphocytes, basophils, and eosinophils. IL-8 is considered to play an important role in allergic responses at local sites of inflammatory skin disease. In this context, it is conceivable that cetirizine exerts its anti-inflammatory effects besides the anti-allergic potential to inhibit IL-8 and MIF production in epidermal keratinocytes, beyond its currently known H1-receptor-antagonistic activity.

In summary, cetirizine is a non-sedating selective H1 histamine antagonist, and is widely used for the treatment of allergic diseases, including perennial rhinitis and urticaria. In addition to these actions, cetirizine has been considered as having anti-inflammatory activity; however, the precise mechanism remains to be investigated. We here showed the potential of cetirizine to lower the levels of IL-8 and MIF. These findings strongly support the suggestion that MIF exerts anti-inflammatory actions in addition to its H1-receptor-antagonistic activity. Therefore, it is expected that cetirizine could be used more effectively to treat allergic diseases such as AD and other diseases involving severe inflammatory reactions.

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