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Original Article

Glucocorticoids induced the production and gene expression of IL-1α through AP-1 and partially NF-κB activation in murine epidermal cells

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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^{-10}–10^{-12} 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

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Introduction

We recently reported that glucocorticoids (GC) augment the inflammatory reaction of both contact hypersensitivity (CHS), irritant response and IgE-induced delayed type reaction[1,2]. 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[3]. 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[4]. 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[5,6]. 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 both CHS and the irritant response in vivo. Our findings demonstrate that GC up-regulated the IL-1α production of the KC, but not that of GM-CSF, TNF-α and IL 6. The activation of NF-κB and AP-1 was enhanced by GC in KC without chemical stimulation. Abrogation of GC activation of NF-κB by antioxidants only partially abrogated IL-1α production, however MAP-kinase inhibitors (PD98059, SB202190) completely abrogated both AP-1 activity and IL-1α production induced by the lower GC concentration.

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 Mannhaim, 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 polyclonalantibody 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 212[7] 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

U/ml), 4 μl of 100mM dithiotheritol, 2 μl of dNTP (2.5 mM each), 4 μl of 20 u/μl ribonuclease inhibitor, 4 μl of 3 u/μl of RAV-2 transcriptase, and 8 μl of water. It was made for every samples and aliquoted at 30 μl/tube with 8 ng/10 μl of total RNA. The tubes were vortexed, spun briefly, and left for 10 min at room temperature and for 60 min at 42°C. Reverse transcriptase was inactivated at 95°C for 5 min. Thus-treated RT mixture was stored at –70°C. The PCR mixture contained reverse-transcribed RNA (0.1 μg total RNA), 5 μl of 10x buffer, 4 μl of dNTP (2.5 mM each), 2.5 μl of 20 μM up-primer and 2.5 μl of 20 μM down-primer, 0.4 μl of 5 u/μl Taq DNA polymerase and 30.1 μl of water. Thereafter, the tubes were transferred to a thermal cycler (DNA amplifier PC-700; ASTEC, Japan). Amplification was conducted for 26 cycles at 94°C for 1 min, at 60°C for 1 min, and at 72°C for 2 min for cDNA of IL-1α and 22 cycles for cDNA of G3PDA, Mice IL-1α primers were 5'-AAG ATG TCC AAC TTC ACC TTC AAG GAG AGC CG-3' upstream and 5'-AAG TCG GTC TCA CTA CCT GTG ATG AGT TTT GG-3' downstream. The mice G3PDH primers were 5'-TGA ACG TCG GTG TGA ACG GAT TTG GC-3' upstream and 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3' downstream. The PCR products were identified by electrophoresis on 1.7% agarose gel containing 0.5 mg/ml ethidium bromide and photographed under UV light. The signal strength was semi-quantitated by using a densitometric scanner (AE-6955, ATTO Tokyo, Japan).

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[8]. After pre-incubation with 2 μg poly (dI-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 GGC GAC TTT CCC AGG C-3' for a mutated NF-κB motif. The sequences of single-stranded oligonucleotides for AP-1 were 5'-CGCTTGACTGACTCAGCCGGAA-3'. The binding reaction mixtures were separated from free oligonucleotide probes by electrophoresis on a native 5% (W/V) polyacrylamide gel as described[9]. 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 cyotokine release from Pam 212 cells was assessed by incubating Pam 212 cells with various concentration of HC. The production of IL-1α was enhanced by stimulation with 10^{-8} to 10^{-10} 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-a was not observed in Pam 212 cells stimulated by HC **(Fig 1B)**. Interestingly, not only IL-1α but GM-CSF was secreted from Pam 212 cells stimulated with 10^{-10} M HC after the cultute 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^{-10} 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 pyrolidine 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^{-4} to 10^{-6} M of HC, whereas it was enhanced in Pam 212 cells by 10^{-10} and

