To investigate the mechanism of the glucocorticoids-induced augmentation of skin response, we have recently reported the modulatory effect of glucocorticoids on the regulation of cytokines production in keratinocytes stimulated with various chemicals in vitro through both NF-κB and AP-1 activation. Further to clarify the mechanism in the glucocorticoids-induced augmentation of cytokines production from keratinocytes, we examined the effect of glucocorticoids to keratinocytes without chemical stimulation.

Glucocorticoids 10^{-4} M inhibited the production of IL-1α from Pam 212 cells. However, lower concentration (10^{-8}–10^{-10} M) of glucocorticoids significantly enhanced the production of IL-1α by Pam 212 cells at both the protein and mRNA levels. In contrast, glucocorticoids had no effect on the production of either TNF-α, IL-6, nor GM-CSF by Pam 212 cells cultured for 6 h. Electrophoretic mobility shift assays (EMSA) revealed that 10^{-8}–10^{-10} M glucocorticoids induced the NF-κB activation in Pam 212 cells, however, augmented AP-1 activation by 10^{-8}–10^{-10} M of glucocorticoids was observed in Pam 212 cells.

Furthermore, pyrrolidine dithiocarbamate (PDTC) partially inhibited the IL-1α production and completely inhibited NF-κB expression by Pam 212 cells. On the other hand, MAP-kinase inhibitors (PD98059, SB202190) completely abrogated not only AP-1 activation but the low concentration glucocorticoids-induced IL-1α production.

These data indicated that lower concentration of glucocorticoids induced the augmentation of IL-1α production from keratinocytes mediated through the AP-1 pathway and partially through NF-κB pathway.

**Key words**: Keratinocytes, contact hypersensitivity, cytokines, glucocorticoids, signal transduction.

**Abbreviations**: GC, glucocorticoids; HC, hydrocortisone; NF-κB, nuclear factor-κB; EC, epidermal cell; KC, keratinocytes; CHS, contact hypersensitivity

**Introduction**

We recently reported that glucocorticoids (GC) augment the inflammatory reaction of both contact hypersensitivity (CHS), irritant response and IgE-induced delayed type reaction. An up-regulation of IL-1α and the inhibition of the IL-10 production in the epidermis at the challenge site has been reported to play a critical role in the GC-induced augmentation of murine CHS. To clarify the mechanism of the GC-induced augmentation of the skin response, we investigated the modulatory effect of GC on the regulation of cytokines.
produced by keratinocytes stimulated with various chemicals in vitro. We demonstrated that the augmented inflammatory reaction by GC may reflect the augmentation of IL-1α production by keratinocytes mediated through the NF-κB and AP-1 pathway. In consistent with our recent reports, several groups demonstrated the augmented inflammatory reaction by GC. These findings suggest that GC may activate nuclear factors such as nuclear factor-κB (NF-κB) to enhance production of epidermal cytokines to modulate the inflammatory reaction of CHS and irritant response.

In the present study, to clarify the mechanism in the GC-induced augmentation of cytokine production from keratinocytes (KC), we tried to examine the modulatory effect of GC to KC without any chemical stimulation. Furthermore, the modulatory effect of GC on the activation of NF-κB and AP-1 in GC-stimulated KC was assessed to confirm the phenomenon that topical GC application augments the inflammatory reaction of CHS and irritant response.

