

DHMEQ, a novel NF-kappaB inhibitor, suppresses growth and type I collagen accumulation in keloid fibroblasts

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Running Title: Effect of DHMEQ on keloid fibroblasts

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Summary

Background: Keloid is a benign dermal tumor characterized by proliferation of dermal fibroblasts and overproduction of extracellular matrix (ECM). Nuclear factor kappa B (NF- κ B) plays an important role in regulation of inflammation, immune response and cell proliferation. Activation of the NF- κ B pathway is thought to be closely linked to abnormal cell proliferation and ECM production in keloid fibroblasts.

Objective: This study was set out to investigate the effects of a novel selective NF- κ B inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), on keloid fibroblasts.

Methods: Primary normal and keloid dermal fibroblasts were used for this study. NF- κ B activity was assessed by DNA-binding assay and immunohistochemistry. The effect of DHMEQ was evaluated by cell viability, cell growth and type I collagen accumulation.

Results: Basal NF- κ B activity was constitutively elevated in keloid fibroblasts, indicating that this pathway is involved in keloid pathogenesis. DHMEQ markedly reduced cell proliferation and type I collagen accumulation in keloid fibroblasts.

Conclusion: The inhibition of NF- κ B by DHMEQ may be an attractive therapeutic

approach for keloids.

1. Introduction

Keloids are fibrous overgrowths due to abnormal wound healing process after skin injury. They are characterized by proliferation of dermal fibroblasts and overproduction of extracellular matrix (ECM). Although keloid is a benign dermal tumor, its management is one of the most challenging clinical problems. Keloids do not regress with time and surgical excision alone results in a high rate of recurrence. Various conservative therapies have been attempted, but definite and effective treatment has not yet been established [1,2].

The ubiquitous nuclear factor kappa B (NF- κ B) transcription factor regulates expression of a wide spectrum of genes involved in immune and inflammatory response, cellular proliferation and apoptosis [3-6]. Activation of the NF- κ B pathway is implicated in a diverse range of diseases including asthma, rheumatoid arthritis, inflammatory bowel disease and human cancer. NF- κ B-targeted therapy has been attempted in these diseases and appears to be an effective and useful modality [7]. Recently, it has been reported that the NF- κ B pathway is also activated in keloid fibroblasts [8].

The NF- κ B family is comprised of NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), p65 (RelA), RelB and c-Rel. Although many dimeric forms of NF- κ B have been identified, the most prevalent activated form of NF- κ B is the heterodimer consisting of DNA binding subunit p50 and transactivation subunit p65. In unstimulated cells, NF- κ B is sequestered in the cytoplasm in an inactive form by its inhibitor proteins called inhibitors of κ B (I κ Bs). A variety of stimuli including pro-inflammatory cytokines, mitogens, viral infection, ultraviolet radiation and free radicals activate a large complex termed I κ B kinase (IKK), which phosphorylates I κ B protein, resulting in its ubiquitination and proteosomal degradation. Then, freed NF- κ B translocates to the nucleus and activates transcription of a vast number of target genes. Some of the proinflammatory cytokines that might have a role in keloid pathogenesis such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF- α) are stimulated by NF- κ B. These cytokines can directly activate the NF- κ B pathway, thus establishing a positive autoregulatory loop. Hence, the blockade of the NF- κ B pathway could be an attractive strategy for the treatment of keloids.

A novel NF- κ B inhibitor dehydroxymethylepoxyquinomicin (DHMEQ), a

derivative of the antibiotic epoxyquinomicin C [9], has been found to specifically inhibit TNF- α -induced nuclear translocation of NF- κ B [10]. Treatment with DHMEQ showed an anti-inflammatory effect on rheumatoid arthritis and renal inflammation in animals. DHMEQ also demonstrated potent anti-cancer activity in various *in vitro* and *in vivo* models such as prostate carcinoma, breast carcinoma, thyroid carcinoma and hematologic malignancies without any apparent toxic side effects [11,12].

In the present study, we report that DHMEQ is effective in blocking growth and type I collagen accumulation in keloid fibroblasts, supporting the concept that inhibitors of the NF- κ B pathway may be useful therapeutic agents for keloids.

