Dendritic cells as targets of corticotropin-releasing hormone

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Dendritic cells as targets of corticotropin-releasing hormone

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Table of Contents

List of Figures

List of Tables

ABSTRACT

Dendritic cell as targets of corticotropin-releasing hormone

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Dendritic cell (DC) plays an important role in the generation and regulation of immune responses, but also is considered to represent the link between allergen uptake and the clinical manifestation of allergic disease, such as atopic dermatitis. Recent evidence suggests that crosstalk between mast cells, nerves and keratinocytes might be involved in exacerbation of the inflammatory conditions by stress, but the mechanism still remains unclear. Corticotropin releasing hormone (CRH), which activates the hypothalamic-pituitary-adrenal axis under stress, also has proinflammatory peripheral effects. However, there have been no reports about CRH receptor expression and functional role of CRH in DC. The purpose of this study was to investigate the expression of CRH receptors and the functional role of CRH in the monocyte-derived DC (MoDC) of atopic dermatitis patients and nonatopic healthy control. In this study, mRNAs for CRH-R1α, 1β, as well as CRH-R1 protein was detected in MoDC. CRH-R2α (but not R2β or R2ɣ) mRNA and CRH-R2 protein were present in MoDC. Exposure of DC to lipopolysaccharide (LPS) or tumor necrosis factor α (TNF-α), which activate DC, didn′t alter the expression of CRH receptors. Exposure of DC to CRH resulted in decrease of IL-18 in both atopic dermatitis patients and nonatopic healthy control. This effect was more prominent in atopic

dermatitis patients. However, CRH didn′t alter the expressions of IL-6, CCL17, CCL18 and CCL22. Therefore, our results demonstrate that CRH could modulate immune responses by acting directly on DC .

Key words: Corticotropin releasing hormone, dendritic cell

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I. INTRODUCTION

Atopic dermatitis, psoriasis and other inflammatory skin diseases are known to be exacerbated by stress[1]. Recent evidence suggests that crosstalk between mast cells, neurons and keratinocytes might be involved in such exacerbation, but the mechanism still remains unclear.

Corticotropin releasing hormone (CRH), a 41-amino acid long peptide is a main trigger of the hypothalamo-pituitary-adrenal (HPA) axis[2-4]. Stress signals in the hypothalamus stimulate expression and release of CRH, resulting in propiomelanocortin (POMC) expression and adrenocorticotropin (ACTH) release by the pituitary[3]. ACTH elicits cortisol release by adrenal cortex that is responsible for attenuation of the stress response at the central and peripheral level[3].

The skin has its own neuroendocrine system, which is tightly linked into systemic neuroendocrine axes, probably in order to coordinate peripheral responses to stress and to maintain cutaneous and global homeostasis[5]. CRH is also produced in the skin, where it can act as a regulatory element of local neuroendocrine interactions[5]. CRH has been described to act as a growth factor[6,7], apoptosis regulator[8] and differentiation factor[9]. CRH also acts as pro-inflammatory factor, since it stimulated degranulation of mast cells and increases vascular permeability[10]. CRH also stimulates vascular endothelial

growth factor release by mast cells[11], IL-6 release by keratinocytes[12] and IL-1β release by monocytes[13]. CRH also has an anti-inflammatory activity since it diminishes NF-kB activation in epidermal melanocytes[14], IL-18 expression in human HaCaT keratinocytes[15]. Thus, effects of CRH are dependent on the cell type and on experimental conditions.

CRH exerts its effects by binding to specific cell surface receptors, of which two receptor subtypes, CRH-R1 and CRH-R2[16]. Mapping of the cutaneous CRH signaling system in humans revealed that the CRH receptor type 1 (CRH-R1) is expressed in all major cellular populations of epidermis, dermis, and subcutis[16]. CRH-R1 appears to be the most prevalent isoform of CRH-R, and the CRH-R2 gene is expressed solely in the dermis and adnexal structures[16]. The pathophysiological relevance of CRH-R1 may be reflected by the observation that CRH-R1 is involved in stress-induced exacerbation of chronic contact dermatitis in rats[17]. Furthermore, afftected skin areas from patients with chronic urticaria have increased expression of CRH-R1[18].

Nevertheless, the functional significance of CRH-R expression in peripheral tissues is still unresolved. Moreover, there have been few studies regarding the signals which regulate the expression of CRH-R.

