Substance P Enhances the Production of Interferon-induced Protein of 10 kDa by Human Keratinocytes in Synergy with Interferon-γ

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A neuropeptide substance P is related to skin inflammation. Interferon-induced protein of 10 kDa (IP-10) chemoattracts T helper 1 cells, and interferon-induced protein of 10 kDa production by keratinocytes is enhanced in inflammatory skin diseases such as psoriasis. We examined the *in vitro* effects of substance P on interferon-induced protein of 10 kDa production by human keratinocytes. Though substance P alone did not induce interferon-induced protein of 10 kDa production, it enhanced interferon-induced protein of 10 kDa secretion, mRNA expression, and promoter activity induced by suboptimal concentrations of interferon-γ. Interferon-stimulated response element and two nuclear factor-κB sites on interferon-induced protein of 10 kDa promoter were responsible for the enhancement by substance P. Substance P alone enhanced transcriptional activity and transcription factor binding through the two nuclear factor-κB sites, whereas it did not alter interferon-γ-induced transcriptional activity and transcription factor binding through interferon-stimulated response element. The effects of substance P on interferon-induced protein of 10 kDa production and nuclear factor-κB activation were inhibited by neurokinin-1 receptor antagonist, phospholipase C inhibitor, intracellular Ca^{2+} chelator, and anti-oxidant. These results suggest that substance P may induce nuclear factor-κB activation and interferon-induced protein of 10 kDa production in synergy with interferon-γ via neurokinin-1 receptor on keratinocytes. These effects of substance P may be mediated via phospholipase C activation, intracellular Ca^{2+} signal, and reactive oxygen intermediates.
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It is known that neuropeptides regulate inflammatory reactions (Ansel *et al*, 1996). Substance P (SP) is a tachykinin family neuropeptide and is released from the sensory C-fiber endings (Ansel *et al*, 1996). SP induces the synthesis of proinflammatory cytokines, such as interleukin-6, tumor necrosis factor-α, or interleukin-12 in monocyte/macrophages (Lotz *et al*, 1988; Kincy-Cain and Bost, 1997), induces histamine release from mast cells (Schanahan *et al*, 1985), and the expression of cell adhesion molecules in endothelial cells (Quinlan *et al*, 1998, 1999b). SP binds to guanine nucleotide-binding protein-coupled neurokinin-1 receptor (NK-1R) on membranes of various cells. SP binding to NK-1R activates phospholipase C and generates inositol 1,4,5-triphosphate and diacylglycerol (**Fig 1**). Inositol 1,4,5-triphosphate increases intracellular Ca^{2+} concentration, which induces the formation of reactive oxygen intermediates (ROI), whereas diacylglycerol activates protein kinase C, which promotes the activities of mitogen-activated protein kinases (**Fig 1**) (Lieb *et al*, 1997; Fiebich *et al*, 2000). SP binding to NK-1R also activates adenylate cyclase and generates cyclic adenosine monophosphate signal, which activates protein kinase A (Christian *et al*, 1994). These SP-induced intracellular signaling pathways lead to the activation of various transcription factors; Ca^{2+} signal activates nuclear factor of activated T cells (Quinlan *et al*, 1999a), ROI activates nuclear factor κB (NF-κB) (Lieb *et al*, 1997), mitogen-activated protein kinases activate nuclear factor of interleukin-6 or activator protein 1 (Fiebich *et al*, 2000), and protein kinase A activates cyclic adenosine monophosphate response element binding protein (Christian *et al*, 1994). The SP-induced signaling pathways thus promote the gene expression dependent on these transcription factors (Christian *et al*, 1994; Quinlan *et al*, 1999a).

Previous studies suggest the relationship between SP and inflammatory skin diseases, such as psoriasis (Naukkanrinen *et al*, 1989; Eedy *et al*, 1991), contact dermatitis (Scholzen *et al*, 2001), or photodermatitis (Scholzen *et al*, 1999). Psoriasis-related reports are conflicting, however; some studies show the increased amounts of SP in psoriatic skin lesions (Naukkanrinen *et al*, 1989; Eedy *et al*, 1991), whereas others show nonaltered or rather reduced amounts of SP in those lesions (Anand *et al*, 1991; Pincelli *et al*, 1992). The lymphocytes infiltrating into psoriatic lesions are predominantly activated T helper 1 cells (Bos and de Rie, 1999), indicating the involvement of chemokines attracting those cells. Interferon-induced protein of 10 kDa (IP-10) is a member of the CXC

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Abbreviations: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester; EMSA, electrophoretic mobility shift assay; IκB, inhibitory nuclear factor-κB; IP-10, interferon-induced protein of 10 kDa; ISRE, interferon-stimulated response element; NF-κB, nuclear factor-κB; NK-1R, neurokinin-1 receptor; PDTC, pyrrolidine dithiocarbamate; ROI, reactive oxygen intermediates; SP, Substance P; STAT, signal transducer and activator of transcription.

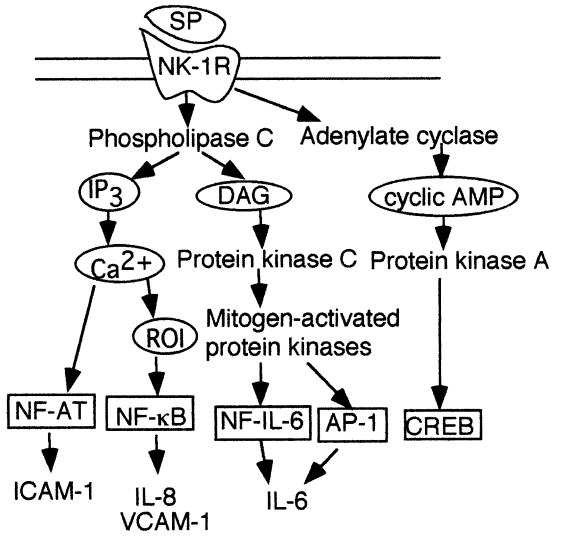

Figure 1. A scheme for SP-induced signaling pathways leading to gene expression. DAG, diacylglycerol; NF-AT, nuclear factor of activated T cells; NF-IL-6, nuclear factor of IL-6; AP-1, activator protein 1; CREB, cyclic adenosine monophosphate response element-binding protein; ICAM-1, intercellular adhesion molecule-1; IL-8, interleukin-8; VCAM-1, vascular cell adhesion molecule-1.

chemokine family and preferentially attracts activated T helper 1 cells through the cell surface CXC chemokine receptor 3 (Kaplan, 2001). Keratinocytes can produce IP-10 in response to interferon (IFN)-γ (Boorsma *et al*, 1998), and the expression of IP-10 is enhanced in keratinocytes of inflammatory skin diseases, such as psoriasis (Rottman *et al*, 2001), contact dermatitis (Flier *et al*, 1999), or delayed type hypersensitivity reaction (Kaplan *et al*, 1987). T helper 1 cells infiltrating into these skin lesions may produce IFN-γ, which induces IP-10 production by keratinocytes, and the produced IP-10 in turn attracts activated T helper 1 cells into the lesional skin. Such a positive feedback loop of IFN-γ/IP-10 may sustain the T helper 1-mediated skin inflammation. As SP acts on keratinocytes and enhances their synthesis of cytokines or cell adhesion molecules related to skin inflammation (Viac *et al*, 1996), it is hypothesized that SP may also enhance IP-10 production by keratinocytes. Besides the recent study reports that SP potentiates IFN-γ-induced major histocompatibility complex class II expression in rat microglia (McCluskey and Lampson, 2001), indicating the synergy between SP and IFN-γ. It is thus anticipated that SP may also potentiate the IFN-γ-induced IP-10 production by keratinocytes; however, it has not been precisely investigated if SP may enhance IP-10 production in keratinocytes, either alone or in combination with IFN-γ.