2. Materials and methods

2. 1. Reagents

Stock solutions of racemic DHMEQ (20 mg/ml) were prepared in dimethylsulfoxide (DMSO) and stored at -20°C until use. Antibodies were obtained from the following sources: anti-p65 polyclonal and β -actin monoclonal from Santa Cruz Biotechnology

(Santa Cruz, CA, USA); anti-collagen type I polyclonal from Rockland
Immunochemicals (Gilbertsville, PA, USA); anti-Hsp47 monoclonal from Stressgen
Assay Designs (Ann Arbor, MI, USA); anti-Poly ADP ribose polymerase (PARP)
polyclonal and secondary horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse
IgG from Cell Signaling Technology (Beverly, MA, USA).

2. 2. Cell culture

Keloid tissue samples were obtained from four different Japanese patients at the time of surgery. Four normal skin tissue samples were obtained from four different Japanese volunteers. No comorbid systemic disorders such as diabetes and cancers, nor taking drugs known to influence the transcriptional response of cells, were identified. All experiments were performed after obtaining hospital ethical committee approval. Informed consent was obtained from each individual. The profile of each sample is summarized in Table. 1. All keloid tissue specimens were pathologically examined to confirm the diagnosis.

Primary cultures of dermal fibroblasts were established as previously described [13].

Explants were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% (w/v) penicillin/streptomycin in 5% CO₂ humidified atmosphere at 37°C. Fibroblasts at passages of 3 to 8 were used in this study. We did not observe any alteration in cell shape, growth rate and sensitivity to DHMEQ until passage 8.

2. 3. DNA-binding assay

Nuclear extracts were prepared as described previously [14]. The multiwell colorimetric assay for phosphorylated p65 protein was performed using the Trans-AM NF-κB p65 Transcription Factor Assay Kit (Active Motif North America, Carlsbad, CA, USA). Briefly, equal amount of nuclear extracts were incubated in 96-well plates coated with immobilized oligonucleotide containing a NF-κB consensus binding site. NF-κB binding to target oligonucleotides was detected by incubation with primary antibodies against the p65 subunit and HRP-conjugated secondary antibody. For quantification of activity, optical densities were measured at 450 nm with a microplate reader ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan).

2. 4. Immunohistochemistry

Immunohistochemical detection of NF- κ B p65 subunit was done using immunoperoxidase technique. Briefly, tissue sections from paraffin-embedded tissue were deparaffinized in xylene and dehydrated in alcohol. Slides were then subjected to antigen retrieval by four times of 5-min microwave heating in 10 mM sodium citrate (pH 6.0) and cooled at room temperature. Endogenous peroxidase was blocked by incubation for 10 minutes with 3% hydrogen peroxidase in deionized water. Nonspecific binding was blocked with 1% BSA for 10 minutes. Sections were then incubated overnight at 4°C with anti-p65 polyclonal antibody. Biotinylated secondary antibody was applied, and sections were incubated with avidin-biotin peroxidase complex, developed with 3, 3'-diaminobenzidine substrate and counterstained with hematoxylin. Positive cells were counted in six high power fields (magnification x400) by two independent investigators in a blinded fashion.

2. 5. Cell viability assay

Cultures were established in 96-well flat-bottomed microtiter plates (Nalge Nunc International, Tokyo, Japan). Cells were counted, plated at 5×10^3 cells/well (100 μ L) and incubated for 24 hours before treatment. Solutions containing DHMEQ were added to each well in 10 μ L of medium at various concentrations, with 6 wells used for each concentration. In control wells, solution of DMSO was added. Cells were incubated at 37°C for 24 hours, and then water-soluble tetrazolium salt (WST)-based assay was done as follows: medium was changed (100 μ L/well), and 10 μ L of Cell Counting Kit-8 solution (Dojindo, Kumamoto, Japan) were added to each well and incubated for 2 hours at 37°C. Optical densities were measured at 450 nm in a microplate reader ImmunoMini NJ-2300.

2. 6. Cell proliferation assay

Cell suspensions (100 μ L, 1×10^3 cells/well) were added to each well of 96-well flat-bottomed microtiter plates and incubated for 24 hours before treatment. Solutions of DHMEQ were added to each wells at the concentration of 5 μ g/ml or 10 μ g/ml. After 24, 48, 96 hours exposure to the drug, WST-based assay was done as described above.