Dendritic cell (DC) is a highly specialized professional antigen-presenting cell usually located at surveillance interfaces of the human body such as the skin or mucosa, and is thought to play an important role in the generation and regulation of immune responses. In particular, DC is considered to represent the link between allergen uptake and the clinical manifestation of allergic disease, such as atopic dermatitis[19]. There have been no reports about CRH-R expression and functional role of CRH in DC.

The purpose of this study is to investigate the expression of CRH-R, the signals which regulate the expression of CRH-R, and the functional role of CRH in the monocyte-derived DC (MoDC).

II. PATIENTS AND METHODS

1. Patients

Blood samples are obtained with informed consent from AD patients according to the criteria of Hanifin and Rajka[20]. Six AD and six nonatopic healthy controls are included. The patients, who have not received any systemic or topical treatment with immunosuppressive drugs for at least four weeks before collection of blood sample are included. The institutional review board approved this study.

2. Culture of human dendritic cell

The culture media containes RPMI 1640 (Gibco laboratories, Grand Island, NY), 2 μM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 10% fetal bovine serum (Hyclone, Logan, UT). Monocytes from study participants are isolated from peripheral blood

mononuclear cells (PBMCs) via the cell attachment method[21]. PBMCs are attached to six-well plates for 40 minutes. The supernatant and the floating cells are discarded, and the attached cells are used for culture. Monocytes are plated in six-well plates at a final concentration of 3×10^6 cells in 3 ml of culture medium. DCs are generated by culturing monocytes for six days in medium supplemented with 500 U/ml GM-CSF, 1000 U/ml IL-4 at days 0, 2, 4, 6[22]. At days 2, 4, and 6, one-third of the medium is removed, and an equivalent volume of fresh medium supplements with the above mentioned cytokines. For activation of DC, cells are treated with either lipopolysaccharide (LPS, Sigma, St Louis, Mo) or tumor necrosis factor α (TNF-α, R&D systems, Minneapolis, MN) for 24h during the last day of culture.

3. RT-PCR analysis

Total RNA was extracted after the seventh day of culture from DCs using

the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Reverse transcription reactions were performed using 1μg of total RNA. Oligonucleotide primers of previously published sequences for CRH-R1 and CRH-R2 isoforms (Table 1) were used. IL-18 primers were: forward, 5'-AGGAATAAAGATGGCTGCTGAAC-3'; reverse, 5'-GCTCACCAC AACCTCTACCTCC-3'.

Receptor Isoforms	PCR	Primer Sequence (5′-3′)
CRH-R1α	Sense	GGCAGCTAGTGGTTCGGCC
	Antisense	TCGCAGGCACCGGATGCTC
CRH-R1β	Sense	GGCCAGGCTGCACCCATTG
	Antisense	TCGCAGGCACCGGATGCTC
CRH-R2α	Sense	ATGGACGCGGCACTGCTCCA
	Antisense	CACGGCCTCTCCACGAGGG
CRH-R2β	Sense	GGGGCTGGCCAGGGTGTGA
	Antisense	CACGGCCTCTCCACGAGGG
CRH-R2γ	Sense	CTGTGCTCAAGCAATCTGCC
	Antisense	CACGGCCTCTCCACGAGGG

Table 1. PCR primers used for human CRH-R1 and CRH-R2

Amplification is performed on a GeneAmp PCR system 2700 (Applied Biosystems, Mountain View, CA). PCR is conducted under the following conditions: denaturation at 94℃ for 1 min, annealing at 67℃ for 1 min, and

extension at 72℃ for 1 min for 40 cycles. Specific PCR fragments are separated on a 1% agarose gel and visualized using ethidium bromide staining. The amounts of PCR products were determined by densitometry using TINA 2.10e software (Raytest, Straubehardt, Germany) and evaluated semi-quantitatively by grading the ratio between the specific products and the β-actin band.

4. Cytokine and chemokine production

For stimulation of cytokine production, DCs are distributed to six-well plates and stimulated with CRH (Sigma) in complete culture medium. CRH was 96% purity and endotoxin was removed from it. DC was stained for 30 min on ice with human anti IL-6, IL-18, mouse anti-CCL17, anti-CCL22, and anti-CCL18 monoclonal antibody (R&D systems) in phosphate-buffered saline containing 0.4% bovine serum albumin. After washing, cells were treated with fluorescein isothiocyanate-conjugated secondary antibody,

followed by fixing and permeabilizing the cells with a formaldehyde/saponin solution (Sigma). Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Results were expressed as the relative fluorescence intensity (RFI) calculated from the mean fluorescence intensity (MFI) as follows: $RFI = (MFI_{parameter\ evaluated} - MFI_{control})/ MFI_{control}$.