In this study, we examined the *in vitro* effects of SP on IP-10 production by human keratinocytes. We found that SP enhanced their IP-10 production in synergy with low levels of IFN-γ at the transcriptional level. We further analyzed the *cis*-regulatory elements responsible for the SP-induced IP-10 transcription.

MATERIALS AND METHODS

Reagents SP, phorbol-12-myristate 13-acetate, pyrrolidine dithiocarbamate (PDTC), and calphostin C were obtained from Sigma (St Louis, MO). GR82334, MEN10207, and [Trp^7, β-Ala^8]neurokinin A-(4–10) were purchased from Peninsula Laboratories (Belmont, CA). 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester (BAPTA-AM), U73122, PD98059, SB202190, and SQ22536 were obtained from Calbiochem (La Jolla, CA). Recombinant human IFN-γ was purchased from R&D (Minneapolis, MN). Antibodies used in the electrophoretic mobility shift assay (EMSA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Culture of normal human epidermal keratinocytes and SV40-immortalized keratinocytes Human neonatal foreskin keratinocytes were cultured in serum-free keratinocyte growth medium (Clonetics, Walkersville, MD) consisting of basal medium MCDB153 supplemented with 0.5 μg hydrocortisone per ml, 5 ng epidermal growth factor per ml, 5 μg insulin per ml, and 0.5% bovine pituitary extract. The cells in third passage were used for the experiments. SV40-immortalized keratinocyte cell line was established from human neonatal foreskin keratinocytes as described (Miquel *et al*, 1996) and cultured in 3 : 1 mixture of Dulbecco minimal Eagle's medium and Ham's F12 with 10% fetal bovine serum, 0.4 μg hydrocortisone per ml, and 10 ng epidermal growth factor per ml. The SV40-immortalized keratinocytes did not show any signs of crisis, and morphologically showed a homogeneous spindle shape similar to that of primary cultured keratinocytes. The cells in eighth passage were used for the experiments.

Measurement of IP-10 secretion Keratinocytes (2×10^4 per well) were seeded in triplicate into 24-well plates in 1 ml keratinocyte growth medium, adhered overnight, then the medium was changed to basal KBM medium (keratinocyte basal medium) depleted of hormones, growth factors, and bovine pituitary extract, and incubated for 24 h. The medium was removed and the cells were pretreated with NK-R antagonists or signal inhibitors for 30 min, then incubated with SP in the presence or absence of IFN-γ at the indicated concentrations for another 24 h. The concentrations of the antagonists or inhibitors were decided on a basis of the reported IC_{50} values. The supernatants were assayed for IP-10 by enzyme-linked immunosorbent assay (HyCult Biotechnology, b.v. Uden, the Netherlands). The sensitivity of the assay was 20 pg per ml.

Reverse transcription–polymerase chain reaction (reverse transcription–PCR) Keratinocytes were incubated as above for 3 h, and the cellular mRNA was extracted using a mRNA purification kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. cDNA was made from RNA samples as described (Tjandrawinata *et al*, 1997). Primers and conditions for amplification were as described (Boorsma *et al*, 1998; Hiramoto *et al*, 1998; Lai *et al*, 2000). The PCR products were analyzed by electrophoresis on 2.5% agarose gels and stained with ethidium bromide, viewed by ultraviolet light, and photographed. Densitometric analysis was performed by scanning the bands into Photoshop and performing densitometry with NIH Image Software.

Plasmids and transfections The firefly luciferase reporter plasmids driven by human IP-10 promoter (–525/ + 97 bp relative to the transcriptional start site) were constructed by PCR and insertion into pGL3 basic vector (Promega, Madison, WI) as described (Majumder *et al*, 1998). Site-specific mutation in IFN-stimulated response element (ISRE) and two NF-κB binding sites of the human IP-10 promoter were created by multiple rounds of PCR using appropriate primers with altered bases as described (Ohmori and Hamilton, 1993). p3xNF-κB1-SV-luc, p3xNF-κB2-SV-luc, and p3xISRE-SV-luc were constructed by inserting three copies of proximal NF-κB (NF-κB1) (5′-GCAACATGGGACTTCCCCAGGAAC-3′, with consensus sequences underlined), distal NF-κB (NF-κB2) (5′-GAGCAGAGGGAAATTCCGTAACTT-3′), and ISRE (5′-CGCTTT-GGAAAGTGAAACCTACCTC-3′) from human IP-10 promoter in front of the heterologous minimal SV40 promoter upstream of firefly luciferase reporter as described (Ohmori and Hamilton, 1993; Ohmori *et al*, 1994). Transient transfections were performed using Lipofectamine Plus reagent (GIBCO/BRL, Grand island, NY) according to the manufacturer's protocol. Keratinocytes were plated in six-well plates and grown to about 80% confluence. Twenty-four hours before the transfection, the medium was changed to basal KBM medium. Keratinocytes were incubated with 1.8 μg of luciferase construct and 0.2 μg of Rous sarcomavirus β-galactosidase vector per well in the mixture of KBM, Plus reagent, and lipofectamine for 5 h, and the cells were washed and incubated in KBM for 18 h. Cells were washed, and pretreated with NK-R antagonists or signal inhibitors in KBM for 30 min, then treated with SP in the presence or absence of IFN-γ at indicated concentrations for 6 h. Cell extracts were prepared and luciferase activities were quantitated using a luciferase assay system (Promega). The same cell extracts were assayed for β-galactosidase activity using chemiluminescent Galacto-Light kit (Tropix, Bedford, MA). All readings were taken using a Lumat 9501 luminometer (Berthold, Wildbach, Germany). The results obtained in each transfection were normalized for β-galactosidase activity and expressed as relative luciferase activity. The transfection and incubation were performed on SV40-immortalized keratinocytes as described above except that serum and growth factor-free Dulbecco minimal Eagle's medium/F12 (3 : 1 mixture) was used.

