

INVESTIGATIVE REPORT

Role of Nerve Growth Factor in RANTES Expression by Keratinocytes

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A role of neurogenic inflammation induced by the neuropeptides and nerve growth factor (NGF) has been attributed to the pathogenesis of several cutaneous disorders such as psoriasis, wound healing and eczematous dermatitis. The underlying mechanisms of the inflammatory process induced by NGF are not clearly established. This study explored whether NGF influences the inflammatory process by inducing chemokines. The effects of NGF were investigated on induction of 2 important chemokines, interleukin-8 and RANTES, which are known to be upregulated in the keratinocytes of various inflammatory conditions. NGF significantly increased RANTES production by the keratinocytes ($p < 0.001$, 2-tailed Student's *t*-test). Induction of RANTES expression in the keratinocytes by NGF provides further insight regarding the role of NGF-NGF receptor system in cutaneous inflammatory conditions. Key words: NGF; chemokines; RANTES; keratinocytes; cutaneous inflammation.

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In addition to major effects on cells of the nervous system, nerve growth factor (NGF) has several functions that promote and maintain an inflammatory response (1–4). Increased levels of NGF and NGF-receptor (NGF-R) have been reported to be associated with various inflammatory conditions (5–7). Recent studies suggest that keratinocytes are a significant source of NGF, and NGF has been attributed to play a key role in the immunological and inflammatory events involved in cutaneous tissue (8–11).

The exact role of NGF in a cutaneous inflammatory reaction is not fully understood. The proposed mechanisms are: proliferation of keratinocytes (12, 13), degranulation of mast cells (4) and activation of T-cells (1, 2). Chemotaxis of leukocytes is a critical event in an inflammatory processes. Chemokines play a pivotal role in the migration and retention of the infiltrating leukocytes.

To elucidate further the regulatory role of NGF on the inflammatory cascades it is important to know whether NGF influences the expression of chemokines. This study investigated the effects of NGF on induction of 2 specific chemokines, RANTES (regulated on activation, normal T-cell expressed and secreted) and interleukin-8 (IL-8), in keratinocytes. RANTES and IL-8 were selected because concurrent expression of NGF, IL-8 and RANTES has been found to be upregulated in several inflammatory diseases (6–8, 14–19). This study also investigated the effects of neuropeptides, e.g. substance P (SP), vasoactive intestinal

peptide (VIP) and calcitonin-gene-regulated peptide (CGRP), which play important role in neurogenic inflammation. The effects were examined of interferon (IFN)- γ and tumor necrosis factor (TNF)- α on RANTES production by keratinocytes along with NGF and neuropeptides, because the IFN- γ and TNF- α are increased in psoriatic lesions.

MATERIAL AND METHODS

Keratinocytes

Normal keratinocytes were prepared from fresh biopsies of normal human skin using a modification of the method described by Normand & Karasek (20). Shave biopsies were taken from 5 normal persons (age range 35–50 years, 1 woman and 4 men). Keratinocytes were cultured in KGM (Clonetics, San Diego, CA, USA), a serum-free medium with 0.15 mM Ca^{2+} . All experiments were performed on the third to fifth passage cultures.

Chemokine assays

Keratinocytes (10^4 cells/well) were cultured individually with NGF (100 ng/ml, optimal dose; Boehringer Mannheim, Indianapolis, IN, USA), NGF-neutralizing monoclonal antibody (Boehringer Mannheim), phytohemagglutinin (PHA; 25 $\mu\text{g}/\text{ml}$; Sigma, St. Louis, MO, USA), IFN- γ (optimal dose 20 ng/ml; Endogen, Woburn, MA, USA), CGRP (optimal dose 1 nM; Sigma), VIP (1 nM; Sigma), IFN- γ (optimal dose 50 ng/ml; Endogen), TNF- α (optimal dose 20 ng/ml; Endogen), IFN- γ neutralizing monoclonal antibody (Endogen) and TNF- α neutralizing monoclonal antibody (Endogen) for 24–72 h. The optimal dose of each reagent was titrated. Supernatant was collected and stored at -70°C . RANTES and IL-8 were assessed by enzyme-linked immunosorbent assay (ELISA; Endogen). The detection limit of RANTES was 0 to 2,000 pg/ml and of IL-8 was 0 to 1,000 pg/ml. The sensitivity of both assays was < 2 pg/ml. Experiments with each reagent (NGF, NGF-neutralizing antibody, PHA, VIP, CGRP, TNF- α and IFN- γ) were repeated at least 10 times (10–15 times). Every time, each reagent was tested in duplicate wells and supernatant from each well was tested in duplicate. Therefore, each time each experiment was tested in quadruplicate for reproducibility.