5. Detection of CRH-R1 and CRH-R2 by Western blot analysis

DCs were lysed, and prepared for Western blot analysis. Samples were resolved on 10% SDS-PAGE and then transferred to nitrocellulose (NC) membranes. The membranes were incubated with goat anti-human CRH-R1 that specifically recognizes CRH-R1, and goat anti-human CRH-R2 that specifically recognizes CRH-R2 (Santa Cruz Biotechnology, Santa Cruz, CA). Following stripping and washing, the membranes were incubated with bovine anti-goat HRP-conjugated serum (Santa Cruz Biotechnology). The protein bands were detected using 3,3,5,5-tetra methylbenzidine (Sigma).

III. RESULTS

1. Expression of mRNA for CRH receptor isoforms in DC

RT-PCR analysis showed that both immature and mature DCs expressed specific mRNAs for CRH-R1α, 1β (Figure 1A, B). CRH-R1β was more abundant than CRH-R1α. RT-PCR for CRH-R2 isoforms showed that DC expressed only CRH-R2α (Figure 2). LPS and TNF-α, which were known to activate DCs, didn't alter the expression of mRNA for CRH-R.

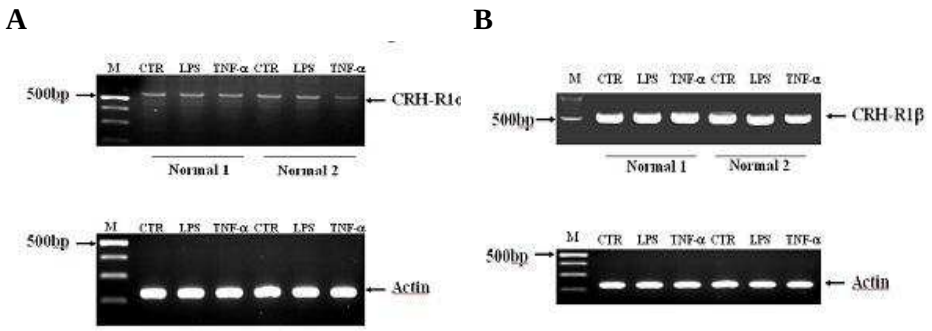


Figure 1. RT-PCR analysis of CRH-R1 isoforms mRNA in DCs of two healthy controls. mRNA for CRH-R1α (A), 1β (B) were detected. For activation, DCs were stimulated for 24h in

complete culture medium with LPS or TNF-α during the last day of DC culture. The control lane (CTR) corresponds to untreated cell population. Both LPS and TNF-α didn't alter the expression level of CRH-R1α, 1β. Results are representative of three independent experiments

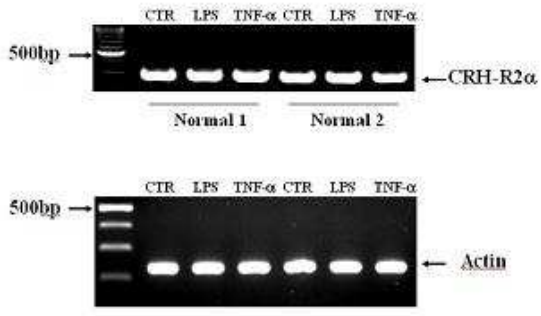


Figure 2. RT-PCR analysis of CRH-R2 isoforms (α, β, γ) mRNA in DCs of two healthy controls. Only mRNA for CRH-R2α was detected. For activation, DCs were stimulated for 24h in complete culture medium with LPS or TNF-α during the last day of DC culture. The control lane corresponds to untreated cell population. Both LPS and TNF-α didn't alter the expression level of CRH-R2α. Results are representative of three independent experiments

2. Western blot analysis of CRH receptor

Western blot analysis was used to detect CRH receptor protein expression in DC of healthy control. SDS-PAGE of whole cell lysates from DC was performed using Abs that specifically recognize CRH-R1 or CRH-R2. The Ab to CRH-R1 yieled a single band of ~50 kDa (Figure 3A). Western blot analysis for CRH-R2 identified a strong band of 49 kDa and a minor band of 51 kDa (Figure 3B). Lipopolysaccharide (LPS) and TNF-α, which were known to activate DCs, didn't alter the expression of CRH receptor protein (Figure 3A, B).