EMSA The probes used were annealed double-stranded DNA containing NF-κB1 site, NF-κB2 site, and ISRE from the human IP-10 promoter. They were labeled by incorporation of [^{32}P]deoxycytidine triphosphate with Klenow DNA polymerase. For gel shift assays, 2–5 μg of nuclear protein extracts were incubated at room temperature for 5 min with a mixture containing 6 mM HEPES (pH 7.9), 0.4 mM ethylenediamine tetraacetic acid, 125 mM KCl, 10% glycerol, 0.05 μg poly dI-dC per μl, 1 mM dithiothreitol, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, 50 μg aprotinin per ml, 50 μg leupeptin per ml. Approximately, 1 ng labeled probe was added and the reactions were incubated at room temperature for another 20 min. In antibody supershift experiments, the nuclear extracts were preincubated with various antibodies on ice for 30 min before the addition of probe. Reactions were then fractionated on a nondenaturing 5% polyacrylamide gel in 0.5 × TBE (25 mM Tris, 0.5 mM Na_3 EDTA, 24.25 mM boric acid). The gels were dried and visualized with PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

The effects of SP on IP-10 secretion in keratinocytes The constitutive IP-10 secretion with medium alone was minimal in keratinocytes (mean ± SEM 51 ± 6 pg per ml, n = 5), and IFN-γ enhanced the IP-10 secretion in a concentration-dependent manner: (i) 98 ± 10 pg per ml by IFN-γ 0.1 ng per ml; (ii) 293 ± 32 pg per ml by IFN-γ 1 ng per ml; (iii) 1780 ± 212 pg per ml by IFN-γ 10 ng per ml (n = 5); and (iv) more than 10 ng per ml of IFN-γ did not further increase the IP-10 secretion. SP alone did not alter the IP-10 secretion, but SP enhanced the IP-10 secretion induced by suboptimal concentrations of IFN-γ (0.1 and 1 ng per ml) (**Fig 2*a***); the significant effect of SP was revealed at 0.1 nM, and increased in a concentration-dependent manner and maximized at 10 nM, whereas at 100 nM the stimulatory effect of SP was reduced. Such bell-shaped dose–responses to SP have been observed in other studies (Quinlan *et al*, 1998, 1999b), and may be caused by receptor desensitization by higher concentrations of SP. Thus 10 nM of SP appeared to be the optimal concentration for the enhancement of IP-10 secretion in synergy with IFN-γ. SP, 10 nM, potentiated IP-10 secretion induced by IFN-γ (0.1 or 1 ng per ml), 5.1-fold or 5.2-fold of controls, respectively. On the other hand, SP did not further enhance the IP-10 secretion induced by a saturating concen-tration of IFN-γ (10 ng per ml). Thus SP enhanced IP-10 secretion in synergy with suboptimal concentrations of IFN-γ. As normal human keratinocytes express NK-1R and NK-2R but not NK-3R (Song *et al*, 2000), we examined which NK-R(s) may be involved in the enhancement of IP-10 production by SP. The synergistic effect of SP on IFN-γ-induced IP-10 secretion was inhibited by the preincubation with NK-1R antagonist GR82334 but not by NK-2R antagonist MEN10207 or NK-3R antagonist [Typ^7,β-Ala^8]neurokinin A-(4–10) (**Fig 2*b***). This indicates that the effects of SP on keratinocytes may be mediated via NK-1R. We then examined if SP may enhance IP-10 mRNA expression induced by suboptimal concentrations of IFN-γ.

The effects of SP on IP-10 mRNA expression A suboptimal concentration (1 ng per ml) of IFN-γ modestly increased IP-10 mRNA level (**Fig 3**). Though SP alone did not increase the IP-10 mRNA level, SP increased the IP-10 mRNA level induced by 1 ng per ml IFN-γ 3.5-fold, and the effect of SP was inhibited by the NK-1R antagonist, GR82334 (**Fig 3**). These results paralleled those in IP-10 secretion (**Fig 2**), and also suggest that the synergy of SP with IFN-γ may occur at a pretranslational level in IP-10 synthesis. We then examined if SP may enhance IP-10 promoter activity induced by suboptimal concentrations of IFN-γ.

The effects of SP on IP-10 promoter activity We transiently transfected human IP-10 promoter-luciferase reporter constructs into keratinocytes, and the promoter activity was assessed by the relative luciferase activities of the cell lysates. IFN-γ (1 ng per ml) increased wild-type IP-10 promoter activity 4.1-fold of controls (**Fig 4*b***, uppermost four columns). SP alone minimally increased

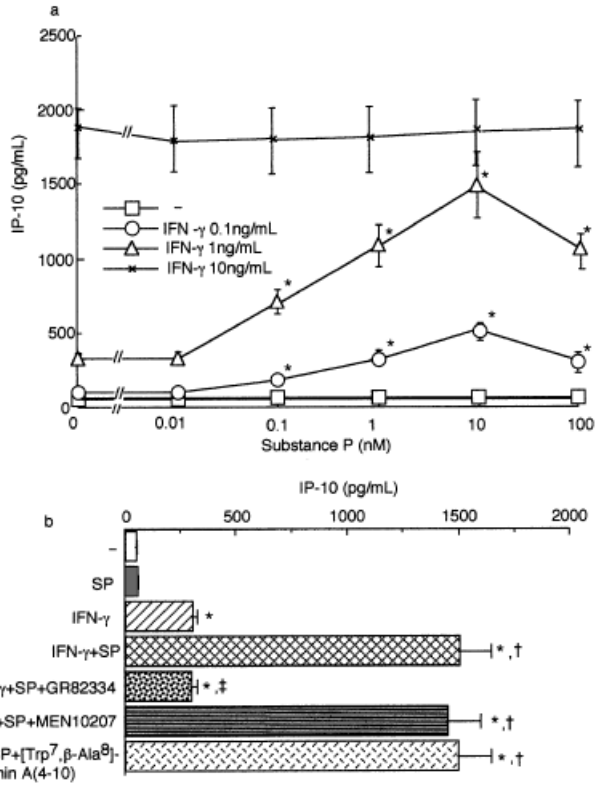

Figure 2. Dose-dependency for the effects of SP on IFN-γ-induced IP-10 secretion in human keratinocytes (*a*) and the inhibition by NK-1R, NK-2R, or NK-3R antagonists on the effects of SP (*b*). (*a*) Human keratinocytes were cultured with indicated concentrations of SP in the presence or absence of indicated doses of IFN-γ. After 24 h, the culture supernatants were assayed for IP-10 secretion. Values are mean ± SD of triplicate cultures. *$p < 0.05$ *vs* control cultures without SP, by one-way ANOVA with Dunnett's multiple comparison test. The data shown in the figure are representative of five separate experiments. (*b*) Keratinocytes were preincubated for 30 min with medium alone, or with GR82334, MEN10207, or [Try^7,β-Ala^8]neurokinin A-(4–10) (each 10 μM), then incubated with SP 10 nM in the presence or absence of IFN-γ (1 ng per ml) for another 24 h. Values are mean ± SEM (n = 5). *$p < 0.05$ *vs* control cultures with medium alone, †$p < 0.05$ *vs* cultures with IFN-γ alone, and ‡$p < 0.05$ *vs* cultures with IFN-γ plus SP, by one-way ANOVA with Scheffe's multiple comparison test.

the IP-10 promoter activity, but synergistically enhanced the promoter activity induced by IFN-γ (1 ng per ml). IFN-γ (0.1 ng per ml), moderately increased the wild-type IP-10 promoter activity 2.5-fold of controls, and the promoter activity by SP 10 nM plus IFN-γ at 0.1 ng per ml was 9.5-fold of controls, indicating the synergistic effect of SP. In contrast, SP did not further increase the IP-10 promoter activity induced by the saturating concentration of IFN-γ; fold increase by IFN-γ (10 ng per ml) was 25.8-fold and that by IFN-γ (10 ng per ml) plus SP 10 nM was 26.0-fold. Thus SP enhanced IP-10 promoter activity in synergy with suboptimal concentrations of IFN-γ. The efficiency of transfection into keratinocytes by the lipofectamine method was low (mean ± SEM 10.5 ± 1.3%; n = 9) as determined by flow cytometry using β-galactosidase vector. It is thus possible that the results above may reflect the reaction of a limited keratinocyte subpopulation. When the transfection by the same method was performed on SV40-immortalized human keratinocytes sharing features of normal human keratinocytes, the transfection efficiency increased to 28.5 ± 2.7% (n = 9). In SV40-immortalized keratinocytes transfected with IP-10 promoter, SP enhanced the promoter activity in synergy with 1 ng IFN-γ per ml in a manner similar to that in normal keratinocytes (data not shown). Thus the results on normal human keratinocytes were reproducible, although associated

