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Effect of PUVA on Nerve Growth Factor Expression in Cultured Keratinocytes

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Nerve growth factor (NGF) is an important autocrine growth factor and also a survival factor for keratinocytes. NGF may act in the hyperproliferative condition, psoriasis. Clinically, the combination of psoralen and UVA (PUVA) has been used in the treatment of a wide variety of cutaneous disorders, such as psoriasis and vitiligo. However, the precise therapeutic mechanism of PUVA on the dermatologic diseases remains unclear. The purpose of this study was to examine whether the expression of NGF in cultured keratinocytes is influenced by PUVA. Thus, normal human keratinocytes were isolated from neonatal foreskin, and the third to fifth-passaged cells were used in this study. The cells were exposed to various doses of UVA (30, 60, 120 mJ/cm^2) after adding 8-methoxypsoralen (8-MOP) to examine the expression of NGF mRNA. The RNA and protein of the cells were extracted at various time points (1, 8, 24 hours) after UVA irradiation to examine the expression of NGF mRNA and production of NGF protein. In keratinocytes, there were no differences in the expression of NGF mRNA between the different doses of UVA irradiation, however, the expression of NGF mRNA in UVA and PUVA groups tended to increase as the time increased. The expression of NGF mRNA was the highest in PUVA group, followed by UVA group and the lowest in 8-MOP group. The expressions of NGF protein at 1 and 8 hours after UVA irradiation were lower in the PUVA group than in the other groups. This study showed that the expression level of NGF protein in keratinocytes was relatively lower in the PUVA groups than in the other groups, suggesting that the therapeutic mechanism of PUVA in psoriasis is related to the decrease of NGF protein.

Key Words: PUVA, NGF, Keratinocyte, Psoriasis

INTRODUCTION

Nerve growth factor (NGF) is a neurotropic polypeptide necessary for the survival and growth of some central neurons, as well as sensory afferent and sympathetic neurons (Levi-Montalcini, 1987). In addition to its actions on the nervous system, it also has a significant biologic effect on cells of the immune-inflammatory compartment (Pearce & Thompson, 1986; Otten et al, 1989; Bischoff & Dahinden, 1992).

Many studies have shown that normal human keratinocytes synthesize and secrete NGF (Di Marco et al, 1991; Di Marco et al, 1993; Pincelli et al, 1994). NGF is able to stimulate gene expression of melanocytes, which is chemotactic for melanocytes, and enhances their dendricity (Yaar et al, 1991). Some studies suggest that NGF is an important autocrine growth factor and survival factor for keratinocytes (Di Marco et al, 1991; Di Marco et al, 1993; Pincelli et al, 1994). As reported in mice (Li et al, 1980) and in other human hyperproliferative condition such as psoriasis (Fantini et al, 1995), NGF probably contributes to the hyperproliferation in wound healing. NGF has also been reported to activate mast cells and T lymphocytes that invade the psoriatic lesion (Pincelli et al, 1994).

Clinically, the combination of psoralen and UVA (PUVA) has been used in the treatment of a wide variety of cutaneous disorders such as psoriasis and vitiligo. However, the precise therapeutic mechanism of PUVA on the dermatologic diseases remains largely unclear.

The purpose of this study was to examine whether the expression of NGF in cultured keratinocytes was influenced by PUVA.

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METHODS

Cell cuture

Normal human keratinocytes were isolated from neonatal foreskin, and were grown in medium 154 (Cascade Biologics Inc., Portland, OR, USA) with human keratinocyte growth supplement (Cascade Biologics Inc.) and 1% penicillin-streptomycin-amphotericin B (10,000 U/ml, 10,000 μg/ml, and 25 μg/ml, respectively; GIBCO BRL., Grand Island, NY, USA) in a humidified atmosphere containing 5% CO_2 at 37°C. Subconfluent primary cultures were passaged in secondary cultures, and the third to fifth-passaged cells were used in this study.

ABBREVIATIONS: NGF, nerve growth factor; PUVA, psoralen and UVA; 8-MOP, 8-methoxypsoralen.

UVA source

The UVA source was a Psoralite (Paul Elder Co., Bryan, OH, USA), which emits UV light between 320 and 400 nm (peak 360 nm). The energy was measured with an IL 1350 research radiometer (International Light Co., Newburyport, MA, USA).

Preparation of 8-MOP

8-MOP (Sigma Chemical Co.) was dissolved in 0.01% DMSO solution at 2×10^{-7} M concentration.

Expression of NGF mRNA according to the UVA doses

Four groups were used for this study. In the DMSO addition group, 0.01% DMSO was added to the medium in order to examine the influence of DMSO in the expression of NGF. The control groups comprised of the 8-MOP addition group and UVA irradiation groups both of which were exposed to different doses of UVA (30, 60, 120 mJ/cm^2), whereas the experimental groups were exposed to UVA (30, 60, 120 mJ/cm^2) after adding 8-MOP (PUVA groups).