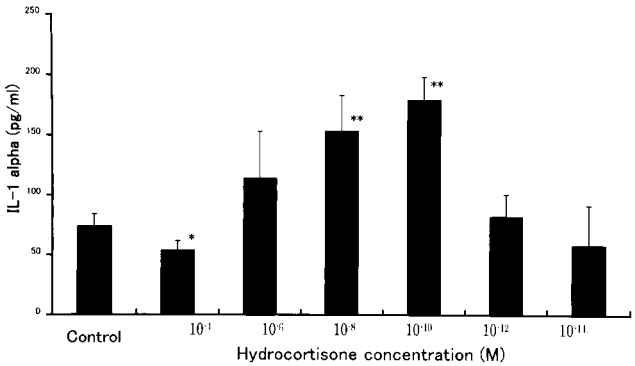
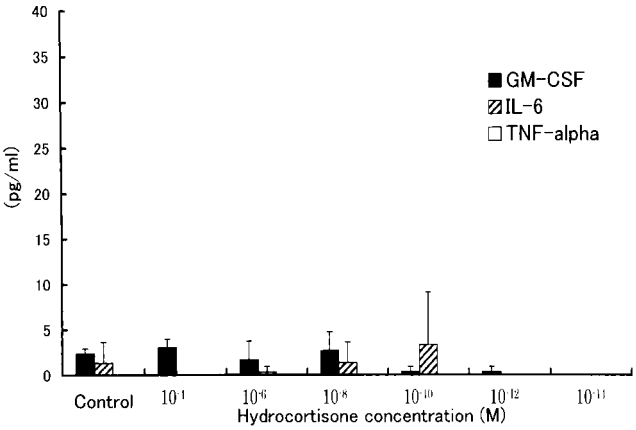


Figure 1. A. Dose response curve of IL-1α production from Pam 212 cells stimulated with various doses of HC.
Pam 212 cells were incubated for 6 hr with the presence or absence of HC (10^{-4} to 10^{-14} M). The culture supernatants were collected and IL-1α was measured by an ELISA assay.
Data represent means ±SD of 4 independent experiments. Statistical analysis was performed using student T-test (**P<0.01, *P<0.05).



B. GM-CSF, IL-6, and TNF-a production from Pam 212 cells stimulated with various dose of HC.
Pam 212 cells were incubated for 6 hr with the presence or absence of HC (10^{-4} to 10^{-14} M). The culture supernatants were collected and GM-CSF, IL-6 and TNF-α were measured by an ELISA assay. The results represent the data of three independent experiments.

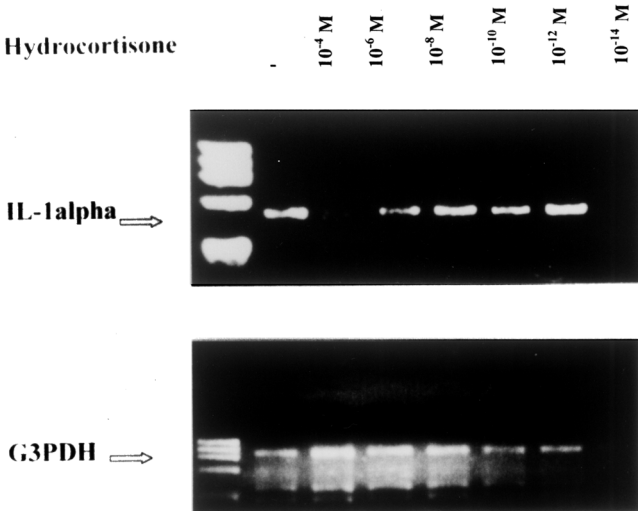


Figure 2A

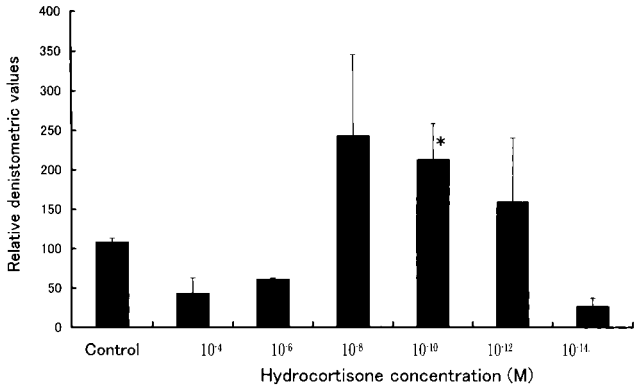


Figure 2B

Figure 2. The expression of IL-1α mRNA in Pam 212 cells stimulated with a various concentration of HC.
Pam 212 cells were prepared as described in the Methods. The cells were stimulated for 5 hr with a various concentration of HC (Fig. 2A). Data are expressed as the relative densitometric values normalized to variations in G3PDH from the same blot (Fig. 2B). The results represent the data of three independent experiments. Values are the mean ±SD three determinations. A statistical analysis was done by Student's T-test, with *p<0.05, **P<0.01 versus the IL-1α mRNA expression in the absence of HC.