Statistical analysis

Differences between the results of experimental treatments were evaluated by means of the 2-tailed Student's *t*-test.

RESULTS

NGF-induced RANTES production by keratinocytes

Normal keratinocytes showed increased production of RANTES when stimulated with NGF, TNF- α or IFN- γ for 24 h compared with unstimulated keratinocytes ($p < 0.001$, 2-tailed Student's *t*-test) (Fig. 1). The peak level of production

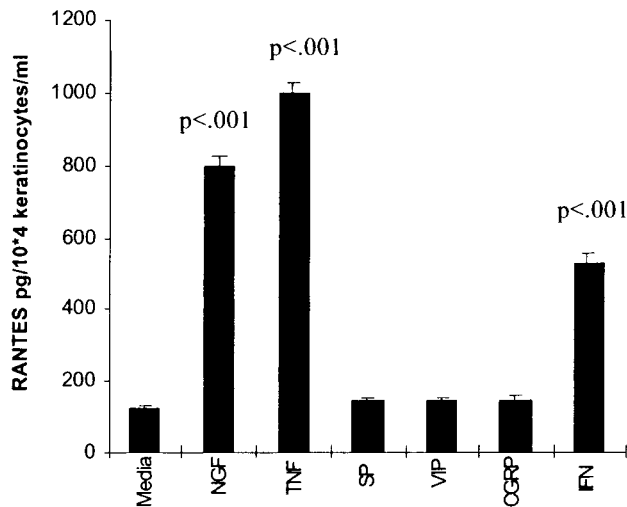


Fig. 1. Effect of NGF and neuropeptides (SP, VIP and CGRP) in RANTES production by keratinocytes. IFN- γ (IFN) and TNF- α (TNF) are positive controls for the experiment. Each bar represents mean \pm SEM. Significant changes are shown as *p*-values on top of the bar.

was at 48 h (Fig. 2A). The dose—response of NGF-induced RANTES production was also examined (Fig. 2B). Maximum RANTES production was observed at 100 ng/ml of NGF. Figure 1 shows that there was no increase in production of RANTES by SP, VIP and CGRP stimulation of keratinocytes. NGF-neutralizing antibody significantly reduced RANTES production by NGF-stimulated keratinocytes ($p < 0.001$, Student's *t*-test) (Fig. 3). This confirms that NGF induces RANTES production in keratinocytes. Anti-TNF- α and anti-IFN- γ antibodies did not inhibit the RANTES induction by NGF (Fig. 3).

Effect of NGF on IL-8 production by keratinocytes

Normal keratinocytes produced very little IL-8. Stimulation with TNF- α (20 ng/ml) for 24 h induced IL-8 production ($p < 0.001$, Student's *t*-test) (Fig. 4). It peaked by 48 h stimulation (data not shown). NGF, SP, CGRP, VIP and IFN- γ induced higher levels of IL-8 compared with culture media only. However, the values were not statistically significant (Fig. 4). The level of IL-8 was more variable than the RANTES levels, which may have affected the statistical evaluation. Increasing the NGF concentration to 1,000 ng/ml and prolonging the stimulation for 5 days failed to elicit significant IL-8 production (data not shown).

DISCUSSION

Resting keratinocytes hardly produce any RANTES in *in vitro* culture. It was previously reported that TNF- α and IFN- γ promote the production of RANTES in oral and cutaneous keratinocytes (21). This is the first study to report that NGF increases the expression of RANTES in keratinocytes.