Keratinocytes were incubated in 100 mm dishes (Costar Co., Cambridge, MA, USA) with the same density of cells per dish for 24 hours. The media were replaced by PBS in the DMSO addition group and UVA irradiation groups. In the 8-MOP addition group and PUVA groups, the media were replaced by PBS containing 8-MOP (2×10^{-7} M). They were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 30 minutes. While UVA irradiation groups and PUVA groups were exposed to UVA, DMSO addition group and 8-MOP addition group were incubated at room temperature without UVA irradiation. Immediately after irradiation, cells were replaced by the respective original medium. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours, and the RNA was then extracted.

Expression of NGF mRNA and production of NGF protein at various length of time after UVA irradiation

Four groups were used for this study, including the DMSO addition group, 8-MOP addition groups, UVA irradiation groups (120 mJ/cm^2), and PUVA groups (120 mJ/cm^2). The RNA and protein of the cells were extracted at various time points (1, 8, 24 hours) after UVA irradiation.

Extraction of RNA

Total RNA was isolated from cultured cells using RNAzol B kit (Tel-Test Inc., Friendswood, TX, USA). The quality and quantity were spectrophotometrically checked by measuring absorbance at 260 and 280 nm.

RT-PCR

The reactions were performed with a First strand cDNA synthesis kit (MBI Fermentas Inc., Amherst, NY, USA) according to the manufacturer's instructions. One microgram of RNA was mixed with 1 μl (0.5 μg/ml) of oligo (dT) 18 primer and distilled water for a total volume of 11 μl. The mixture was heated at 70°C for 5 minutes, and chilled on ice for 5 minutes. Four microliters of 5x reaction buffer, 1 μl of ribonuclease inhibitor, and 2 μl of 10 mM dNTP were mixed together, and reacted at 37°C for 5 minutes, and 2 μl (20 U/ml) of M-MuLV reverse transcriptase was added to the total volume of 20 μl. The PCR was carried out at 37°C for 1 hour, at 70°C for 10 minutes, and at 4°C for 5 minutes.

For cDNA amplication, 45 μl of PCR master mix containing 1x PCR buffer, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase (MBI Fermentas Inc.), 20 pmol of sense and antisense NGF primers (Table 1, Bioneer, Cheongwon, Chungbuk, Korea) were added to 5 μl of cDNA. The reaction mixtures were subjected to 35 cycles of an amplication program that consisted of denaturation at 94°C for 1 minute (5 minutes for the first cycle), annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes (5 minutes for the last cycle) in the Thermal cycler (Perkin Elmer, USA).

The amplified PCR products (10 μl) were combined with 2 μl of 6x loading buffer and electrophoresed on a 2% agarose gels in 0.5 x TBE buffer at 90 V for 1 hour, and the bands were visualized (NGF; 264 bp, β-actin; 267 bp) by a UV transilluminator.

Southern blotting of PCR product

The agarose gel was denatured in 0.5 N NaOH/1.5 M NaCl and electroblotted onto a nylon membrane (Amersham, Bukinghamshire, UK) using Turboblotter (Schleicher & Schull, Dassel, Germany). The internal oligonucleotide probe (Table 2, Bioneer) was end-labeled with [γ-^{32}P] dATP (>3,000 Ci/mmol) using the end labelling method. The membrane was subjected to prehybridization in the QuikHyb hybridization solution (Stratagene, La Jolla, CA, USA).

QuikHyb hybridization solution was mixed with a radioactively labeled probe, and 100 μg/ml of ssDNA and the membrane was hybridized at 65°C for 1 hour. The filters were absorbed by 3M absorbent paper, and autoradiography was done at −70°C. The intensities of the bands on x-ray film (Konica Co., Tokyo, Japan) were measured by a densitometer (Bio-Rad, Hercules, CA, USA) and were expressed as intensities relative to β-actin.