10^{-12} M of HC **(Fig 3A)**.

Pyrrolidine 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^{-8} and 10^{-10} M of HC

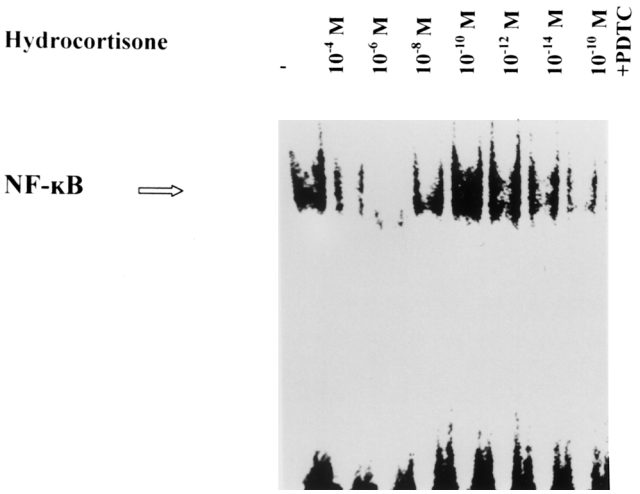


Figure 3A

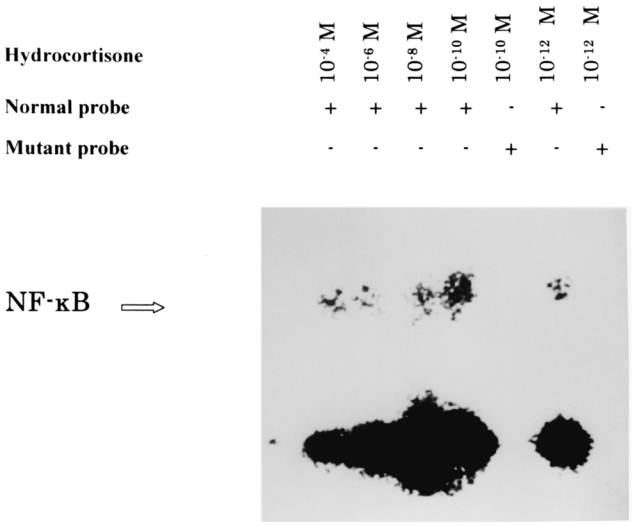


Figure 3C

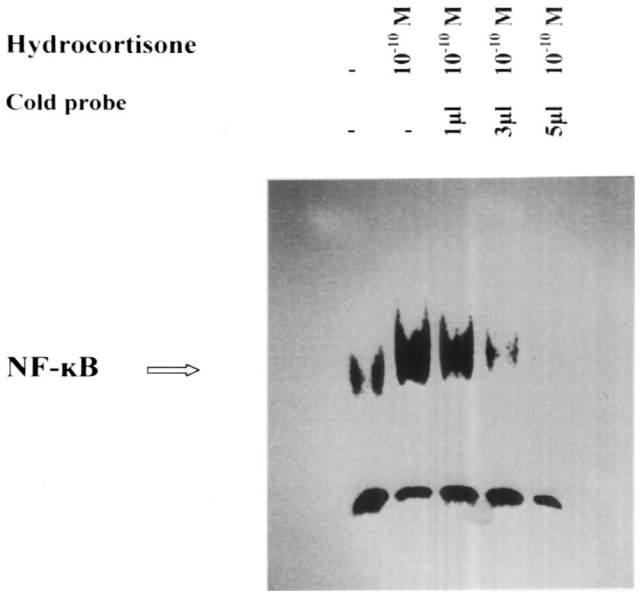


Figure 3B

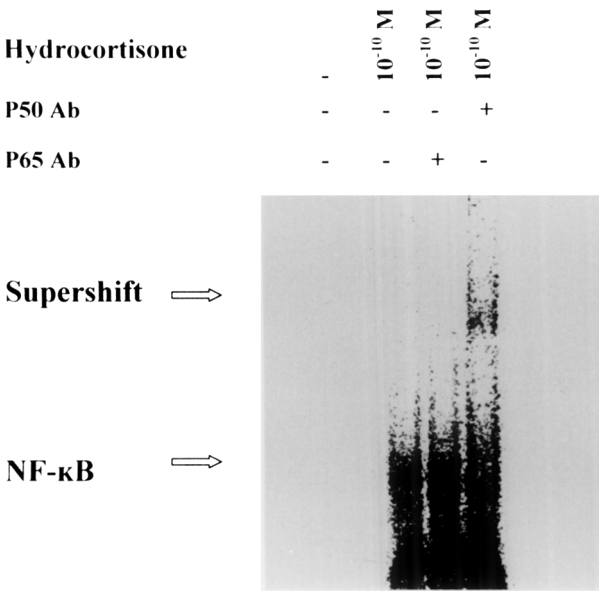


Figure 3D

Figure 3. Activation of transcription factor NF-κB of Pam 212 cells stimulated with various concentrations of HC.
Pam 212 cells were stimulated with various concentration of HC. Nuclear protein extracts were prepared and incubated with radiolabeled oligonucleotide probes. The resulting protein-DNA complexes were analyzed by gel electrophoresis mobility shift assays (Fig. 3A). To examine the specificity of NF-κB activation, Pam 212 cells were treated with 100 μM PDTC for 1/2 hr and 10^{-10} HC was then incubated for 1 hr (Fig. 3A). Increasing concentrations of cold probe (10–200 fold) were simultaneously incubated with the mix reaction (Fig. 3B). Oligonucleotides corresponding to the mutant version of the NF-κB motif were induced in the binding reaction (Fig 3C). Anti-P50 and P65 monoclonal antibodies were subsequently incubated with the mix reaction for 20 mins (Fig. 3D). These results represent the findings of four independent experiments.

(Fig 4A).

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 of HC was supershifted by both polyclonal antibody

