The Development of a Wound Healing Model Using Nerve Growth Factor(NGF) Expression in Raft Culture

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Background: **The wound healing process is impaired or delayed in aged patients. The development of a new wound healing model is needed. Nerve growth factor (NGF) plays a special role in wound healing because NGF is expressed only in proliferating tissues such as wounds.**

Objective: **The aim of our study was to develop a wound healing model using a 3-dimensional culture system, raft culture, by comparing the level of NGF expression according to the wound stage after an artificial wound was made to the raft samples. We tried to specifically localize the site of NGF expression both in mRNA and protein level.**

Methods: **Raft culture using normal human keratinocytes was done and a 2 mm slit wound was made in the center of the raft samples. Raft samples of no wound, 4 d, 7 d, and 9 d after wounding were prepared. In situ RT-PCR and immunohistochemistry were performed to detect and localize NGF expression after making wounds and the addition of substance P (SP).**

Results: **We failed to localize NGF mRNA expression in raft samples by in situ RT-PCR. Immunohistochemistry showed NGF staining throughout the epidermis although a little more dense staining was found in the basal layer. NGF(+) cells tended to increase until 7 d after wounding, but there were no significant differences according to the wounding days. There was a tendency that the SP(+) group showed more NGF(+) cells than the SP(-) group, but there were no statistical differences.**

Conclusion: **We think that our in vitro raft wound model using NGF expression could be used, at least in part, as an objective indicator for wound healing. In our raft model lacking nerve, NGF may not be suitable for representing wound healing process because this model can not reflect the interaction between the skin and the nervous system. Expression of growth factors or cytokines other than NGF need to be applied to our raft culture system.**

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Key Words : Nerve growth factor, Wound healing model, Raft culture

Recently the aged population has increased with a concomitant increase of chronic debilitating diseases such as diabetes mellitus. The wound healing process is impaired or delayed in these patients and this inevitably leads to the increased incidence of chronic ulcers. So a new therapeutic modality or the development of a new wound healing model is needed.

Wound healing involves many intermingled, interdependent, and orderly processes such as inflammatory response, epithelization, fibroplasia, and collagen remodeling or wound contraction. Many growth factors released from inflammatory cells or keratinocytes have been known to play important roles in wound healing[1-3].

Nerve growth factor (NGF) was originally reported to play a crucial role in the development and maintenance of sensory and sympathetic neu-

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rons[4,5]. However, NGF is released in increasing levels by growing colonies and in lower quantities by confluent keratinocytes. Also it appears that only keratinocytes from the basal, growing layer of the epidermis are able to make NGF in substantial quantities, suggesting a special role of NGF during wound healing[6].

When any tissue innervated by the sensory nervous system is injured, an immediate orthodromic signal is elicited by the sensory nerves from the tissue to dorsal root ganglia and to the central nervous system, allowing the host to quickly sense and respond to the injury. In addition to this orthodromic response, the sensory nerves are capable of a second impulse in the reverse direction, so called antidromic response. With this antidromic response, neuropeptides such as substance P (SP) are released into the injured tissue[7]. In this way so called neurogenic inflammation occurs and helps wound healing.

A recent report suggested that there is bi-directional cross-talk between NGF-producing cells in the skin and SP secreting sensory fibers[8]. NGF can regulate the expression of neuropeptides in sensory neurons and it was found that SP was capable of augmenting NGF mRNA in murine keratinocytes. In this context the interaction of skin and nervous system is very important in wound healing processes. Growing keratinocytes by secreting increasing amounts of NGF might regulate skin reinnervation during wound healing[9].

Various authors[2,10,11] reported in vivo animal wound model, but there were many problems with the animal model because many factors such as animal handling and wounding methods could result in bias and objective measurement of certain growth factors might be very difficult. Our study was done to develop in vitro a wound healing model using 3-dimensional culture system, raft culture, by comparing the expression of NGF according to the wound stage after an artificial wound was made to the raft samples. We tried to specifically localize the site of NGF expression both in mRNA and protein level.