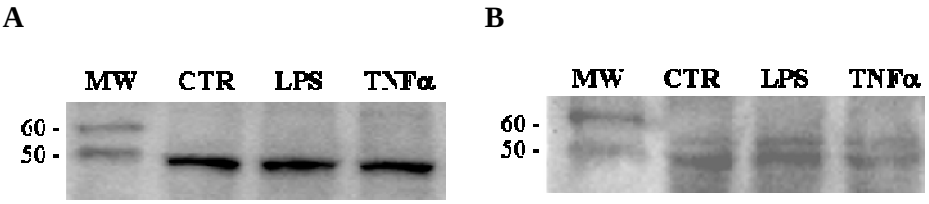


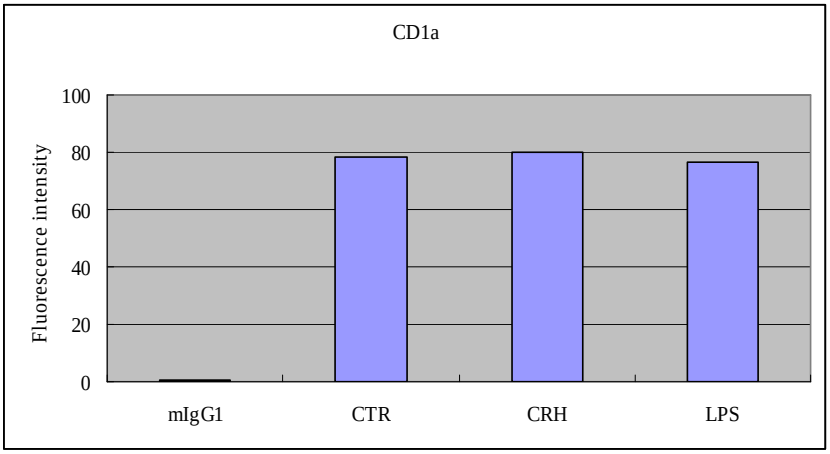
Figure 3. Western blot analysis of CRH-R1 (A) and CRH-R2 (B) in DC of healthy control. DC was collected, lysed and samples of the whole cell lysates were run on 10% SDS-PAGE, using

Abs that specifically recognize CRH-R1 or CRH-R2. The Ab to CRH-R1 yieled a single band of ~50 kDa (A). Western blot analysis for CRH-R2 identified a strong band of 49 kDa and a minor band of 51 kDa (B). For activation, DC was stimulated for 24h in complete culture medium with LPS or TNF-α during the last day of DC culture. The control lane (CTR) corresponds to untreated cell population. LPS and TNF-α didn't alter the expression of CRH receptor protein. Results are representative of three independent experiments MW, molecular weight marker.

3. The effect of CRH on phenotyping of DC during in vitro generation

Phenotypic analysis of MoDC was performed by using flow cytometry. To investigate the effect of CRH on phenotyping of DC, cells were exposed to CRH at 50nM for 24h during the last day of culture. Expression of CD1a wasn′t affected by both CRH and LPS (Figure 4A). Although CRH didn′t alter the expression of CD83, LPS increased CD83 markedly (Figure 4B).

A



B

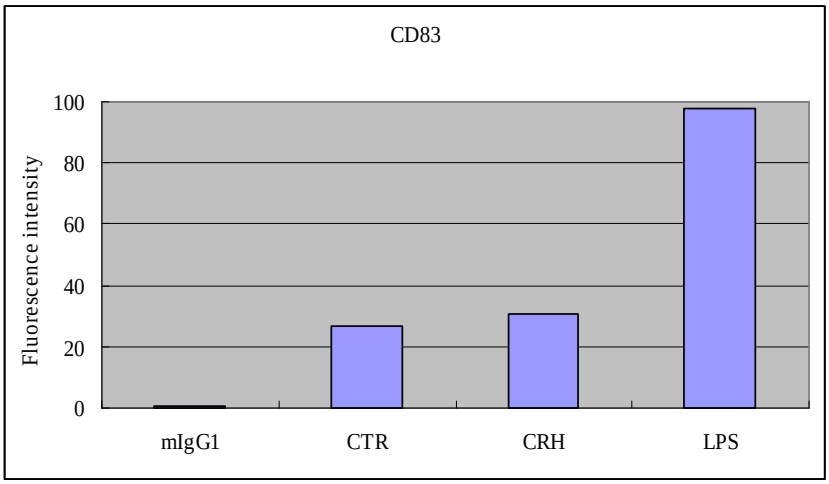


Figure 4. Phenotypic analysis of MoDC was performed by using flow cytometry. To investigate the effect of CRH on phenotyping of DC, cells were exposed to CRH at 50nM for 24h during the last day of culture. LPS was used for positive control. The control (CTR) corresponds to untreated cell population. Expression of CD1a wasn't affected by both CRH and LPS (Figure

4A). Although CRH didn′t alter the expression of CD83, LPS increased CD83 markedly (Figure 4B).