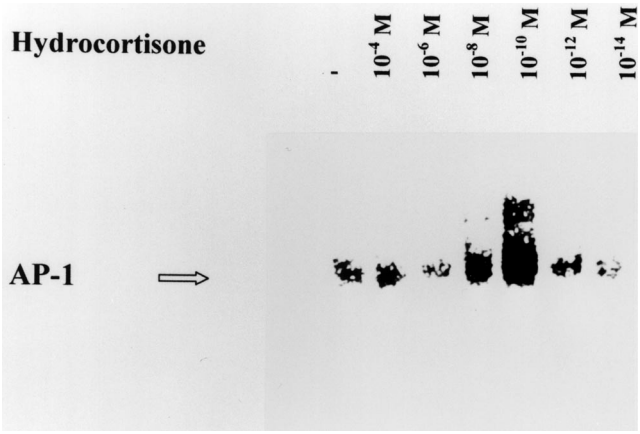


Figure 4A

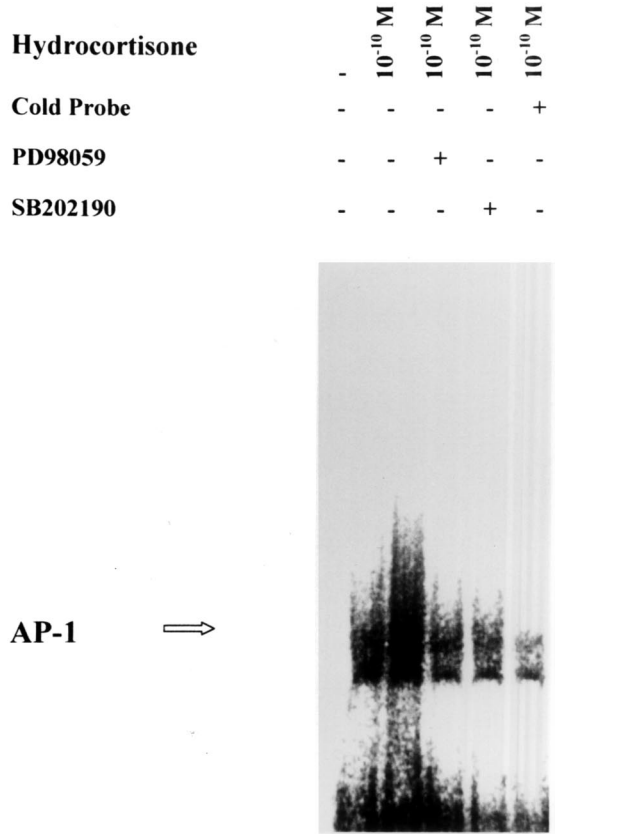


Figure 4B

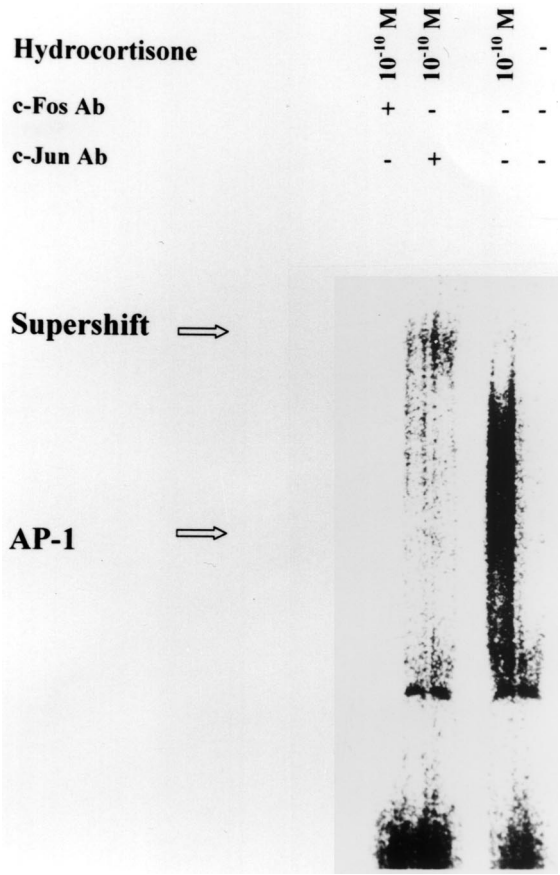


Figure 4C

Figure 4. Activation of transcription factor AP-1 of Pam 212 cells stimulated with various dose of HC.
A. Pam 212 cells were cultured for 1 h in the presence or the absence of HC (10^{-4}–10^{-14} M). Nuclear protein extracts were prepared and incubated with radiolabeled oligonucleotide probes. The resulting protein-DNA complexes were analyzed by gel electrophoresis mobility shift assays. Figure 4A shows that 10^{-8}–10^{-10} M HC enhanced the activation of AP-1 in Pam cells. B. Pam 212 cells were incubated for 1 hour in the presence of 10–10 M HC with or without pretreatment with MAP-kinas inhibitors SB202190 (5 μM) or PD098059 (5 μM) as inhibitors of the P38 and P42/44 enzymes. C. The supershift assays with monoclonal antibodies agaimst c-Fos or c-Jun or cold probe (100 fold). These results represent the findings of three independent experiments.

against c-Fos and polyclonal antibody aginst c-Jun **(Fig. 4C)**. These supershift data indicated that the AP-1 complexs 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-1α 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 lecvel. Inhibitory effect of PDTC on NF-