MATERIALS AND METHODS

1. mouse keratinocyte cell line, PAM212

We first performed the experiment with PAM212 to know the optimal concentration of SP to be used in normal human keratinocyte because it is easy and quick to culture PAM212 cells.

1) culture of PAM212

PAM212 was cultured in DMEM supplemented with 10% fetal calf serum and 5% PSF (penicillin, streptomycin, and fungizone)[12]. 150 mm culture dish was used to secure large number of cells for detecting NGF mRNA signal. When the confluency reached about 80%, neutral endopeptidase (NEP) inhibitor, thiorphan (Sigma), was added at 10 M concentration to keep SP from being degraded by endogenous NEP. After 30 min, SP (10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) (Peninsula Lab, California) was added to the culture and the culture was maintained for 2 h. PMA (50 ng/ml) was used as a nonspecific stimulant for a positive control.

2) Northern blot for murine NGF after adding SP

mRNA was extracted using poly-A RNA extraction kit (Boehringer Mannheim, Germany). About 5 μg of mRNA was extracted from at least 2 $\times 10^8$ cells. The ß-NGF murine cDNA probe was prepared from plasmid SP64. 3 μg of mRNA was electrophoresed in 1% agarose and 6.6% formaldehyde gel, and transferred to Hybond-N nylon membrane (Amersham, UK).

Blots were prehybridized for 30 min at 65℃ in a roller bottle containing 5 ml of Rapid-hyb buffer (Amersham, UK). The probe was labelled with 32[P]dCTP using Rediprime DNA labelling system (Amersham, UK) and purified using Quick SpinTM (TE) Columns (Boehringer-Mannheim, Germany). Specific activities of 1-3 $\times 10^8$ dpm/μg were achieved. Denatured labelled probe was added directly to the Rapid-hyb buffer and blots were hybridized for 2 h at 65℃ . Washing was done in 2 × SSC/0.1% SDS at room temperature for 20 min, 0.1 × SSC/0.1% SDS at room temperature for 15 min, and 0.1 × SSC/0.1% SDS at 65℃ for 15 min. All films were autographed overnight on RX film (Fuji Medical Systems USA Inc) with intensifying screens at -70℃.

2. normal human keratinocyte

1) monolayer culture and RT-PCR

Normal human foreskin was obtained from circumcisions. Skin keratinocytes were isolated as previously described[13], and cultured in keratinocyte growth medium (KGM, Clonetics, San

Diego, CA) composed of MCDB-153 medium supplemented with epidermal growth factor (10 mg/ml), bovine pituitary extract (70 μg/ml), hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), penicillin (100μg/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml). In these experiments, second-passage cells were used. When the confluency reached about 80%, total RNA was extracted using RNA extraction kit (Promega).

RT was performed using RT kit (Promega) as directed in the kit. Synthesized cDNA was amplified by PCR using human NGF primers[14] (forward primer: 5'-TCATCATCCCATCCCATCTT-3' and reverse primer: 5'-CTTGACAAAGGTGTGAGTCG-3'). Expected size of the PCR fragment is 264 bp. The conditions for PCR were as follows; initial denaturation at 94℃ for 2 min followed by 40 cycles of denaturation at 94℃ for 1 min, annealing at 50℃ for 1 min, and extension at 72℃ for 1 min. PCR product was loaded on to a 1% agarose gel in 1 x TBE buffer and stained with ethidium bromide. RT-PCR was done to confirm the NGF signal in normal human keratinocytes instead of doing Northern blot.