4. Effect of CRH on IL-18 mRNA production in DC of atopic dermatitis patients and nonatopic healthy controls

To study the effect of CRH on IL-18 mRNA expression in DC, the cells were exposed to CRH at 50nM for 6h. Total RNA was extracted and RT-PCR for IL-18 were performed. IL-18 mRNA expression was reduced by CRH in both atopic dermatitis patients and nonatopic healthy control (Figure 5A). However, the effect was more prominent in atopic dermatitis patients than nonatopic healthy control (Figure 5B).

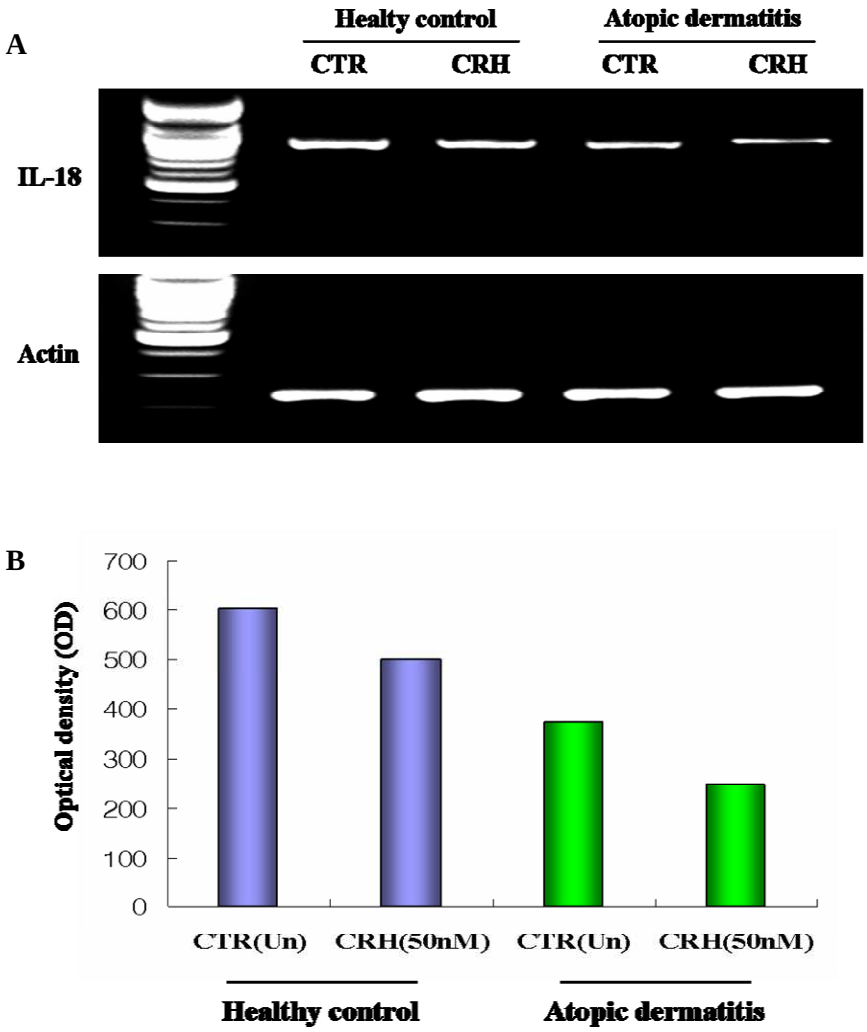


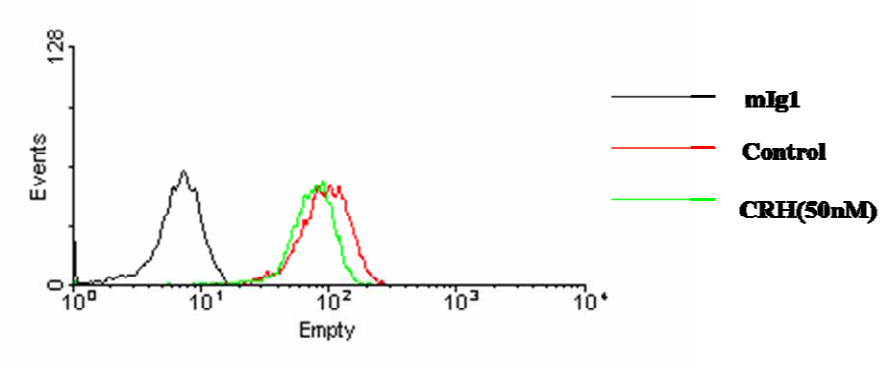
Figure 5. RT-PCR analysis of IL-18 mRNA in DC of atopic dermatitis patients and nonatopic healthy control. For studying effect of CRH, DCs were treated for 6h in complete culture medium with CRH at 50nM during the last day of DC culture. The control lane corresponds to untreated cell population. IL-18 mRNA expression was reduced by CRH in both atopic dermatitis patients and healthy control (Figure 5A). However, the effect was more prominent in