Table 1. Oligonucleotides used in the RT-PCR

Primers	Sequence (5'→3')	Size of product (bp)
NGF		
Sense	TCATCATCCCATCCCATCTT	264
Antisense	CTTGACAAAGGTGTGAGTCG	
β-actin		
Sense	TACCTCATGAAGATCCTCA	267
Antisense	TTCGTGGATGCCACAGGAC	

Table 2. Sequence of probe specific for PCR product of NGF

Probe	Sequence (5'→3')
NGF	ACTGTTTGAATACACTGTTGTTAATGTTCACCTCTCCCAA
β-actin	AGGCAGCTCGTAGCTCTTCTCCAGGGA

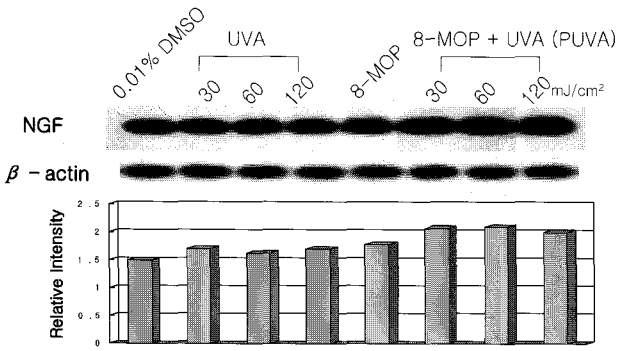

Fig. 1. Southern blotting of PCR product of NGF mRNA in keratinocytes according to the UVA doses. There were no differences in the expression between the different doses of UVA irradiation. The expression of NGF mRNA was higher in the PUVA groups than in the 8-MOP addition group or the UVA irradiation groups. Similar results were obtained in two independent experiments.

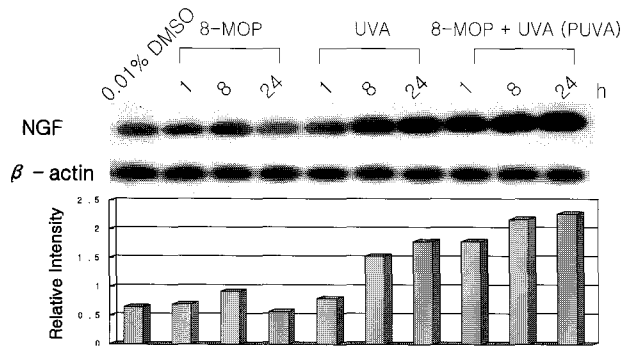

Fig. 2. Southern blotting of PCR product of NGF mRNA in keratinocytes at various length of time after UVA irradiation. The expression had a tendency to increase in the UVA and PUVA groups as time increased. The expression of NGF mRNA was the highest in the PUVA, followed by the UVA and the lowest in the 8-MOP groups. Similar results were obtained in two independent experiments.

Western blotting

Fifty micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and was blotted onto nitrocellulose paper (Amersham). The blots were incubated with antibody to NGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by horseradish peroxidase-conjugated secondary antibody. Bound antibody was detected using an Enhanced Chemiluminescence plus kit (Amersham). The intensities of bands on x-ray film were measured by a densitometer.

RESULTS

Expression of NGF mRNA in keratinocytes according to the UVA doses

There were no differences in the expression of NGF mRNA between different doses of UVA irradiation (Fig. 1). However, the expression of NGF mRNA was higher in the PUVA group than in the 8-MOP addition group or the UVA irradiation group.

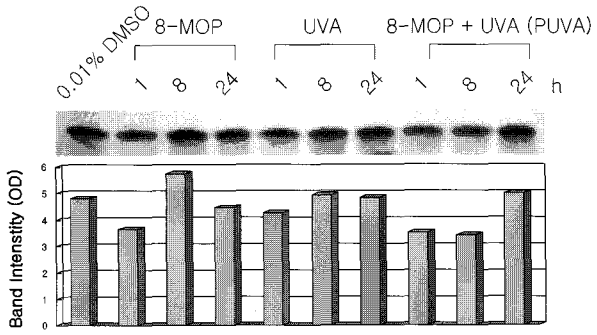

Fig. 3. Expression of NGF protein in keratinocytes at various length of time after UVA irradiation. The expression of NGF protein was lower in the PUVA groups than in the other groups at 1 and 8 hours after UVA irradiation.

Expression of NGF mRNA in keratinocytes at various length of time after UVA irradiation

The expression of NGF mRNA had a tendency to increase in the UVA and PUVA groups as the time progressed (Fig. 2). The expression of NGF mRNA was the highest in the PUVA group, followed by the UVA group and the lowest in the 8-MOP group.

Expression of NGF protein in keratinocytes at various length of time after UVA irradiation

The expressions of NGF protein at 1 and 8 hours after UVA irradiation were lower in the PUVA group than in the other groups (Fig. 3).