2) raft culture and wounding

Reconstruction of RE-DED(reconstructed epidermis on de-epidermized dermis) RE-DED was reconstructed by culturing keratinocytes on de-epidermized dermis (DED). DED was prepared as described previously[15]. Briefly, strips of skin obtained from plastic surgery were maintained in PBS at 37℃ and the epidermis was removed after 5 to 7 days. The tissue was killed by 10 successive freezings and thawings. The pieces of dead DED were kept at -20 ℃ About 10mm diameter of DED was plated into a 12mm polycarbonate filter chamber (3.0 μm Millicell-pc; Millipore Co., Bedford, MA, USA) in six well culture dishes. Skin keratinocytes were seeded at a density of 5×10^5 cells/Millicell on the top of this DED and cultured in submerged state for 2 days and then at the air-liquid interface for 10 days. The cultures were maintained with growth medium consisting of a 3:1 mixture of DMEM and Ham's nutrient mixture F12 medium containing 10% FCS(fetal calf serum), 5 μg/ml insulin, 1×10^{-10} M cholera toxin, 0.4 μg/ml hydrocortisone, 5 μg/ml transferrin, and 1×10^{-11} M triiodothyronine.

Wounding was made in the center of the raft sample with a 2 mm slit using No. 15 blade. No wound sample was used as (-) control. 4 d, 7 d, and 9 d after wounding was made, raft samples were fixed in 10% buffered formalin for 6 h and were made into paraffin blocks. Thiorphan (10 μM) and SP (10^{-6} M) were added to the other 4 samples and total 8 samples were made.

3) In situ RT-PCR

In situ RT-PCR was done with slight modification as previously described[16]. Fixed paraffin blocks were cut into 4 m thick pieces. Each section was placed on silanized glass slides (Perkin Elmer) and deparaffinized. After digesting with trypsin (2 mg/ml) for 5-10 min, RNase-free DNase solution in buffer (50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$) was treated overnight at 37℃. Next day in situ RT-PCR reaction was done using above human NGF primers. RT reaction was carried out using RT kit from Promega as directed. cDNA was amplified on the slide itself using Perkin Elmer GeneAmp In Situ PCR System 1000. Basically in situ PCR protocol is similar to tube PCR except that higher concentration of MgCl2 (4.5 mM) and higher amount of Tag enzyme (5U/50 μl reaction) were used. 1 mM dig dUTP was mixed to the PCR reaction and dig-labeled nucleic acid was detected with anti-dig-AP conjugate antibody as directed in the Dig Nucleic Acid Detection Kit (Boehringer Mannheim, Germany).

4) Immunohistochemistry

Commercially available rabbit polyclonal NGF antibody (Santa Cruz, CA) was used as a primary antibody (1:100 dilution). Formalin-fixed raft samples were sectioned at 4 μm, deparaffinized, and rehydrated through a graded series of alcohol. After quenching endogenous peroxidase activity with 3% hydrogen peroxide for 5 min, sections were blocked with blocking reagent and incubated with primary antibody for 1-2 h at room temperature or overnight at 4℃. Following two buffer washes, the secondary biotinylated goat anti-rabbit antibody linked with peroxidase-labelled streptavidin (DAKO LSAB kit, CA) was applied for 30 min at room temperature. The reaction product was visualized using diaminobenzidine. Finally, the sections were counterstained with Meyer's hematoxylin.