Materials and Methods

Reagents

Hydrocortisone (HC) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, Pyrrolidine dithiocarbamate (PDTC) and Tween-20 from the Sigma Chemical Co., Darmstadt, Germany, FITC conjugated I-Ad antibody from PhaMingen, San Diego, USA, Dulbecco’s minimal essential medium (DMEM) from Nissui pharmaceutical Co. Ltd, Tokyo, Japan, fetal calf serum (FCS) from Flow Laboratories, Rockville, Maryland, USA, hexanucleotide mixture from Boehringer Mannheim, Germany, RAV-2 transcriptase, Taq DNA polymerase and ribonuclease inhibitor from Takara Co., Japan, single-stranded oligonucleotides for NF-κB or AP-1 and anti-NF-κB p50 goat polyclonal antibody, anti-NF-κB p65 goat polyclonal antibody, anti-c-Fos goat polyclonal antibody and anti-c-Jun goat polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA, USA and horseradish peroxidase-conjugated goat anti-rabbit immunogloblin from DAKO, Denmark. MAP kinase inhibitor, SB202190( 4-(4-fluorophenyl))-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole; FHPI) and PD98059 (2'-amino-3'-methoxyflavone) from Calbiochem, Bioscience, Inc. La Jolla, CA, USA.

Epidermal cell line

An epidermal cell line derived from a BALB/c mouse, Pam 2127 was used throughout the experiment. Pam 212 cells were cultured in 6-well culture plates (Corning, 25810) in DMEM supplemented with 7% FCS, 5 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. All experiments were performed by using Pam 212 cells at the semi-confluent stage. Langerhans cells were not detectable in Pam 212 cells after staining with FITC-conjugated I-Ad antibody.

In vitro stimulation of Pam 212 cells with hydrocortisone

Pam 212 cells were cultured for 1 to 18 hr at 37°C with varying concentration (10⁻⁴ M to 10⁻¹⁴ M) of HC, followed by washing and incubation in DMEM with 7% FCS. Pyrrolidine dithiocarbamate (PDTC), PD98059 or SB202190 were added to the culture flasks 30 min before the addition of HC. The culture supernatants were collected and prepared for ELISA assay after 6 hr or 18 hr. Nuclear extracts of Pam 212 cells were prepared from cells cultured for 1 hr with or without HC.

Enzyme-linked immunosorbent assay (ELISA) of cytokines

Pam 212 cells were incubated in 6-well culture plates with 10⁻⁴ M to 10⁻¹⁴ M hydrocortisone at 37°C for 6 hr or 18 hr. The culture media were collected and stored at –70°C. The release of IL-1α, GM-CSF, IL-6 and TNFa into the culture media was estimated by using ELISA assay kit (Genzyme, Minneapolis, USA) according to the supplier’s instructions. The sensitivity of the kits was 10 pg/ml.

RNA extraction, reverse transcription (RT) and polymerase chain reaction (PCR)

RNA was extracted from Pam 212 cells cultured in serum free DMEM for 5 hr with or without either chemicals or HC by using RNA-zol (Biotex CS101). The RT mixture was prepared by mixing 4 μl of 10 x buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl2), 4 μl of hexanucleotide mixture (62.5 A260
was precipitated and stored at –80°C. Nonidet P-40, 0.5 mM DTT and 1 mM PMSF and were dissolved in a buffer containing 20 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1% Triton X-100. The cells were subsequently lysed in a high salt extraction buffer containing 0.5 M NaCl, 100 mM KCl with approximately 10,000 cpm of 32P-labeled probe for 30 min at room temperature. The sequences of single-stranded oligonucleotides for AP-1 were 5′-CGCTTGACT-3′ downstream, 5′-AAG TCG GTC TCA CTA CCA C-3′ upstream and 5′-GAC TGG GAC TTT CCC AGG C-3′ for the binding site for NF-κB. The cytoskeleton of Pam 212 cells before and after the experiments was maintained at more than 98% when assessed by trypan blue dye exclusion test.

Electrophoretic mobility shift assays (EMSA)