atopic dermatitis patients than nonatopic healthy control (Figure 5B). Results are representative of three independent experiments.

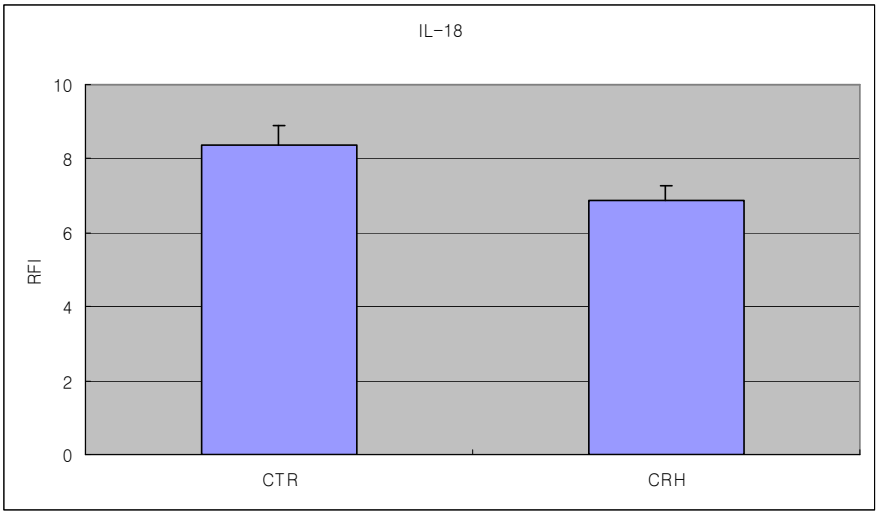
5. Effect of CRH on cytokine and chemokine production in DC of atopic dermatitis patients.

To investigate the effect of CRH on cytokine and chemokine protein expression in DC of atopic dermatitis patients, the cells were subjected to flow cytometric analyses using human anti IL-6, IL-18, mouse anti-CCL17, anti-CCL22, and anti-CCL18 monoclonal antibody (R&D systems, Minneapolis, MN). Cells were exposed to CRH at 50nM for 24h during the last day of culture. IL-18 expression was reduced by CRH in atopic dermatitis patients (Figure 6A, B). However, there was no change in expression of IL-6, CCL17, CCL18 and CCL22 (Figure 6C).