NGF positive cells were counted in 5 randomly se-

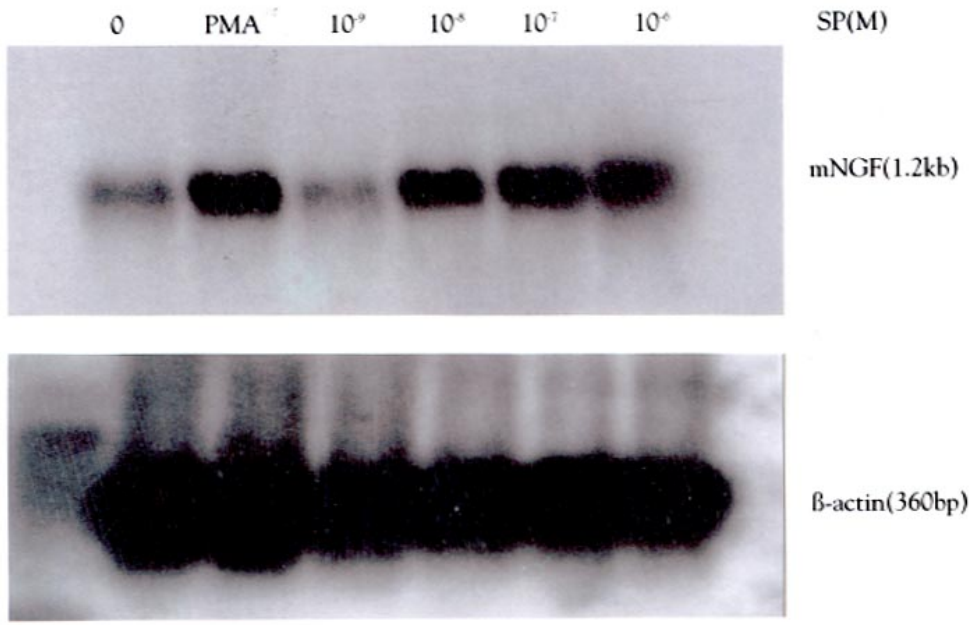

Fig. 1. Expression of murine NGF (mNGF) in PAM212 cells detected by Northern blot. mNGF signal increase was quite dependent on SP concentration.

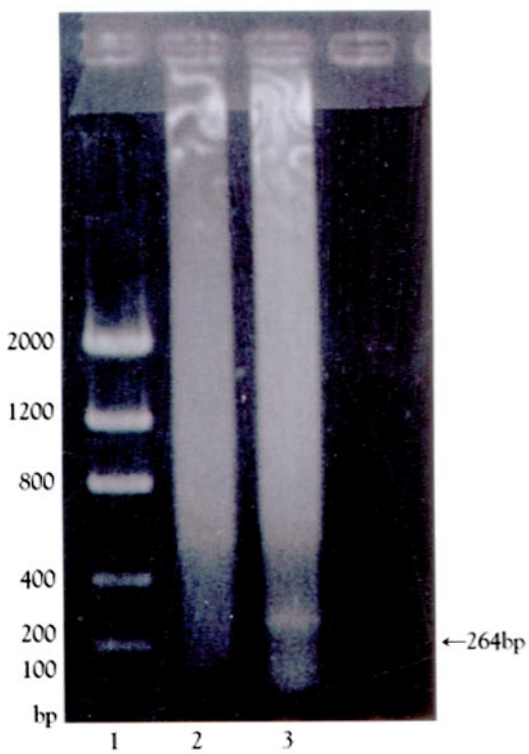

Fig. 2. 264bp RT-PCR product was detected on 1% agarose gel after 40 cycles of PCR. 1:marker(low DNA mass ladder), 2:(-) control, 3:human NGF

lected fields under × 100 magnification and analyzed by Image analyzer using soft ware BM1 plus version 1.20. Statistical analysis was done using t-test.

RESULTS

1. Determination of optimal concentration of SP in PAM212 cell

NGF expression increased quite in proportion to the concentration of SP (Fig. 1). 10-6 M concentration of SP was taken as optimal and was used in the experiment with normal human keratinocytes. Quite a large amount of mRNA (about 3 μg) was necessary to detect NGF signal by Northern blot.

2. Confirmation of NGF mRNA expression in normal human keratinocytes by RT-PCR

The expression level of NGF is usually very low and it is very difficult to detect NGF signal from a small amount of RNA by Northern blot. Rather we performed RT-PCR just to confirm the expression of NGF mRNA before going further to the wounding experiment with raft culture. 264 bp RT-PCR product was detected after 40 cycles of PCR quite long compared to the usual PCR cycles (Fig. 2).