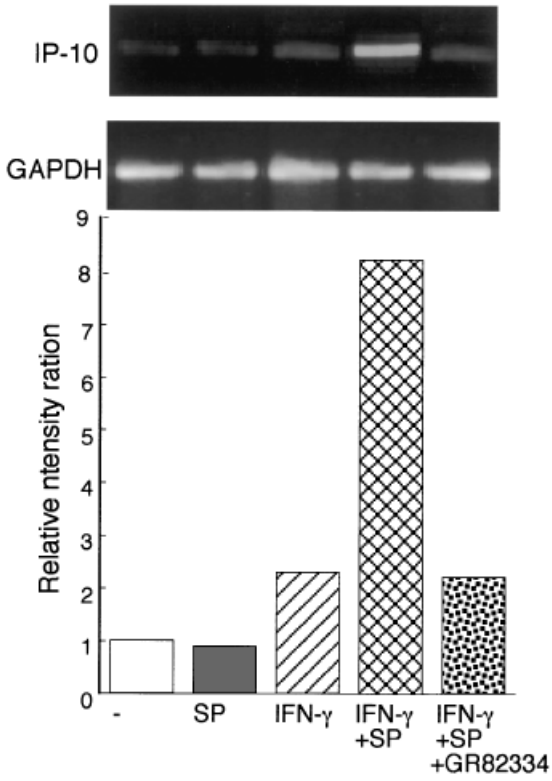

Figure 3. The effects of SP on IFN-γ-induced IP-10 mRNA expression in human keratinocytes. Keratinocytes were preincubated with medium alone, or with 10 μM GR82334 for 30 min, then incubated with 10 nM SP in the presence or absence of IFN-γ (1 ng per ml) for 3 h. RNA was isolated, and reverse transcription–PCR was performed. The intensity of the band for IP-10 was corrected to that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The lower graph shows the corrected intensities relative to that in control cells cultured with medium alone (set as 1.0). The results shown in the figure are representative of five separate experiments.

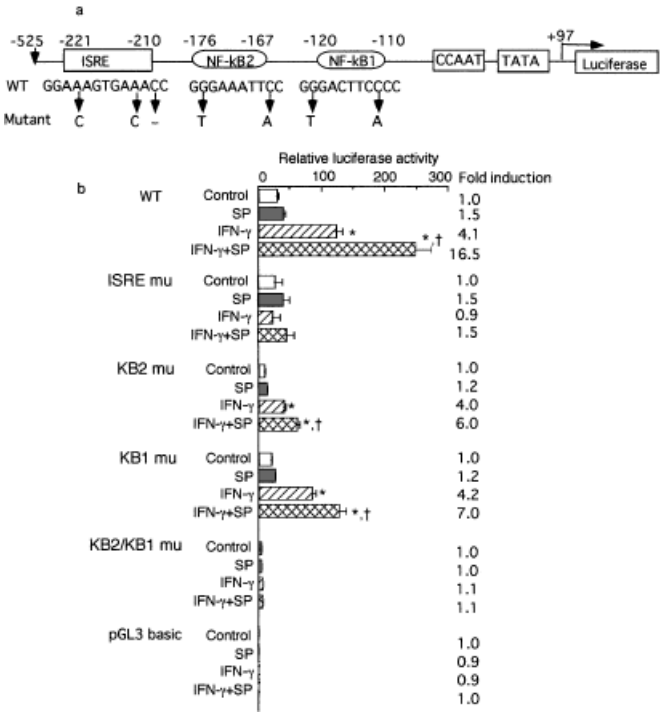

Figure 4. The effects of SP on the basal or IFN-γ-induced activities of wild-type or mutated IP-10 promoters in human keratinocytes. (*a*) Schematic representation of human IP-10 promoter. The locations of ISRE, NF-κB2, and NF-κB1 are shown with their sequences, and substituted or deleted bases for mutation are indicated. The nucleotide positions are relative to the transcriptional start site. (*b*) Keratinocytes were transiently transfected with wild-type (WT) or mutated human IP-10 promoter linked to luciferase reporter together with β-galactosidase vector. After transfection, cells were allowed to recover for 18 h and treated with 10 nM SP in the presence or absence of IFN-γ (1 ng per ml) for 6 h. Cells were harvested and luciferase and β-galactosidase activities were measured. Relative luciferase activities normalized to β-galactosidase activities were shown. The data are mean ± SEM ($n = 4$). Values at right indicate the fold induction *vs* basal promoter activity. *$p < 0.05$ *vs* control values and †$p < 0.05$ *vs* values with IFN-γ alone, by one-way ANOVA with Scheffe's multiple comparison test.

with low transfection efficiency. Further transfection studies were thus performed on normal human keratinocytes followed by confirming the results in SV40-immortalized keratinocytes.

It is known that IFN-γ-induced IP-10 transcription required the cooperation of ISRE with at least one of the two NF-κB sites on the IP-10 promoter (Ohmori and Hamilton, 1993; Ohmori *et al*, 1997). We thus examined which of these elements may be responsible for the synergy of SP with IFN-γ, using promoters with mutation in each of these elements. The mutation of ISRE completely abrogated the IP-10 promoter induction by IFN-γ and the synergistic effect of SP (**Fig 4*b***, the second four columns), suggesting that ISRE is necessary for both IFN-γ-induced promoter activation and the synergistic effect of SP with IFN-γ. When either NF-κB2 (distal NF-κB site) or NF-κB1 (proximal NF-κB site) was mutated, the basal promoter activity was reduced, and the fold-induction by SP plus IFN-γ also decreased, whereas the fold induction by IFN-γ alone was retained. These indicate that both NF-κB sites may be responsible for the constitutive promoter activity, and also for the synergy of SP with IFN-γ. When both NF-κB2 and NF-κB1 sites were mutated, basal promoter activity was mostly abolished and IFN-γ-induced activation and synergistic effect of SP with IFN-γ were lost even if ISRE was intact. When SV40-immortalized keratinocytes were transfected with a series of mutated promoters, the results were similar to those in normal keratinocytes (data not shown). These suggest that IFN-γ-induced promoter activation may require at least one of the two NF-κB sites in addition to ISRE. Thus all of the ISRE, NF-κB2, and NF-κB1 sites may be involved in the synergistic effect of SP with IFN-γ on IP-10 promoter activation. We then analyzed if SP may alter the activity of transcription through ISRE, NF-κB2, or NF-κB1 motifs either alone or in synergy with IFN-γ.