Chemokines are a large superfamily of structurally and functionally related molecules. Among the various subfamilies, CXC (IL-8, GRO- α , IP-10, etc.) and C-C (RANTES, MIP, MCP, etc.) chemokines have been studied most extensively in inflammatory diseases (14, 19, 22–24). These

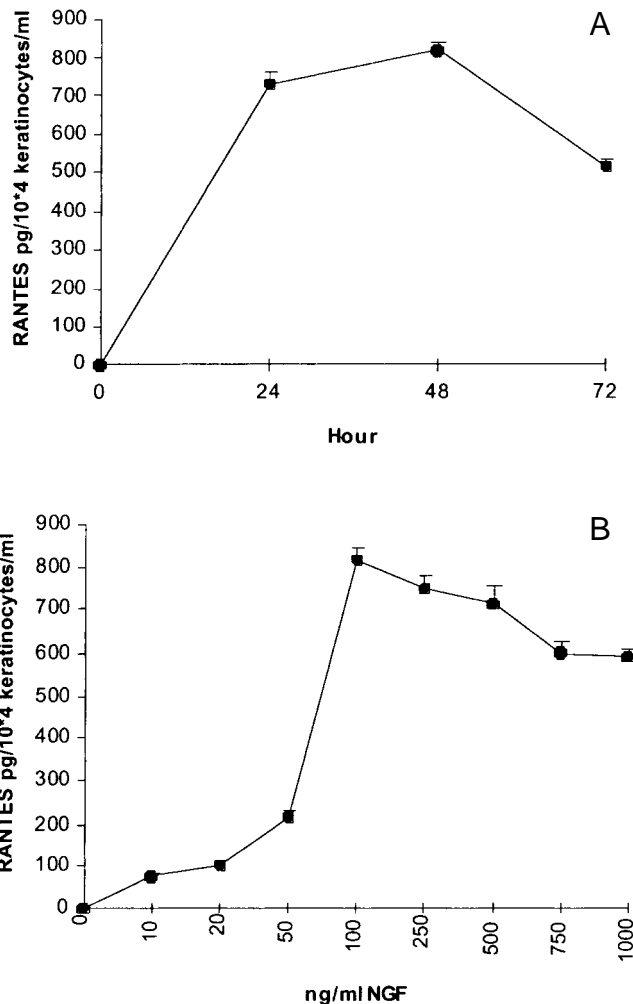


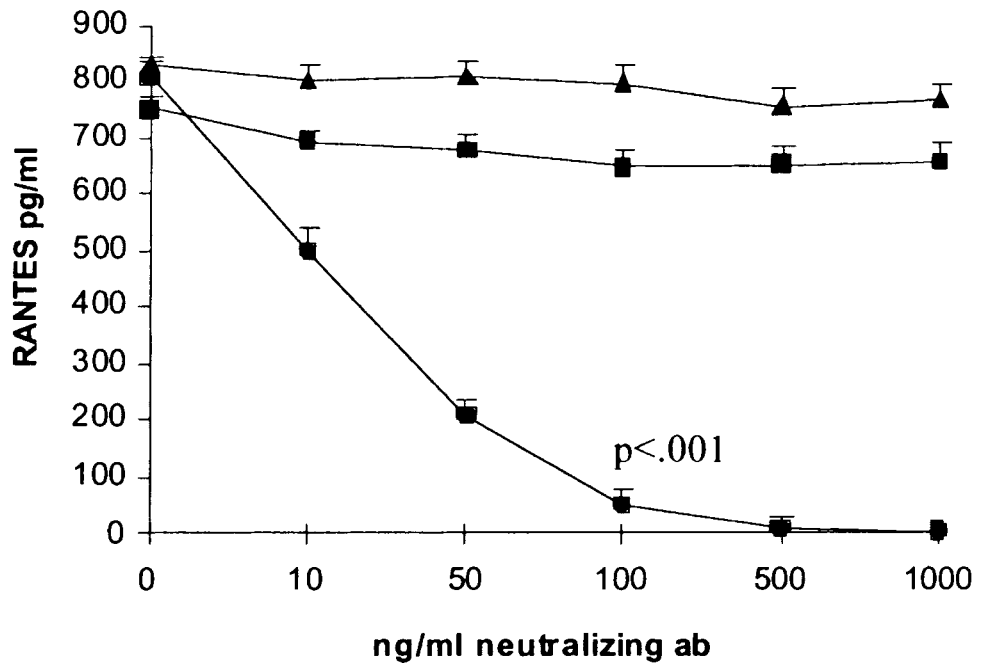
Fig. 2. (A) Kinetics and (B) dose response of NGF-induced RANTES production by keratinocytes. NGF dose in the kinetics study was 100 ng/ml. Time of stimulation is 24 h for the dose—response curve. Results are expressed as mean \pm SEM.

chemokines are produced by several cell populations under the influences of inducing factors. Understanding about the cellular sources and the regulatory factors involved in the expression of different chemokines is essential to elucidate the molecular mechanisms involved in cell trafficking. The 2 chemokines studied in this report, IL-8 and RANTES, are found to be overexpressed in several inflammatory diseases such as psoriasis, eczematous dermatitis and rheumatoid arthritis, along with NGF (6–8, 14–19, 25).