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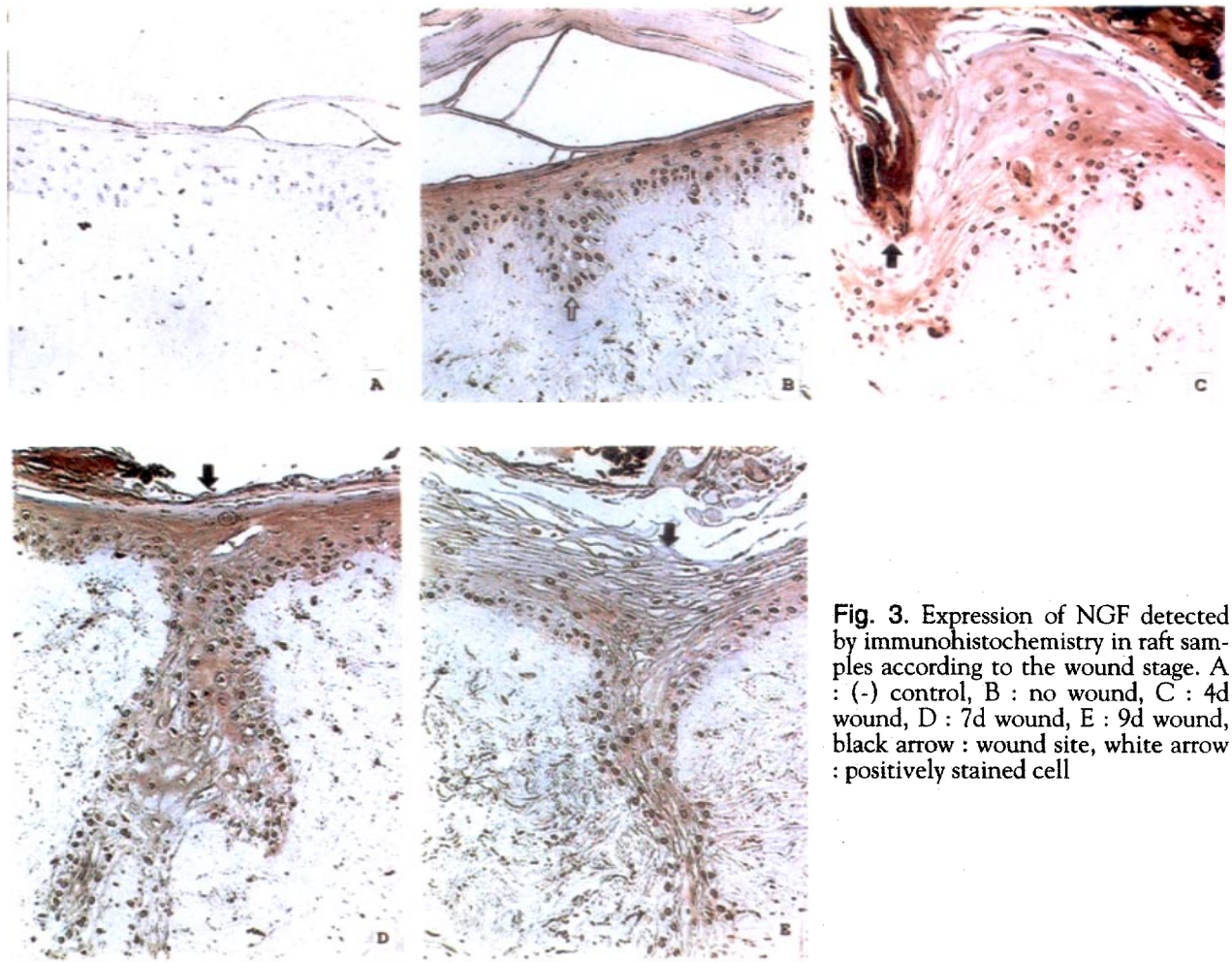

Fig. 3. Expression of NGF detected by immunohistochemistry in raft samples according to the wound stage. A : (-) control, B : no wound, C : 4d wound, D : 7d wound, E : 9d wound, black arrow : wound site, white arrow : positively stained cell

3. NGF expression in raft culture

We failed to localize NGF mRNA expression in raft samples by in situ RT-PCR because the majority of tissue sections on the slides came off and only a portion remained. In in situ RT-PCR the positive signal is detected in bluish purple nuclear staining. We found that in situ RT-PCR was working because RT (+) sample showed purplish color change on the remaining tissue while RT (-) sample showed no color change.