DISCUSSION

NGF is essential for the regulation and protection of neural tissue (Levi-Montalcini, 1987). It also has a regulatory function and pro-inflammatory properties for skin as well as immune and endocrine systems (Pearce & Thompson, 1986; Otten et al, 1989; Bischoff & Dahinden, 1992). Non-neural cells expressing NGF mRNA include keratinocytes, fibroblasts, smooth-muscle cells, mast cells, macrophages and some T lymphocytes (Di Marco et al, 1991; Di Marco et al, 1993; Pincelli et al, 1994). Cultured human keratinocytes express NGF mRNA and protein, particularly during the active phase of growth. It is also an autostimulant and mitogen to keratinocytes (Di Marco et al, 1991; Di Marco et al, 1993; Pincelli et al, 1994). NGF contributes to the hyperproliferation in wound healing, as reported in mice (Li et al, 1980), and in other human hyperproliferative conditions such as psoriasis (Fantini et al, 1995).

Psoriatic lesions are characterized by an accelerated turnover of neural elements and by denser innervation (Weddel et al, 1965; Armagni et al, 1979). In particular, an imbalance of the neuropeptide (NP) content is an universal finding in the psoriatic skin (Pincelli et al, 1992). Although the mechanisms leading to biochemical alterations in the peptidergic neurons in psoriasis are not well

understood, there is evidence that NGF could play an important role. In the skin, when NGF content is increased in the epidermis, the expression of neuropeptides is the highest (Lindsay & Harmar, 1989). Therefore, NGF could play a role in neurogenic inflammation through the regulation of NP and in keratinocyte proliferation which are known to be involved in the pathogenesis of psoriasis (Haegerstrand et al, 1989; Lindsay & Harmar, 1989; Donnerer et al, 1992; Pincelli et al, 1992; Donnerer et al, 1993; Pincelli et al, 1993; Pincelli, 1998).

In addition, NGF prevents keratinocyte apoptosis by upregulation of Bcl-xL. It also takes part in the pathogenesis of the psoriatic lesion. Any dysfunction of apoptosis could lead to pathological conditions characterized by epidermal thickening (Goldsmith, 1986; Wrone-Smith et al, 1995). Recently, it has been shown that keratinocytes derived from psoriatic plaques are more resistant to apoptosis than normal skin (Wrone-Smith et al, 1997). NGF protein levels are increased in psoriatic skin as compared to non-lesional and normal skin (Fantini et al, 1995), and psoriatic keratinocytes express higher amounts of NGF than normal keratinocytes (Raychaudhuri et al, 1998). Moreover, NGF activates T lymphocytes and recruits mast cells and lymphocytes, which would initiate the inflammatory process of psoriasis (Aloe & Levi-Montalcini, 1977; Pearce & Thompson, 1986; Thorpe, 1987; Otten et al, 1989; Bischoff & Dahinden, 1992).

Clinically, the combination of PUVA has been used in the treatment of psoriasis. The therapeutic mechanisms of PUVA on psoriasis are based on the fact that PUVA induces photoconjugation of psoralens to DNA and subsequent suppression of mitosis, DNA synthesis, and cell proliferation (Pathak et al, 1974; Pohl & Christophers, 1979). PUVA is able to deplete affected skin of immune-competent cells, notably T cells (Vallat, 1994; Krueger, 1995). However, the precise therapeutic mechanism of PUVA on psoriasis is not yet clear. It is necessary to clarify the relationship between the growth factors, especially NGF, and PUVA. Some authors reported that the expression of NGF mRNA and protein in keratinocytes is modulated by UVB irradiation (Tron et al, 1990; Gillardon et al, 1995; Marconi et al, 1999; Saade et al, 2000). Although UVB initially inhibited the expression of keratinocyte NGF mRNA, an induction of NGF mRNA was seen at 24 hours. This report was consistent with the observation of Gillardon et al (1995) that UVB irradiation increased the levels of NGF immunoreactive material within the epidermis 12 hours later. Moreover, Saade et al (2000) recently showed that exposure to UVB elicited significant upregulation of NGF. To the contrary, however, Marconi et al (1999) showed that UVB dramatically downregulated the synthesis and release of NGF in normal human keratinocytes, and this downregulation of NGF function plays an important role in the mechanism of UVB-induced apoptosis in human keratinocytes. Our results showed an increased expression of NGF mRNA after UVA irradiation or PUVA. The expression of NGF mRNA in keratinocytes had a tendency to increase in UVA and PUVA groups as the time passed. The expression of NGF mRNA was the highest in PUVA, followed by the UVA and the lowest in the 8-MOP groups. However, the expression of NGF protein was lower in the PUVA groups than in the other groups at 1 and 8 hours after UVA irradiation. Even though the expression level of NGF mRNA in the PUVA groups was high, the level of the NGF protein expression was remarkably lower in the PUVA groups than in the other groups. The reason of discrepancy between the levels of expression of the NGF mRNA and protein in the present study is not obvious. Nevertheless, taking all the facts into consideration, we suggest that the therapeutic mechanism of PUVA in the treatment of psoriasis is related to the decrease of NGF protein in keratinocytes.

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