Pam 212 cells were cultured in a 50 ml culture flask with or without hydrocortisone for 1 hr. The cells were then subsequently lysed in a high salt extraction buffer containing 20 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 0.5 mM DTT and 1 mM PMSF and were incubated for 30 min on ice. The crude nuclear extract was precipitated and stored at –80°C. After pre-incubation with 2 μg poly (dl-dC) as nonspecific competitor DNA, 15 μg of the crude nuclear extract was incubated in a binding buffer containing 10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 2 mM DTT, 1 mM PMSF, 5% glycerol, and 100 mM KCl with approximately 10,000 cpm of annealed 32P-labeled probe for 30 min at room temperature. The sequences of single-stranded oligonucleotides for NF-κB were 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ for the binding site for NF-κB and 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ for the binding site for NF-κB. The sequences of single-stranded oligonucleotides for AP-1 were 5′-CGCTTGACT-GACTCAGCGCGGAA-3′. The binding reaction mixtures were separated from free oligonucleotide probes by electrophoresis on a native 5% (W/V) polyacrylamide gel as described. For supershift assay, 4 μg of anti-NF-κB p50 antibody, anti-NF-κB p65 antibody or nonimmune rabbit serum were added to the reaction mixture simultaneously with the nuclear extract. The reaction mixture was subjected to electrophoresis as previously described and thereafter the gels were then dried and autoradiographed.

Results

IL-1α but not GM-CSF, IL-6 nor TNF-α is secreted from Pam 212 cells by the stimulation of HC

The cytokine release from Pam 212 cells was assessed by incubating Pam 212 cells with various concentrations of HC. The production of IL-1α was enhanced by stimulation with 10⁻⁴ to 10⁻⁸ M of HC and inhibited by stimulation with 10⁻⁴ M of HC (Fig 1A). In contrast, the enhanced production of either GM-CSF, IL-6 or TNF-α was not observed in Pam 212 cells stimulated by HC (Fig 1B). Interestingly, not only IL-1α but also GM-CSF was secreted from Pam 212 cells stimulated with 10⁻⁴ M HC after the culture for 18 h (data not shown). The amount of IL-1α detected in the culture media was not due to cell death caused by HC since viability of Pam 212 cells before and after the experiments was maintained at more than 98% when assessed by the trypan blue dye exclusion test.

HC enhances the expression of IL-1α mRNA in Pam 212 cells

As shown in Fig. 2A, the production of IL-1α protein by Pam 212 cells was up-regulated by the HC treatment. RT-PCR was performed to detect the mRNA expression of IL-1α by Pam 212 cells stimulated with various concentrations of HC. As shown in Fig 2B, the stimulation of Pam 212 cells with 10⁻⁸ to 10⁻⁶ M HC for 5 hr resulted in a 2.5 and 2-fold up-regulation of the IL-1α mRNA expression (Fig 2A,B).

HC induced NF-κB activation and NF-κB activation was inhibited by pyridoline dithiocarbamate

An electrophoretic mobility shift assay (EMSA) was performed to investigate whether or not HC was involved in the activation of NF-κB. NF-κB activation was inhibited in Pam 212 cells by 10⁻⁹ to 10⁻⁶ M of HC, whereas it was enhanced in Pam 212 cells by 10⁻⁶ and 10⁻⁴ M HC.
Pyrrrolidine dithiocarbamate (PDTC) is an antioxidant to act as a radical scavenger. It is a potent inhibitor of NF-κB activation. One hundred μM PDTC led to a strong inhibition of HC-inducible DNA-binding of NF-κB (Fig 3A). A 5 μl 100-fold molar excess of the NF-κB oligonucleotide completely eliminated binding to the extract from HC-treated Pam 212 cells (Fig 3B). In contrast with NF-κB oligonucleotide, the mutant NF-κB oligonucleotide did not bind to NF-κB (Fig 3C). The NF-κB complex activated by lower concentration of HC was supershifted by polyclonal antibody against p50 but not by polyclonal antibody against p65 (Fig. 3D).

**HC induced AP-1 binding activation and AP-1 activation was inhibited by MAP kinase inhibitors**

An electrophoretic mobility shift assay (EMSA) was performed to investigate whether or not HC was involved in the activation of AP-1. AP-1 activation was enhanced in Pam 212 cells by 10⁻¹² and 10⁻¹⁰ M of HC.
PD98059 and SB202190 are MAP kinase inhibitors. PD98059 led to a partial inhibition of low concentration HC-inducible DNA-binding of AP-1 (Fig 4B), whereas SB202190 induced a strong inhibition of low concentration HC-inducible DNA-binding of AP-1. The AP-1 complex activated by low concentration HC was supershifted by both polyclonal antibody (Fig 4A).
against c-Fos and polyclonal antibody against c-Jun (Fig. 4C). These supershift data indicated that the AP-1 complexes contained both c-Jun and c-Fos.