By immunohistochemistry NGF staining was detected throughout the epidermis although a little more dense staining was found in the basal layer. Epidermal cells proliferated a little around the slit wound and the wound quite recovered 9 d after wounding (Fig 3). NGF(+) cells were counted using Image Analyzer in 5 randomly selected fields (Table 1, Fig. 4) and the mean ± SD of counted cells were compared statistically. NGF(+) cells tended to increase until 7 d after wounding, but there were no significant differences according to the wounding days. In terms of SP effect there was a tendency that SP(+) group showed more NGF(+) cells than SP(-) group, but there were no statistical differences (Fig. 4).

DISCUSSION

Various growth factors or cytokines released from inflammatory cells or epidermal cells in and around the injury site have been reported to be involved in wound healing. It is debatable that which one of these is the most important factor to accelerate wound healing. These factors may interact together to help the wound healing process[1-3].

NGF was originally reported to be a crucial polypeptide in the survival and maintenance of cutaneous sensory nerves. In addition to this ac-

Table 1. Number of NGF(+) cells in each raft sample

	NGF (+) cells[a]
no wound	89.60±14.54
no wound + SP	101.20±12.56
4 d wound	94.60±10.24
4 d wound + SP	113.00±58.43
7 d wound	106.80±16.18
7 d wound + SP	115.00±31.26
9 d wound	85.60±35.12
9 d wound + SP	91.00±38.99

[a]The numbers are expressed as mean ± SD. NGF(+) cells tended to increase until 7 d after wounding. SP(+) group showed more NGF(+) cells than SP(-) group. But there were no statistically significant differences according to the wound stage or SP.

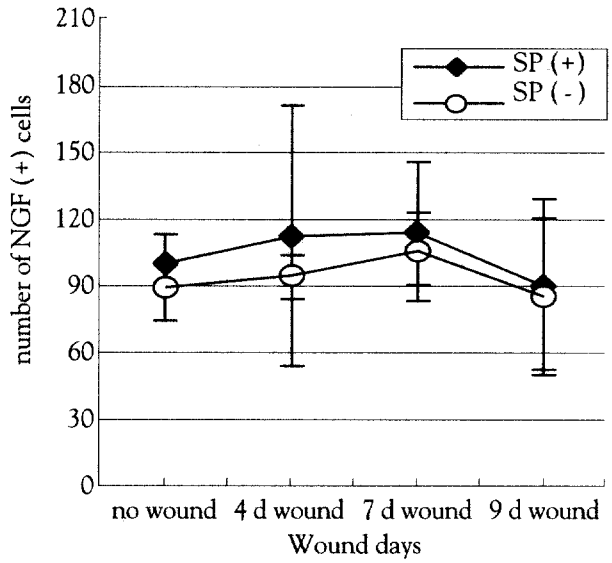

Fig. 4. NGF(+) cells increased until 7 d after wounding, but there were no significant differences according to the wounding days. In terms of SP effect SP(+) group showed more NGF(+) cells than SP(-) group, but there were no statistical differences.

tivity, NGF might play a special role in wound healing because NGF is expressed only in proliferating tissues such as the wound. Liu10 designed a wound chamber from a dissected nerve in a rat and said that NGF level from the wound chamber fluid increased. Thus several authors tried to see NGF as an objective indicator reflecting the wound healing process[17-19].

Keratinocytes are the main cellular component of skin and dense arborization of newly formed nerve fibers is observed just beneath or adjacent to the injured epidermis[6,20]. So the main source of NGF is presumed to be the keratinocytes. In fact it was reported that keratinocytes are the main source of NGF in the skin[21,22] and the other cells including fibroblasts and lymphocytes release NGF[8,23,24].