The effects of SP on transcription through ISRE, NF-κB2, or NF-κB1 motifs Keratinocytes were transiently transfected with plasmids containing three repeats of ISRE, NF-κB2, or NF-κB1 linked to the heterologous minimal SV40 promoter and luciferase reporter, and were incubated with SP alone or together with IFN-γ (1 ng per ml). The transcriptional activity through each element was evaluated by the relative luciferase activity in the lysates of the transfected cells. The enhancerless SV40 promoter-luciferase vector produced very low background luciferase activity, which was not altered by SP, IFN-γ, either alone or combination (**Fig 5**, the lowermost four columns). SP alone increased the transcriptional activities through NF-κB2 and NF-κB1 motifs, whereas 1 ng per ml of IFN-γ did not increase the constitutive and SP-induced transcriptional activities through both motifs. The constitutive transcriptional activity through ISRE was much lower than those through two NF-κB motifs, and IFN-γ enhanced the ISRE activity in a concentration-dependent manner; 2.3-fold of controls by 0.1 ng per ml, 5.1-fold by 1 ng per ml, and 16.3-fold by 10 ng per ml of IFN-γ, respectively. SP did not increase the constitutive and IFN-γ-induced transcriptional activities through ISRE. When transfection was performed on SV40-immortalized keratinocytes, SP also increased the transcriptional activities through

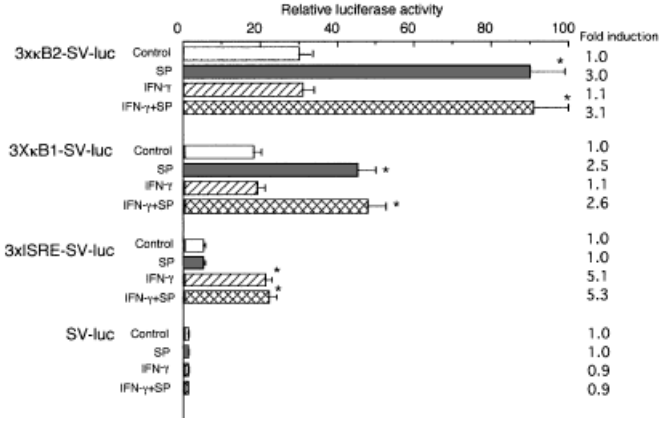

Figure 5. SP and/or IFN-γ-induced activation of transcription through NF-κB2, NF-κB1, or ISRE motifs. Keratinocytes were transiently transfected with luciferase reporter plasmids driven by NF-κB2, NF-κB1, or ISRE linked to heterologous SV-40 minimal promoter together with β-galactosidase vector. The cells were allowed to recover for 18 h and incubated with 10 nM SP in the presence or absence of IFN-γ (1 ng per ml) for 6 h. The cells were then harvested and luciferase and β-galactosidase activities were assayed. The results are shown as relative luciferase activities normalized for β-galactosidase activities, and represent mean ± SEM (n = 4). Values at right indicate the fold induction *vs* basal activity. * $p < 0.05$ *vs* control values with medium alone, by one-way ANOVA with Scheffe's multiple comparison test.

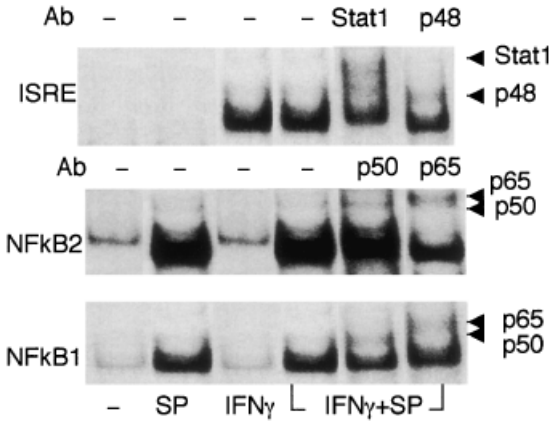

Figure 6. SP and/or IFN-γ-induced transcription factor binding to ISRE, NF-κB2 or NF-κB1 motifs in human keratinocytes. Keratinocytes were incubated with 10 nM SP in the presence or absence of 1 ng IFN-γ per ml for 30 min, and nuclear extracts were prepared. The nuclear extracts were incubated with ^{32}P-labeled oligonucleotides containing NF-κB2, NF-κB1, or ISRE motifs from human IP-10 promoter. In supershift assays, antibodies against transcription factors were incubated for 30 min before the addition of the probe. *Arrows* indicate the supershifted complexes. The results shown in the figure are representative of four separate experiments.

NF-κB2 and NF-κB1 motifs without altering that through ISRE in a manner similar to that in normal keratinocytes (data not shown). Thus SP enhanced the transcriptional activity through the two NF-κB sites, whereas IFN-γ enhanced that through ISRE, and each effect was independent. We then examined if SP may alter transcription factor binding to each element either alone or together with IFN-γ.

The effects of SP on transcription factor binding to ISRE, NF-κB2, or NF-κB1 sites Nuclear extracts were obtained from keratinocytes incubated with SP and/or IFN-γ, and EMSA was performed using ISRE, NF-κB2, or NF-κB1 probes. Incubation of keratinocytes with SP alone did not induce the DNA–protein complexes with the ISRE probe, whereas IFN-γ alone induced the complex with the ISRE probe (**Fig 6**, the uppermost panel, lanes 2 and 3). The addition of SP did not affect the IFN-γ-induced complex with the ISRE probe (**Fig 6**, the uppermost panel, lane 4). The antibodies against signal transducer and activator of transcription (STAT) 1α and 48 kDa ISRE-binding protein (p48) supershifted the IFN-γ-induced complex with the ISRE probe (**Fig 6**, the uppermost panel, lanes 5 and 6), indicating the presence of STAT1α and p48 in the complex. Incubation of keratinocytes with SP alone induced the DNA–protein complexes associated with NF-κB2 and NF-κB1 probes (**Fig 6**, the middle and lowermost panels, lane 2). The addition of IFN-γ did not affect the SP-induced complexes with both NF-κB probes (**Fig 6**, the middle and lowermost panels, lane 4). The anti-NF-κB p50 and p65 antibodies supershifted the complexes with NF-κB2 and NF-κB1 probes (**Fig 6**, the middle and lowermost panels, lanes 5 and 6), indicating the presence of p50 and p65 in the complexes. Thus SP induced transcription factor binding to two NF-κB sites whereas IFN-γ induced that to ISRE, and each effect was independent. Though SP alone induces transcription factor binding and transcriptional activity at two NF-κB sites on IP-10 promoter, SP *per se* cannot induce IP-10 promoter activity. This indicates that the NF-κB activation by SP may be necessary but insufficient for the IP-10 promoter activation, and the cooperation with IFN-γ-induced STAT-1α/p48 may be required for the significant promoter activation.