In recent years the contributory roles of neuropeptides (SP, VIP, CGRP) and NGF in an inflammatory process (neurogenic inflammation) have been a major focus of research. In the present study neuropeptides did not have any significant effect on RANTES or IL-8 production, whereas NGF increased the production of RANTES in keratinocytes. It was also observed that NGF might not have a significant effect on IL-8 expression. In contrast to the chemotactic effects of RANTES on T-lymphocytes, the major chemotactic effect of IL-8 is on neutrophils (26, 27).

NGF has been attributed as a key regulatory molecule in the inflammatory cascades of various cutaneous disorders such as psoriasis, wound healing and eczematous dermatitis

Fig. 3. Inhibition of NGF-induced RANTES production by NGF-neutralizing antibody (●). TNF- α (■) and IFN- γ (▲) neutralizing antibodies did not inhibit RANTES production. Results are expressed as mean \pm SEM. Significant inhibition, *p*-value is shown.



(5, 8, 10, 25). The induction of RANTES by NGF in the keratinocytes as observed in this study provides new information regarding the pro-inflammatory functions of NGF. Part of the cutaneous inflammatory reaction cascade could be: NGF induces RANTES in the keratinocytes which, in turn, is chemotactic to resting CD4 memory T-cells and activated naive and memory T-cells (28).

Several functions of NGF are particularly relevant to the inflammatory and proliferative processes of psoriasis. There are reports of increased levels of NGF and NGF-R in psoriatic lesions (5, 8, 26). NGF is mitogenic to keratinocytes (12, 13). NGF recruits mast cells and promotes their degranulation (4, 27). Keratinocyte proliferation and mast cell degranulation are early events in a developing lesion of psoriasis. In addition, NGF activates T-lymphocytes and

recruits inflammatory cellular infiltrates (1–3). Recently, it was reported that keratinocytes in psoriatic plaques express high levels of RANTES (15, 16). The increased levels of RANTES in keratinocytes provide an explanation for the epidermotropism of activated T-cells in psoriatic tissue (29–31). It is possible that higher levels of NGF in a developing psoriatic lesions (8) act in an autocrine pathway on keratinocytes to induce RANTES. This helps to maintain the vicious cycle of proliferative and inflammatory processes typical of psoriasis.

Keratinocytes play a key role in the induction and maintenance of an inflammatory process. Keratinocytes are sources of growth factors, cytokines and chemokines (32–34). The observation in this study that NGF induces increased expression of RANTES in keratinocytes provides further insight into the role of the NGF–NGF-R system in various inflammatory dermatoses.

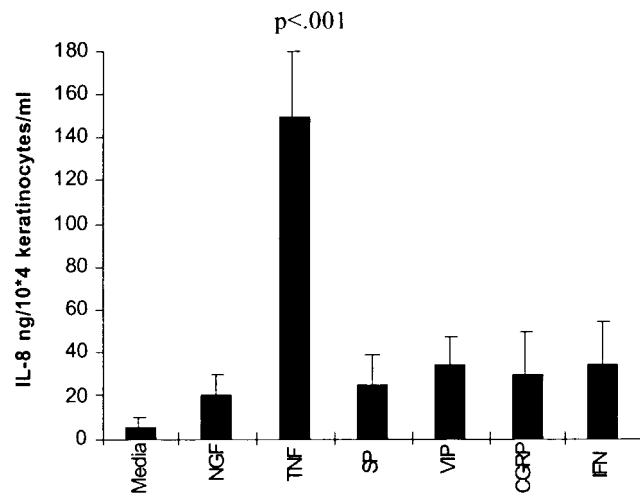


Fig. 4. Effect of NGF, neuropeptides (SP, VIP and CGRP) and IFN- γ (IFN) in IL-8 production by keratinocytes. TNF- α (TNF) is a positive control for the experiment. Time of stimulation is 24 h. Results are expressed as mean \pm SEM. Significant change is shown as a *p*-value on top of the bar.

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