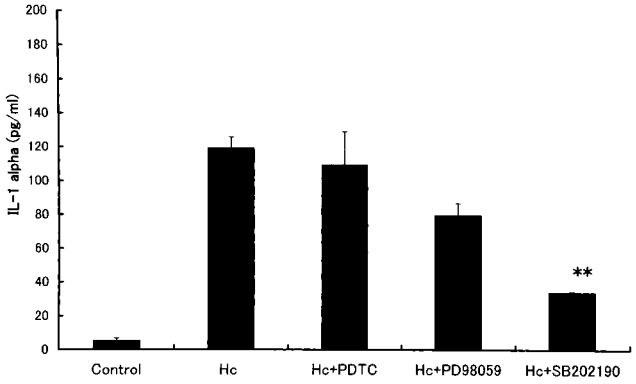


Figure 5A

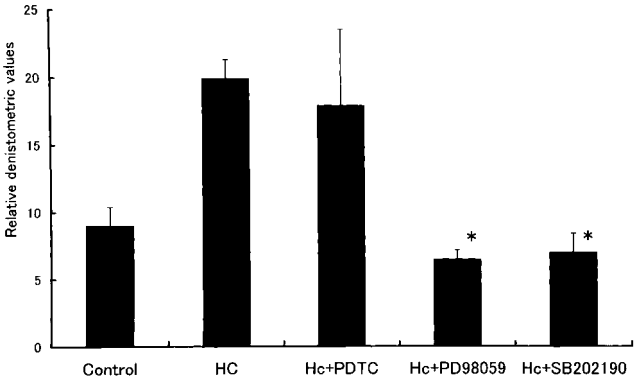


Figure 5C

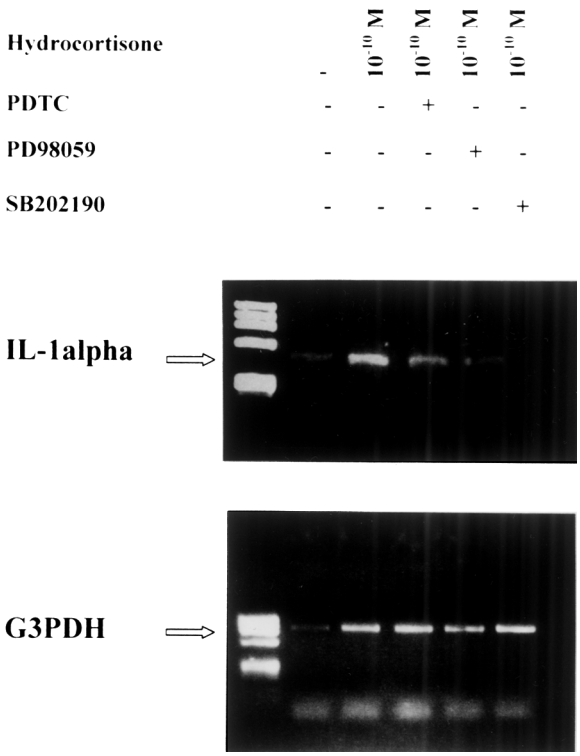


Figure 5B

Figure 5. IL-1α production induced by HC was inhibited MAP kinase inhibitor but not PDTC in both protein and mRNA level.
Pam 212 cells were treated with 100 μM PDTC, 5 μM PD98059, or 5 μM SB202190 for 30 min, washed, and then incubated for 6 h with vehicle alone (Control) or with 10-10.
A. The culture supernatants were collected and protein level of IL-1α was measured by ELISA. Data represent mean ± SD of 4 independent experiments. Statistical analysis was performed using student T-test (**P<0.01, *P<0.05).
B. RNAs were extracted by appropriate extraction kit after harvesting supernatants and cDNAs were prepared and amplification was performed using primers for mIL-1α and G3PDH to standardize the results.
C. Data are expressed as the relative densitometric values normalized to variations in G3PDH from the same blot. Data represent mean ± SD of 3 independent experiments. Statistical analysis was performed using student T-test (**P<0.01, *P<0.05).

κB 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[10], higher concentration (10^{-4} 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}–10^{-12} M HC to the chemical-stimulated keratinocytes augmented the IL-1α production[4]. 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[13,14]. It has also recently been demonstrated that the topical application of GC enhances both allergic and non-allergic cutaneous reaction[1,2,15], 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[15]. 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[3].

The inducible expression of IL-1α is regulated via transcription factors, mainly NF-κB and AP-1[16].