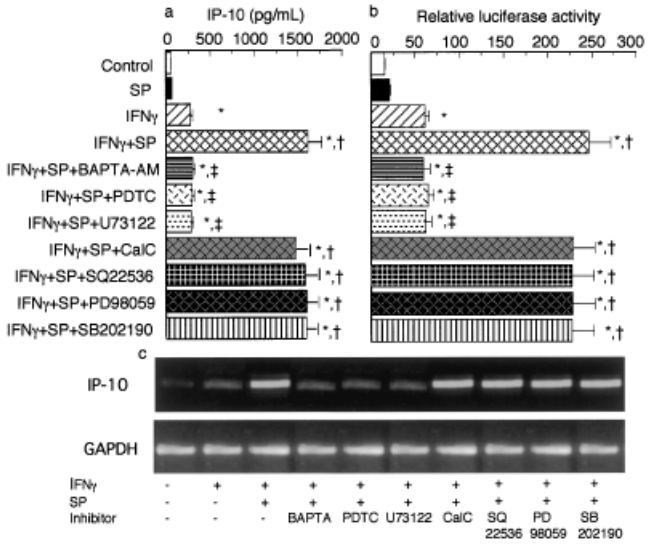

Figure 7. The inhibition by several signal inhibitors on the enhancement by SP of IFN-γ-induced IP-10 secretion (*a*), promoter activity (*b*), and mRNA expression (*c*) in human keratinocytes. (*a,c*) Keratinocytes were preincubated with medium alone, or with 50 μM BAPTA-AM, 200 μM PDTC, 1 μM U73122, 1 μM calphostin C, 200 μM SQ22536, 10 μM PD98059, or 10 μM SB202190 for 30 min, then incubated with 10 nM SP in the presence or absence of 1 ng IFN-γ per ml. IP-10 secretion was analyzed after 24 h, or RNA was extracted after 3 h. (*b*) Keratinocytes were transiently transfected with human IP-10 promoter linked to luciferase reporter plasmid, and recovered for 18 h, and incubated as above. After 6 h, luciferase activities of the cell lysates were analyzed and were normalized for β-galactosidase activities. (*a,b*) Values are mean ± SEM (n = 5). * $p < 0.05$ *vs* control values with medium alone, † $p < 0.05$ *vs* values with IFN-γ alone, and ‡ $p < 0.05$ *vs* values with IFN-γ plus SP, by one-way ANOVA with Scheffe's multiple comparison test. The results shown in (*c*) are representative of five separate experiments.

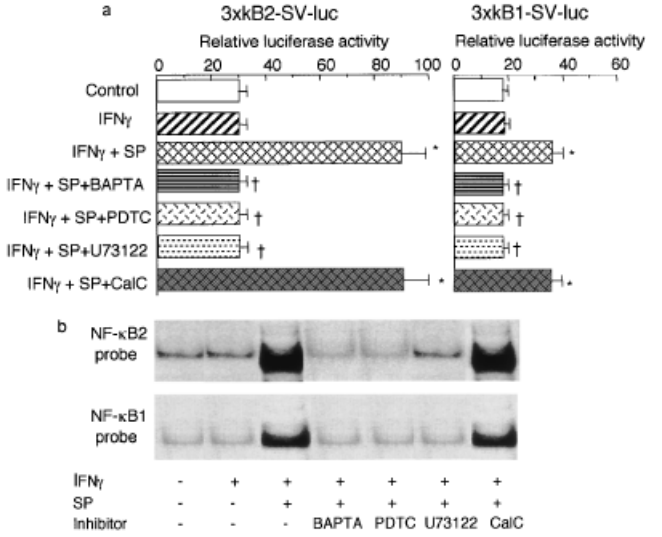

Figure 8. The inhibition by several signal inhibitors on SP plus IFN-γ-induced transcriptional activities (*a*) or DNA binding (*b*) of NF-κB. (*a*) Keratinocytes were transiently transfected with luciferase reporter vectors driven by NF-κB2 or NF-κB1 motifs linked to minimal SV40 promoter together with β-galactosidase vector. Cells were preincubated with medium alone, or with 50 μM BAPTA-AM, 200 μM PDTC, 1 μM U73122, or 1 μM calphostin C (CalC) for 30 min, then incubated with 10 nM SP plus 1 ng IFN-γ per ml for 6 h. Luciferase activities of the cell lysates were analyzed and normalized for β-galactosidase activities. The data are mean ± SEM (n = 5). $^{*}p < 0.05$ *vs* control values with medium alone, and †$p < 0.05$ *vs* values with SP alone, by one-way ANOVA with Scheffe's multiple comparison test. (*b*) Keratinocytes were preincubated with agents above, then incubated with 10 nM SP plus 1 ng IFN-γ per ml. After 30 min, nuclear extracts were obtained and analyzed for EMSA using NF-B2 or NF-B1 probes. The results shown in the figure are representative of five separate experiments.

The SP-induced signals required for NF-κB activation and IP-10 production SP is known to activate a variety of intracellular signaling pathways (**Fig 1**), and we aimed to know which of the signals may be responsible for the SP-induced NF-κB activation and resultant enhancement of IP-10 synthesis in synergy with IFN-γ. Keratinocytes were preincubated with several signal inhibitors, then incubated with SP in the presence of IFN-γ (1 ng per ml). Intracellular Ca^{2+} chelator BAPTA-AM, anti-oxidant PDTC, and phospholipase C inhibitor U73122 inhibited the enhancement by SP on IFN-γ-induced IP-10 secretion (**Fig 7*a***), mRNA expression (**Fig 7*c***), and promoter activation (**Fig 7*b***). In contrast, protein kinase C inhibitor calphostin C, adenylate cyclase inhibitor SQ22536, p42/44 mitogen-activated protein kinase inhibitor PD98059, or p38 mitogen-activated protein kinase inhibitor SB202190 did not suppress the effects of SP. When IP-10 promoter was transfected into SV40-immortalized keratinocytes, the effects of signal inhibitors on SP plus IFN-γ-induced promoter activation were similar to those in normal keratinocytes (data not shown). These suggest that phospholipase C activity, Ca^{2+} signal, and ROI formation may be responsible for the enhancement by SP on IFN-γ-induced IP-10 synthesis. On the other hand, the effect of SP may not involve the activation of protein kinase C, adenylate cyclase, p42/44, or p38 mitogen-activated protein kinases. We then analyzed if the above-mentioned signal inhibitors may suppress the SP-induced NF-κB activation. SP plus IFN-γ-induced enhancement of NF-κB2 or NF-κB1 transcriptional activity was abrogated by BAPTA-AM, PDTC, and U73122, and not by calphostin C in normal human keratinocytes (**Fig 8*a***) and in SV40-immortalized keratinocytes (data not shown), which paralleled the inhibition on IP-10 synthesis. SP plus IFN-γ-induced transcription factor binding to NF-κB2 or NF-κB1 probes was inhibited by BAPTA-AM, PDTC, and U73122 (**Fig 8*b***, lanes 3, 4, and 5) but not by calphostin C (**Fig 8*b***, lane 6). These results suggest that SP-induced NF-κB activation may require phospholipase C activity, Ca^{2+} signal, and ROI formation.

DISCUSSION

In this study, SP enhanced IFN-γ-induced IP-10 transcription via NF-κB activation in human keratinocytes. Transfection into normal human keratinocytes using lipofectamine was associated with low transfection efficiency (11%). All the results of transfection studies in normal keratinocytes, however, could be reproduced in SV40-immortalized keratinocytes with higher transfection efficiency (29%) and sharing features of normal human keratinocytes. These suggest that the results in transfection studies on normal human keratinocytes may reflect the reaction by a considerable population of keratinocytes. We are now testing if the results can also be reproduced in normal keratinocytes transfected by a method using Effectene (Zellmer *et al*, 2001) with higher transfection efficiency (41%).