A


128
Events
0
10^0
10^1
10^2
10^3
10^4
Empty
mIg1
Control
CRH(50nM)

B


IL-18
10
8
6
4
2
0
RFI
CTR
CRH

C

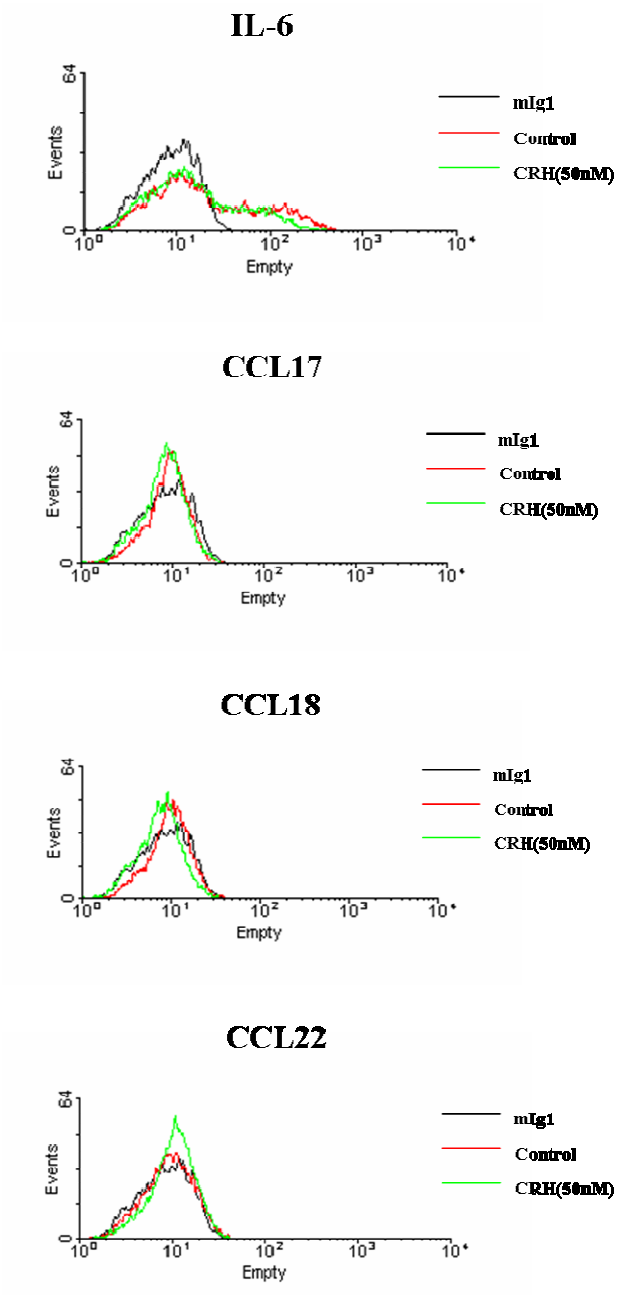

IL-6
Events
64
0
10^0
10^1
10^2
10^3
10^4
Empty
mIg1
Control
CRH(50nM)
CCL17
Events
64
0
10^0
10^1
10^2
10^3
10^4
Empty
mIg1
Control
CRH(50nM)
CCL18
Events
64
0
10^0
10^1
10^2
10^3
10^4
Empty
mIg1
Control
CRH(50nM)
CCL22
Events
64
0
10^0
10^1
10^2
10^3
10^4
Empty
mIg1
Control
CRH(50nM)

Figure 6. Flow cytometric analysis of IL-18 protein in DCs of atopic dermatitis patients. For studying effect of CRH, DCs were treated for 24h in complete culture medium with CRH at 50nM during the last day of DC culture. The control (CTR) corresponds to untreated cell population. IL-18 expression was reduced by CRH in atopic dermatitis patients (Figure 5A, B). There was no change in expression of IL-6, CCL17, CCL18 and CCL22 (Figure 6C). Results are representative of three independent experiments.

* RFI (relative fluorescence intensity) = $(MFI_{parameter\ evaluated} - MFI_{control})/ MFI_{control}$.

IV. DISCUSSION

To our knowledge, this is the first report that mRNA of CRH receptor isoforms is expressed in human DCs. We detected mRNA for CRH-R1α, 1β, as well as CRH-R1 protein. CRH-R2α (but not R2β or R2ɣ) mRNA and CRH-R2 protein were present in MoDC. CRH-R1 mRNA is widely expressed in mammalian brain and pituitary and is responsible for activation of the POMC gene and ACTH and β-endorphin release from the anterior pituitary[23]. In human peripheral tissues, CRH-R1 is expressed in a wide range of tissues such as the testis, ovary, endometrium, myometrium, placenta, adrenal gland, adipose tissue, skin, spleen, heart, and specific cells of the immune system[4]. The gene for human CRH-R1 contains 14 exons, and the complete gene product is a 444 amino acid protein, termed CRH-R1β, that exhibits impaired agonist binding and signaling properties[24]. Excision of exon 6, which encodes for a 29 amino acid insert in the first intracellular loop, results in expression