2. 7. Western blotting

Cells were washed twice with ice-cold PBS, collected in 1 ml PBS and centrifuged for 3 minutes at 3,000 rpm. For total cell extracts, each pellet was resuspended in 200 μ L of a buffer containing 20 mM HEPES (pH 7.5), 0.35 M NaCl, 20% glycerol, 1% NP40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). After incubated for 15 minutes on ice, lysates were centrifuged for 15 minutes at 15,000 rpm at 4°C, and the supernatants were collected and stored at -80°C. To obtain the secreted ECM proteins, culture media was collected and concentrated using a Speed Vac CVE 200D (EYELA, Tokyo, Japan). Protein concentrations were determined with a Bichinonic Acid Assay Kit (Sigma, St Louis, MO, USA). Equal amount of proteins were resolved by SDS- PAGE and transferred onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA) by semidry blotting. After incubation with appropriate primary antibody, the antigen-antibody complexes were visualized using HRP-conjugated secondary antibody and enhanced chemiluminescence reagents (Amersham, Piscataway, NJ, USA). For

quantitation of band intensities, image analysis was done with Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD, USA).

2. 8. Statistical analysis

All data were expressed as the mean \pm SD. Differences between groups were examined for statistical significance with the Mann-Whitney U test and/or one-way ANOVA followed by Fisher's PLSD where appropriate. A p value not exceeding 0.05 was considered statistically significant.

3. Results

3. 1. NF- κ B is activated in keloid fibroblasts

We first examined basal DNA-binding activity of NF- κ B in normal and keloid fibroblasts. For this purpose, primary fibroblasts derived from four different patients and healthy volunteers were established. Consistent with the previous report [8], there was elevated basal level of NF- κ B activity in keloid fibroblasts as compared to normal

fibroblasts (Fig. 1).

To further confirm activation of the NF- κ B pathway in keloids, we performed immunohistochemistry using keloid tissue specimens. As shown in Fig. 2, intense immunoreactivity for p65 in both cytoplasm and nucleus was observed in keloid tissue (Fig. 2B, D). By contrast, normal skin tissue specimen showed very weak immunoreactivity (Fig. 2A, C). The number of positive cells in keloid tissue was significantly higher than that in normal tissue (Fig. 2E). Note that epidermis of the keloid tissue section also showed diffuse and intense immunostaining for p65, suggesting that NF- κ B is also activated in keloid keratinocytes. Thus, activation of NF- κ B in keloids were confirmed both *in vitro* and *in vivo*.

3. 2. DHMEQ suppresses NF- κ B in keloid fibroblasts

To examine the pharmacological effect of DHMEQ on primary normal and keloid fibroblasts, DNA-binding assay was performed. The addition of DHMEQ was found to suppress basal DNA binding of the p65 protein in both normal and keloid fibroblasts; however, keloid fibroblasts were more sensitive to DHMEQ (Fig. 3). Although the

mean basal NF- κ B activity was approximately 1.7-fold higher in keloid fibroblasts compared to normal fibroblasts ($p < 0.05$), it decreased to the same level as in normal fibroblasts after treated with 5 $\mu\text{g/ml}$ of DHMEQ. This result suggests that DHMEQ successfully suppressed NF- κ B activity in keloid fibroblasts.

3. 3. Cytotoxic effect of DHMEQ

To determine the cytotoxic effect of DHMEQ, normal and keloid fibroblasts were treated with various concentrations of DHMEQ, and cell viability was estimated by WST assay. The response varied between the cells obtained from different individuals, but viability of keloid fibroblasts was decreased stronger than that of normal fibroblasts at same concentration of DHMEQ (Fig. 4). Average ED_{50} was calculated to be 27.3 ± 4.0 $\mu\text{g/ml}$ for normal fibroblasts and 20.1 ± 2.9 $\mu\text{g/ml}$ for keloid fibroblasts ($p < 0.05$). To explore whether the cytotoxic effect of DHMEQ was due to apoptosis, we performed Western blotting for PARP. Cleavage of PARP along with DNA laddering and membrane inversion are known to be the most characteristic events in apoptosis. As shown in Fig. 4B, apparent PARP cleavage was detected from the concentration of 25

$\mu\text{g/ml}$ in both normal and keloid fibroblasts. DHMEQ did not induce any cytotoxic effect at lower than $15 \mu\text{g/ml}$ in normal fibroblasts. Thus, we used DHMEQ at the concentration of 5 and $10 \mu\text{g/ml}$ for further experiments.