SP-induced NF-κB activation required Ca^{2+} and ROI as second messengers. Our results are consistent with the previous report; SP increased interleukin-8 expression via NF-κB activation in human astrocytoma U373 MG cells dependently on the second messengers above (Lieb *et al*, 1997). It is supposed that ROI may induce certain tyrosine kinase(s) to tyrosine-phosphorylate inhibitory NF-κB (IκB)-α, which may dissociate IκB-α from inactive IκB-α-NF-κB complex, leading to the nuclear translocation and DNA binding of active free *NF-κB p65/p50* (Imbert *et al*, 1996; Kang *et al*, 2000). ROI include superoxide, hydrogen peroxide, hydroxyl radical, or lipid peroxide (Shi *et al*, 1999), and certain enzymes to generate ROI, such as superoxide-generating NADPH oxidase, are activated by Ca^{2+} signal (Tauber, 1987). It is reported that SP induces Ca^{2+} mobilization from intracellular store via phospholipase C in keratinocytes (Koizumi *et al*, 1994; Mochizuki-Oda *et al*, 1994; Li *et al*, 1997). It is thus hypothesized that SP-induced Ca^{2+} signal may induce NF-κB activation via ROI formation in keratinocytes. This possibility should be investigated further.

Phospholipase C generates diacylglycerol, which activates protein kinase C (**Fig 1**). It is known that protein kinase C directly or indirectly induces the serine phosphorylation and degradation of IκB-α and resultant NF-κB activation (Imbert *et al*, 1996). In SP-stimulated keratinocytes, however, protein kinase C-independent pathways appeared to activate predominantly NF-κB.

SP preferentially binds to NK-1R, but neurokinin receptors are promiscuous and SP interacts with NK-2R in murine keratinocytes (Song *et al*, 2000; Burbach *et al*, 2001). Furthermore, a non-neurokinin receptor for SP has been detected in monocytes (Kavelaars *et al*, 1994), and receptor-independent action of SP has also been reported (Kavelaars *et al*, 1993). At least in human keratinocytes, however, SP appears to act predominantly through NK-1R. Burbach *et al* (2001) also reported that SP induced nerve growth factor production in human keratinocytes mainly via NK-1R.

SP potently activated NF-κB, but SP alone only minimally induced IP-10 promoter, and IFN-γ-induced p48/STAT1α activation was required for the promoter activation by SP to occur. IFN-γ induces the tyrosine phosphorylation and homodimerization of STAT1α, which combines with p48, and is directed to ISRE (Bluyssen *et al*, 1995). Possibly NF-κB p65/p50 bound to two NF-κB sites may cooperate with p48/STAT1α bound to ISRE on IP-10 promoter and synergistically enhance the transcription. Such cooperation between NF-κB site and IFN-responsive element occurs in the expression of intercellular adhesion molecule-1, interferon-regulatory factor-1 (Ohmori *et al*, 1997), or major histocompatibility complex class I heavy chain (Johnson and Pober, 1994). The cooperation between NF-κB and

STAT1α may be mediated by the summing up of the interaction of individual transcription factors with basal transcriptional components such as TATA-binding protein. Such multiprotein interactions may facilitate the assembly of a pre-initiation complex, and thus promote the transcriptional initiation and elongation (Sauer *et al*, 1995). Alternatively, the transcriptional synergy may be mediated by the direct interaction of NF-κB and STAT1α, which may stabilize the DNA binding of individual transcription factors and thus create a highly stable multiprotein complex (Thanos and Maniatis, 1995). As IFN-γ alone activated IP-10 promoter via ISRE, IP-10 promoter activity may predominantly depend on ISRE in human keratinocytes. The mutation of both NF-κB sites, however, abrogated the response to IFN-γ, although ISRE was intact. This is possibly because the mutation of two NF-κB sites changed the conformation of IP-10 promoter and interfered with the p48/STAT1α binding or transcriptional activity or because the mutation disrupted the constitutive binding of NF-κB and abrogated its constitutive cooperation with STAT1α.

This study showed that SP *in vitro* enhanced IP-10 synthesis induced by suboptimal concentrations of IFN-γ in human keratinocytes. Thus SP may *in vivo* potentiate the effect of low levels of endogenous IFN-γ, possibly derived from T helper 1 cells infiltrating into inflammatory skin lesions such as contact dermatitis or photodermatitis as well as psoriasis. The *in vitro* effects of SP, however, may not be significantly manifested *in vivo* as SP is rapidly metabolized in tissues. Further studies should elucidate if direct cutaneous injection of SP may *in vivo* enhance IP-10 synthesis in synergy with IFN-γ.

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REFERENCES

Anand P, Springall DR, Blank MA, Sellu D, Polak JM, Bloom SR: Neuropeptides in skin disease: increased VIP in eczema and psoriasis but not axillary hyperhidrosis. *Br J Dermatol* 124:547–549, 1991

Ansel JC, Kaynard AH, Armstrong CA, Olerud J, Bunnett N, Payan D: Skin–nervous system interactions. *J Invest Dermatol* 106:198–204, 1996

Bluyssen HAR, Muzaffar R, Vlieststra RJ, *et al*: Combinatorial association and abundance of components of interferon-stimulated gene factor 3 dictate the selectivity of interferon responses. *Proc Natl Acad Sci USA* 92:5645–5649, 1995

Boorsma DM, Flier J, Sampat S, *et al*: Chemokine IP-10 expression in cultured human keratinocytes. *Arch Dermatol Res* 290:335–341, 1998

Bos JD, de Rie MA: The pathogenesis of psoriasis: immunological facts and speculations. *Immunol Today* 20:40–46, 1999

Burbach GJ, Kim KH, Zivony AS, *et al*: The neurosensory tachykinins substance P and neurokinin A directly induce keratinocyte nerve growth factor. *J Invest Dermatol* 117:1075–1082, 2001

Christian C, Gilbert M, Payan DG: Stimulation of transcriptional regulatory activity by substance P. *Neuroimmunomodulation* 1:159–164, 1994

Eedy DJ, Johnston CF, Shaw C, Buchanan KD: Neuropeptides in psoriasis: an immunocytochemical and radioimmunoassay study. *J Invest Dermatol* 96:434–438, 1991

Fiebich BL, Schleicher S, Butcher RD, Craig A, Lieb K: The neuropeptide substance P activates p38 mitogen-activated protein kinase resulting in IL-6 expression independently from NF-κB. *J Immunol* 165:5606–5611, 2000

Flier J, Boorsma DM, Bruynzeel DP, *et al*: The CXCR3 activating chemokines IP-10, Mig, and IP-9 are expressed in allergic but not in irritant patch test reactions. *J Invest Dermatol* 113:574–578, 1999

Hiramoto M, Aizawa S, Iwase O, *et al*: Stimulatory effects of substance P on CD34 positive cell proliferation and differentiation in vitro are mediated by the modulation of stromal cell function. *Int J Mol Med* 1:347–354, 1998

Imbert V, Rupec RA, Livolsi A, *et al*: Tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α. *Cell* 86:787–798, 1996

Johnson DR, Pober JS: HLA class I heavy-chain gene promoter elements mediating synergy between tumor necrosis factor and interferons. *Mol Cell Biol* 14:1322–1332, 1994

Kang JL, Pack IS, Hong SM, Lee HS, Castranova V: Silica induces nuclear factor-κB activation through tyrosine phosphorylation of IkB-α in RAW264.7 macrophages. *Toxicol Appl Pharmacol* 169:59–65, 2000

Kaplan AP: Chemokines, chemokine receptors and allergy. *Int Arch Allergy Immunol* 124:423–431, 2001