PDTC inhibited low concentration HC-induced NF-κB activation but not IL-1α production from HC-stimulated Pam 212 cells, however MAP-kinase inhibitor inhibits both AP-1 activation and IL-1α production from HC-stimulated Pam 212 cells

PDTC inhibited strongly NF-κB activation induced by low concentration HC (Fig. 3A). PDTC is considered to inhibit augmentation of IL-1 α release and NF-κB activation by HC. As shown in Fig 5A, C, 100 μM PDTC partially but not significantly inhibited IL-1α production by Pam 212 cells stimulated by 10⁻¹⁰ M HC in both protein and mRNA level. Inhibitory effect of PDTC on NF-κB activation is depicted in Fig 6A and C.
kB activation in Pam 212 cells stimulated by low concentration HC was more prominent than that on IL-1α production, whereas AP-1 inhibition was almost parallel to that on IL-1α production in the case of HC-stimulated Pam 212 cells inhibited by MAP-kinase inhibitors (Fig 5). MAP-kinase inhibitors inhibited strongly both AP-1 activation and IL-1α production induced by HC (Fig. 4B, Fig. 5).

Discussion

In consistent with Kupper's report\(^{19}\), higher concentration (10\(^{-7}\) M) of HC inhibited the production of IL-1α, whereas interestingly lower concentration (10\(^{-8}\) to 10\(^{-10}\) M) of HC augmented the production of IL-1α but not IL-6, GM-CSF or TNF-α from cultured murine Pam 212 cells without chemical stimulation (Fig 1A, B). Low concentration of GC also up-regulated the expression of the mRNA for IL-1α in Pam 212 cells (Fig 2). These findings indicate that a low dose of GC has ability to augment CHS through up-regulation of IL-1α production of KC. We have already demonstrated that the addition of 10\(^{−6}\) to 10\(^{−12}\) M HC to the chemical-stimulated keratinocytes augmented the IL-1α production\(^{5}\). In line with our observations, the administration of low dose GC has been reported to up-regulate the number of MIF-positive cells in contact dermatitis\(^{11}\), while significantly enhancing skin DTH reaction and produced a significant increase in the number of T cells in the lymph nodes draining of the DTH reaction site\(^{12}\). GC enhance the production of cytokines to modulate the
skin reaction. Further, GC up-regulate the expression of such cytokines receptors as IL-6R, IL-1R and GM-CSF-R. It has also recently been demonstrated that the topical application of GC enhances both allergic and non-allergic cutaneous reaction, despite the fact that the topical application of GC diminishes the number of resident I-A+ Langerhans cells in the epidermis for a prolonged period of time. Recently we demonstrated that the topical application of GC induced the up-regulation of IL-1α and down regulation of IL-1Ra and IL-10 in the challenge site of contact hypersensitivity.

The inducible expression of IL-1α is regulated via transcription factors, mainly NF-κB and AP-1.

10^{-8} M HC enhanced the DNA binding activity of p65 protein, one of the NF-κB elements, by Pam 212 cells stimulated with either TNBS or DNBS. Dexamethasone also has been reported to enhance phorbol ester (PE)-induced IL-1β gene expression and NF-κB activation in a monocyctic leukemia cell line. GC may have an ability to enhance NF-κB activation of chemically stimulated cells including keratinocytes and monocytes. In this paper, we examined whether GC has an ability to activate NF-κB without chemical stimulation. The enhancement of NF-κB binding to nuclear was detected in Pam 212 cells cultured in the presence of 10^{-10}–10^{-12} M HC without chemical stimulation (Fig. 3A), whereas the enhancement of IL-1α production was observed at a range of HC between 10^{-6} and 10^{-10} M. A relatively lower concentration of HC activates NF-κB to transcribe the IL-1α gene on one side while the higher dose of HC may induce various transcriptional signals other than NF-κB to synthesize IL-1α molecules on the other side. In contrast with these data, 10^{-6} to 10^{-8} M HC enhanced the activation of NF-κB in chemical-stimulated Pam 212 cells. These data suggest that there is a difference of a signal transduction between the modulated effect of GC to chemical stimulated KC by GC and direct effect of GC on non-treated KC.