of CRH-R1α mRNA. This appears to be the main functional CRH-R1 receptor variant containing 415 amino acids, which primarily mediates CRH actions[4]. Concerning CRH-R2 variants, CRH-R2α and 2β are found in both human and rodents, and 2γ has so far been found only in the limbic regions of the human CNS[25-27]. These three variants differ only in their N-terminal extracellular domains. The different N termini do not significantly alter agonist binding and signaling properties of the various CRH-related peptides, although the CRH-R2β is about 10-fold more potent in second messenger activation compared with CRH-R2α or R2γ[27]. These variants, however, do exhibit significant differences in their tissue distribution. Slominski *et al*.[28] reported that none of CRH-R2 subtypes was detected in epidermal normal and malignant keratinocytes, normal and malignant melanocytes, dermal fibroblasts, adipose tissue. By contrast, CRH-R2α was detectable in cells derived from human scalp adnexal structures (hair follicle keratinocytes and hair follicle papilla fibroblasts). Tissue-specific distribution and various

mRNA splicing mechanisms of CRH-R might explain many different biological actions of CRH in various tissues.

CRH profoundly influences the function of the immune system indirectly, through the activation of the HPA axis and sympathetic system, and directly, through the local modulatory actions on inflammatory responses[3]. Epidemiological and experimental studies suggest that stress and stress hormones influence the development, course, and pathology of certain allergic, autoimmune/inflammatory, infectious, and neoplastic diseases, mainly by stimulating Th2 instead of Th1-type immune response, which means by enhancing humoral and suppressing cellular immunity[29]. Atopic dermatitis, psoriasis, and other inflammatory skin diseases are known to be exacerbated by stress. It was recently reported that CRH regulates the expression of IL-18 in HaCaT cell[15]. However this is the first time that CRH decreases expression of IL-18 in DCs. Moreover, this effect of CRH on IL-18 production was more prominent in atopic dermatitis patients than nonatopic healthy controls.

Because IL-18 functions primarily as an IFN-γ inducer and a promoter of Th1 responses in T cells, our results not only supports the previous hypothesis that stress downregulates cellular immunity, but also address that CRH could modulate immune responses by acting directly on DCs.

V. CONCLUSION

In conclusion, DCs express mRNAs for CRH-R1α, 1β and CRH-R2α, but not those for R2β and R2ɣ. Both CRH-R1 and CRH-R2 protein were also present in DCs. Furthermore, it was found that CRH regulates the expression of IL-18 in DCs via specific CRH-Rs. These findings suggest that CRH could modulate immune responses by acting directly on DCs and may provide an insight into the pathophysiology of neuroinflammatory skin disease such as psoriasis and atopic dermatitis.

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Abstract (In Korean)

수지상세포에서 Corticotropin releasing hormone 의 작용

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스트레스가 아토피피부염, 건선, 지루성 피부염, 원형 탈모증 등 여러 피부과적 질환의 경과에 영향을 준다는 것은 일반적으로 받아들여지는 사실이나, 어떠한 면역학적 기전으로 작용하는지는 아직 명확히 밝혀져 있지 않다. Corticotropin releasing hormone (CRH)은 대표적인 스트레스 매개 물질로 각질형성세포, 비만세포 등 다양한 세포에 영향을 준다는 것이 최근 보고되고 있으나 아토피피부염 환자의 병인에 중요한 세포 중 하나인 수지상 세포에서의 역할에 대해서는 연구된 바가 없다. 본 연구에서는 수지상세포에서 CRH 수용체(CRH-R)의 발현양상과 CRH 가 싸이토카인 및 케모카인 발현에 미치는 영향을 알아보고자 하였다.

연구결과 말초혈액 단핵세포에서 배양한 수지상세포에 존재하는 CRH-R 아형은 CRH-R1α, 1β 와 CRH-R2α 였다. CRH-R 의 발현은

수지상세포를 활성화시키는 것으로 알려진 lipopolysaccharide (LPS)나 tumor necrosis factor α (TNF-α)의 영향을 받지 않았다. CRH 는 아토피피부염 환자와 정상인 모두에서 수지상세포의 interleukin 18 (IL-18) 발현을 감소시켰으며, 이러한 결과는 아토피피부염 환자에서 더 뚜렷하였다. Thymus and activation-regulated chemokine (TARC/CCL17), macrophage-derived chemokine (MDC/CCL22) 및 CC chemokine ligand 18 (CCL18)의 발현은 CRH 의 영향을 받지 않았다. 이상의 결과로 수지상세포에는 CRH 특이 수용체가 존재하며, CRH 는 이를 통해 IL-18 감소 등 면역반응 조절에 관여함을 확인할 수 있었다.

핵심되는 말: Corticotropin releasing hormone, 수지상세포