As stated in the introduction, skin actively participates in wound healing through the interaction with the nervous system, not as a passive sensory organ. SP is a neuropeptide belonging to the tachykinin family. SP is released from sensory C-fibers of the cutaneous nervous system after injury. There is growing evidence that the release of SP is capable of mediating a wide range of proliferative and inflammatory activities termed neuroinflammation[25-27]. By this way SP appears to play an important role in wound healing. More importantly, NGF must be released locally for the survival and maintenance of sensory nerve fibers.

The problem with detecting NGF signal especially in vivo is that NGF expression in a normal situation is usually too low to be detected by conventional methods including Northern blot. No one has ever succeeded in detecting NGF signal in in vivo. Recently increased signal of murine NGF after acetone irritation to the mouse skin was reported by Northern blot. But in this report the entire skin of the mouse trunk was used to extract a large amount of RNA and this is not suitable for in vivo wound model[28].

The main purpose of our study was to detect and localize the changing pattern of NGF signal in raft culture after making a wound. Before doing the raft experiment we needed to reconfirm simply by RT-PCR whether human keratinocytes express NGF. We did not perform Southern blot to see if our PCR band was real NGF signal. 264 bp product was clearly visualized and this band was considered to be NGF signal.

In our study the same concentration of SP (10^{-6}M), the optimal concentration of which was determined in PAM212 cells, was added to each raft sample to be able to detect increasing NGF message compared to SP(-) controls. The expression of NGF mRNA is normally very low and the detection of NGF mRNA signal in tissue by in situ hybridization is very difficult. Recently Staecker et

al[16] reported that they detected NGF mRNA in tissue by in situ RT-PCR method. Actually we also succeeded in localizing murine NGF mRNA signal in vivo by the same method (data not shown). Basically in situ RT-PCR is also a PCR and exact comparison of the expression level was quite difficult although increased expression of murine NGF mRNA could be detected around the proliferating or thickened epidermis. Based on this experience, we performed in situ RT-PCR to localize NGF signal in raft samples, but failed to detect it. Our problem with the raft culture with human keratinocyte in vitro was that the sections on the silanized slides easily came off when doing 40 cycles of in situ PCR. We tried in situ RT-PCR many times with different conditions as we changed the digestion time with trypsin, fixation time with buffered formalin or PCR cycles.

We could localize NGF protein in the raft samples quite well by immunostaining. NGF signal was detected almost exclusively in the epidermis. It appeared that NGF was expressed especially in the basal layer of the epidermis and NGF expression increased around the proliferating epidermis where the slit wound was made. NGF(+) cells tended to increase until 7 d after wounding, but there were no statistically significant differences according to the wounding days. Anyway we could observe wound healing process in the epidermis of the raft culture and the recovering epidermis was observed 9 d after wounding. We added SP because the interaction between the skin and the nervous system is very important and SP released from the sensory nerve terminal was reported to upregulate NGF expression. However, in our experiment we could not find the augmenting effect of SP on the expression of NGF. There was a tendency that SP(+) group showed more NGF(+) cells than SP(-) group, but there were no statistical differences.

The problems with our in vitro raft wound model were as follows; 1) it was quite difficult to make the same and consistent slit wounds to the raft samples, and the wounding method could cause biases to the results, 2) our in vitro model lacks the nervous system and the wounding injury may not be sufficient to detect the changing pattern of NGF expression, and this factor might explain why we could not detect significant upregulation of NGF after adding SP

In conclusion we think that our in vitro raft wound model using NGF expression could be used, at least in part, as an objective indicator for wound healing. A more sophisticated method for making consistent wounds should be developed. In our raft model lacking nerve NGF may not be suitable as an objective indicator for the wound healing process because this model can not reflect the interaction between the skin and the nervous system. Expression of growth factors or cytokines other than NGF, normal expression of which is quite abundant, need to be applied to our raft culture system.

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