Kaplan G, Luster AD, Hancock G, Cohn ZA: The expression of a gamma interferon-induced protein (IP-10) in delayed immune responses in human skin. *J Exp Med* 166:1098–1108, 1987

Kavelaars A, Jeurissen F, Kunzel FD, Jr, Roijen HV, Rijkers GT, Heijnen CJ: Substance P induces a rise in intracellular calcium concentration in human T lymphocytes in vitro: evidence of a receptor-independent mechanism. *J Neuroimmunol* 42:61–70, 1993

Kavelaars A, Broeke D, Jeurissen F, *et al*: Activation of human monocytes via a non-neurokinin substance P receptor that is coupled to Gi protein, calcium, phospholipase D, MAP kinase, and IL-6 production. *J Immunol* 153:3691–3699, 1994

Kincy-Cain T, Bost KL: Substance P-induced IL-12 production by murine macrophages. *J Immunol* 158:2334–2339, 1997

Koizumi H, Yasui C, Fukaya T, Ueda T, Ohkawara A: Substance P induces inositol 1,4,5-triphosphate and intracellular free calcium increase in cultured normal human epidermal keratinocytes. *Exp Dermatol* 3:40–44, 1994

Lai J-P, Zhan G-X, Campbell DE, Douglas SD, Ho W-Z: Detection of substance P and its receptor in human fetal microglia. *Neuroscience* 101:1137–1144, 2000

Lieb K, Fiebich BL, Berger M, Bauer J, Schulze-Osthoff K: The neuropeptide substance P activates transcription factor NF-κB and κB-dependent gene expression in human astrocytoma cells. *J Immunol* 159:4952–4958, 1997

Lotz M, Vaughan JH, Carson DA: Effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science* 241:1218–1221, 1988

Majumder S, Zhou LS-H, Chaturvedi P, Babcock G, Aras S, Ransohoff RM: p48/STAT-1α-containing complexes play a predominant role in induction of IFN-γ-inducible protein, 10 kDa (IP-10) by IFN-γ alone or in synergy with TNF-α. *J Immunol* 161:4736–4744, 1998

McCluskey LP, Lampson LA: Local immune regulation in the central nervous system by substance P *vs* glutamate. *J Neuroimmunol* 116:136–146, 2001

Miquel C, Gagnoux-Palacios L, Durand-Clement M, Marinkovich P, Ortonne JP, Meneguzzi G: Establishment and characterization of cell line LSV5 that retains the altered adhesive properties of human junctional epidermolysis bullosa keratinocytes. *Exp Cell Res* 224:279–290, 1996

Mochizuki-Oda N, Nakajima Y, Nakanishi S, Ito S: Characterization of the substance P receptor-mediated calcium influx in cDNA transfected Chinese hamster ovary cells. *J Biol Chem* 269:9651–9658, 1994

Naukkanrinen A, Nickoloff BJ, Farber EM: Quantification of cutaneous sensory nerves and their substance P content in psoriasis. *J Invest Dermatol* 92:126–129, 1989

Ohmori Y, Hamilton TA: Cooperative interaction between interferon (IFN) stimulus response element and κB sequence motifs controls IFNγ- and lipopolysaccharide-stimulated transcription from the murine IP-10 promoter. *J Biol Chem* 268:6677–6688, 1993

Ohmori Y, Tebo J, Nedospasov S, Hamilton TA: κB binding activity in a murine macrophage-like cell line. Sequence-specific differences in κB binding and transcriptional activation functions. *J Biol Chem* 269:17684–17690, 1994

Ohmori Y, Schreiber RD, Hamilton TA: Synergy between interferon-γ and tumor necrosis factor-α in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor κB. *J Biol Chem* 272:14899–14907, 1997

Pincelli C, Fantini F, Romualdi P, Sevignani C, Lesa G, Benassi L, Giannetti A: Substance P is diminished and vasoactive intestinal peptide is augmented in psoriatic lesions and these peptides exert disparate effects on the proliferation of cultured human keratinocytes. *J Invest Dermatol* 98:421–427, 1992

Quinlan KL, Song I-S, Bunnett NW, *et al*: Neuropeptide regulation of human dermal microvascular endothelial cell ICAM-1 expression and function. *Am J Physiol* 275:C1580–C1590, 1998

Quinlan KL, Naik SM, Cannon G, Armstrong CA, Bunnett NW, Ansel JC, Caughman SW: Substance P activates coincident NF-AT- and NF-κB-dependent adhesion molecule gene expression in microvascular endothelial cells through intracellular calcium mobilization. *J Immunol* 163:5656–5665, 1999a

Quinlan KL, Song I-S, Naik SM, *et al*: VCAM-1 expression on human dermal microvascular endothelial cells is directly and specifically up-regulated by substance P. *J Immunol* 162:1656–1661, 1999b

Rottman JB, Smith TL, Ganley KG, Kikuchi T, Krueger JG: Potential role of the chemokine receptors CXCR3, CCR4, and the integrin αEβ7 in the pathogenesis of psoriasis vulgaris. *Lab Invest* 81:335–347, 2001

Sauer F, Hansen SK, Tjian R: Multiple TAFIIs directing synergistic activation of transcription. *Science* 270:1783–1788, 1995

Schanahan F, Denburg JA, Fox J, Bienenstock J, Befus D: Mast cell heterogeneity: effects of neuroenteric peptides on histamine release. *J Immunol* 135:1331–1337, 1985

Scholzen TE, Brzoska T, Kalden DH, O'Reilly F, Armstrong CA, Luger TA, Ansel JC: Effect of ultraviolet light on the release of neuropeptides and neuroendocrine hormones in the skin: mediators of photodermatitis and cutaneous inflammation. *J Invest Dermatol Symp Proc* 4:55–60, 1999

Scholzen TE, Steinhoff M, Bonaccorsi P, *et al*: Neutral endopeptidase terminates substance P-induced inflammation in allergic contact dermatitis. *J Immunol* 166:1285–1291, 2001

Shi X, Dong Z, Huang C, *et al*: The role of hydroxyl radical as a messenger in the activation of nuclear transcription factor NF-κB. *Mol Cell Biochem* 194:63–70, 1999

Song I-S, Bunnett NW, Olerud JE, *et al*: Substance P induction of murine keratinocyte PAM 212 interleukin 1 production is mediated by the neurokinin 2 receptor (NK-2R). *Exp Dermatol* 9:42–52, 2000

Tauber AI: Protein kinase C and activation of the human neutrophil NADPH-oxidase. *Blood* 69:711–720, 1987

Thanos D, Maniatis T: Virus induction of human IFN-β gene expression requires the assembly of an enhanceosome. *Cell* 83:1091–1100, 1995

Tjandrawinata RR, Dahiya R, Hughes-Fulford M: Induction of cyclo-oxygenase-2 mRNA by prostaglandin E_2 in human prostatic carcinoma cells. *Br J Cancer* 75:1111–1118, 1997

Viac J, Gueniche A, Doutremepuich JD, Reichert U, Claudy A, Schmitt D: Substance P and keratinocyte activation markers: an in vitro approach. *Arch Dermatol Res* 288:85–90, 1996

Zellmer S, Gaunitz F, Salvetter J, Surovoy A, Ressig D, Gebhardt R: Long-term expression of foreign genes in normal human epidermal keratinocytes after transfection with lipid/DNA complexes. *Histochem Cell Biol* 115:41–47, 2001