NF-κB activation is generally controlled by reactive oxygen species and the cellular redox state, and can be inhibited by antioxidant. In this experiment, GC induced the direct binding activation of NF-κB. This activation was markedly inhibited by PDTC (Fig 3A), but not by anti-TNF α mAb or anti IL-1α mAb (data not shown). Further, supershift assay and the inhibition assay by cold probe revealed the specificity of NF-κB binding band. Our findings indicate that the redox-dependent process partially involves in the GC dependent enhancement of IL-1α gene transcription. As the inhibition of IL-1α mRNA expression and IL-1α production by PDTC was incomplete and not significant in our experiment (Fig. 5), it is likely that other transcriptional factors are also involved in the IL-1α gene transcription of HC-induced IL-1α neosynthesis. Since then AP-1 is considered to be the most probable candidate of other transcriptional factor in IL-1α enhancement. The AP-1 activity in Pam 212 cells was also examined by EMSA. As we speculated, an augmented AP-1 activation was observed in Pam 212 cells in the presence of a lower doses (10^{-9}–10^{-10}) of GC (Fig 4A). MAP-kinase inhibitor inhibited more than 75% of IL-1α production and 100% of IL-1α mRNA expression (Fig. 5), and also inhibited AP-1 activation completely (Fig. 4B). These data indicated that GC augmented the production of IL-1α in the Pam 212 cells through mainly the activation of AP-1 and partially NF-κB. How then do GC enhance the NF-κB and AP-1 activation? GC induced the IL-1α release from chemical stimulated Pam 212 cell and activated both NF-κB and AP-1. Since GC enhance these transcription factors both in the presence of haptens or irritants but not SEB, GC must strongly interact with the hapten-or irritants-induced signal transduction pathway to overcome the effect of what on the NF-κB or AP-1 expression, however another mechanism should play a role since GC activated both NF-κB and AP-1 without chemical stimulation. One possible mechanism is that GC may modulate a coactivator like CREB binding protein which then interact with the activation of NF-κB. The another possible mechanism is that GC would induce cross-coupling of NF-κB and AP-1 transcription factor previously reported. The formation of that complex could explain the potent ability of MAP-kinase inhibitors in abolishing both the IL-1α protein and mRNA expression and also in inhibiting both AP-1 and the NF-κB signal transduction at the same time (Fig. 3, 4). Interestingly, GC activate NF-κB p65 protein in the presence of chemicals, although without chemical stimulation, GC induced binding activation of NF-κB p50 protein since monoclonal antibody against NF-κB p50 but not p65 induced supershift (Fig 3C). The cross coupling between the Fos and Jun element with the p65 may be a possible factor of inhibiting the DNA binding with antibody against p65 in supershift assay. However, the exact mechanism in the signal transduction pathway which is enhanced by GC without chemical stimulation, is still unclear. To clarify these mechanisms, further investigations should be done in...
the near future.

In conclusion, (1) GC induced the production of IL-1α but not GM-CSF, IL-6 nor TNF-α from Pam 212 cells without chemical stimulation, and (2) the activation of NF-κB and AP-1 is involved in the augmentation of IL-1α production of murine keratinocytes induced by GC.

Based on these findings, the augmented inflammatory reaction by GC may reflect the augmentation of IL-1α production by keratinocytes mediated through AP-1 activation and partially through the activation of the NF-κB pathway.

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