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[4]. Dexamethasone also has been repoted to enhance phorbol ester(PE)-induced IL-1β gene expression and NF-κB activation in a monocytic leukemia cell line[6]. 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^{-8} 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[4]. 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[16,17], 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^{-8}–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[4]. 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[4], 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[18]. 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[4], 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 shupershift assay[18]. 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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References

1. Igawa K, Katayama I, Minatohara K, Satoh T, Yokozeki H, Nishioka K. : Topical glucocorticoid augments both allergic and non-allergic cutaneous reactions in mice when applied at afferent stage of contact sensitivity. *Allergology Int.* 1997;**46**: 33-41.
2. Katayama I, Igawa K, Minatohara K et al: Topical glucocorticoid augments IgE-mediated passive cutaneous anaphylaxis in BALB/c mice and mast cell deficient WBB6F1 v/v mice. *Clin Exp Allergy*. 1997; **27**:1477.
3. Igawa K, Yokozeki H, Miyazaki Y, Minatohara K, Satoh T, Katayama I, Nishioka K. : Topical glucocorticoids application induced an augmentation in the expression of IL-1α while inhibition the expression of IL-10 in the epidermis in murine contact hypersensitivity. *Clinical Exp Allergy*. 2001; **31**: 485-494.
4. Miyazaki Y, Yokozeki H, Awad S et al: Glucocorticoid augment the chemically induced production and gene expression of Interleukin-1α through NF-κB and AP-1 activation in murine epidermal cells. *J Invest Dermatol.* 2000; **115**:746-752.
5. Grabbe S, and Lugar AT: Removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of murine contact hypersensitivity. *J.Immunol.* 1955; **155**: 4207-4217.
6. Wang Y, Zhang JJ, Dai W, Lei KY, Pike W.: Dexamethasone potently enhances phorbol ester-induced IL-1β gene expression and nuclear factor NF-κB activation. *J. Immunol.* 1997; **459**: 534-537.
7. Yuspa SH, Hawley-Nelson P, Koehler B, Stanley JR: A survey of transformation markers in differentiating epidermal cell lines in culture. *Cancer Res.* 1980; **40**: 4694-4703.
8. Schlze-Osthoff K, Beyaert R, Vandeboorde V, Haegeman G, Fiers W: Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of *TNF. EMBO J.* 1993; **12**: 3095-3104.
9. Schreiber EP, Matthais MM, Schaffner W: Rapid detection of octameric binding proteins with “mini-extracts” prepared from a small number of cells. *Nucleic Acids Res.* 1989; **17**: 6419.
10. Kupper TS, Mcguire J: Hydrocortisone reduces both constitutive and UV-elicited release of epidermal thymocyte activating factor (ETAF) by cultured keratinocytes. *J. Invest. Dermatol.* 1986; **87**:570-573.
11 Malorny U., Knopp J, Burmeister G, Sorg C: Immunohistochemical demonstration of migration inhibitory factor (MIF) in experimental allergic contact dermatitis. *Clin. Exp. Immunol.* 1988; **71**: 164.
12. Dhabhar FS, McEwen BS: Enhancing versus suppressive effect of stress hormones on skin immune function. *Proc Natl Acad Sci USA* 1999; **96**:1059-1064.
13. Snyers L, Wit DL, Content J: Glucocorticoid up-regulation of high-affinity interleukin 6 receptors on human epithelial cells. Proc. Natl. Acad. Sci. 1990; **87**: 2838-2842.
14. Almawi WY, Beyhum HN, Rahmen AA, Rieder MJ: Regulation of cytokine and cytokine receptor expression by glucocorticoid, J Leuko Biol, 1996; **60**:563-572.
15. Aberer W. Stingl L, Pogantsch S, Stingl G: Effect of glucocorticosteroids on epidermal cell-induced immune responses. *J. Immunol.* 1984; **133**: 792-797.
16. Lee WY, Butler AP, Locniskar MF, Fisher SM: Signal transduction pathways involved in phorbol ester and autocrine induction of interleukin-1α mRNA in murine keratinocytes. *J. Biol. Chem.* 1994; **269**: 17971-17980.
17. Corsini E, Schubert C, Marinovich M, Galli CL: Role of mitochondria in tributyltin-induced Interleukin-1 production in murine keratinocytes. *J.Invest.Dermatol.* 1996; **107**: 720-725.
18. Stein B, Baldwin AS, Ballard DW et al: Cross-coupling of the NF-κB p65 and Fos/Jun transcription factors produces potential biological function. *The EMBO Journal.* 1993; **12**: 3879-3891.