3. 4. Effect of DHMEQ on cell growth

We next examined the effect of DHMEQ on cell proliferation. Cells were treated with DHMEQ at the concentration of 5 or $10 \mu\text{g/ml}$ and cultured for up to 4 days. Although the response to DHMEQ varied between the cells, most of normal fibroblasts were affected only slightly (Fig. 5A), and the DHMEQ treatment had no significant influence on average cell proliferation of normal fibroblasts (Fig. 5B). By contrast, the growth of keloid fibroblasts was significantly inhibited compared to the normal fibroblasts (Fig. 5A, B) in a dose dependent manner. It has been reported that DHMEQ causes only a transient NF- κB inhibition [15], but DHMEQ inhibited the growth of keloids during the observed period.

3. 5. Effect of DHMEQ on type I collagen accumulation

To study the effect of DHMEQ on ECM, we performed Western blotting to assess both secreted and cellular type I collagen. The culture media or whole cell lysates were used for this purpose. After 24 hours treatment with DHMEQ, expression of both secreted and cellular type I collagen was reduced in keloid fibroblasts in a dose dependent manner, while normal fibroblasts showed no decrease (Fig 6).

HSP47 is a collagen-specific molecular chaperone that interacts with procollagen during the process of folding, assembly and transport from the endoplasmic reticulum [16]. The expression of HSP47 is closely correlated with that of collagen in fibrotic disorders including keloids. However, the exact mechanism of how HSP47 is regulated remains unclear. Therefore, we performed western blotting to investigate whether DHMEQ affects HSP47 expression. Consistent with a previous report [17], very strong immunoreactive bands at 47 kDa were detected in all keloid samples compared to normal samples. However, DHMEQ treatment did not show any effect on HSP47 expression (Fig. 6), indicating that the regulation of HSP47 is independent of NF- κ B signaling pathway.

4. Discussion

In the present study, we demonstrated the constitutive elevation of the NF- κ B activity in keloid fibroblasts in both DNA-binding assay (Fig. 1) and immunohistochemical analysis (Fig. 2), suggesting that this signaling pathway may play a role in keloid pathogenesis. Since NF- κ B family protein is found in virtually all cell types, normal fibroblasts are also affected by DHMEQ. However, cell viability assay showed no significant cell number decrease at lower than 15 μ g/ml of DHMEQ in normal fibroblasts (Fig. 4A), and also cell proliferation assay showed that only keloid fibroblasts displayed significant growth reduction at low doses of DHMEQ (Fig. 5). Presumably, this could be due to low basal NF- κ B activity in normal fibroblasts since low concentration of DHMEQ hardly affected NF- κ B activity in normal fibroblasts but strongly reduced in keloid fibroblasts (Fig. 3). These results suggest that survival and growth of keloid fibroblasts are largely dependent on high basal activity of NF- κ B signaling.

Two mechanisms can be considered for how DHMEQ suppresses proliferation of

keloid fibroblasts. One is through down-regulation of genes involved in cell cycle progression. Cyclins D1 and D2 are downstream targets of NF- κ B pathway and directly regulate G1-S transition [18]. Thus, the down-regulation of those cyclins by DHMEQ may lead to growth arrest in keloid fibroblasts. The other is through suppression of inflammatory cytokine IL-6. IL-6 is also a downstream target of NF- κ B pathway and is involved in keloid pathogenesis. Because IL-6 peptide induces proliferation of fibroblasts [19], inhibition of this cytokine by DHMEQ may also lead to decreased proliferation of keloid fibroblasts.

The effect of NF- κ B activation on type I collagen, a principal component of excessive matrix in keloids, seems to be complicated. Type I collagen is a heterotrimeric protein composed of two α 1 chains and one α 2 chain encoded by the *COL1A1* and *COL1A2* genes, respectively. Several reports have proposed that NF- κ B regulates type I collagen negatively. NF- κ B binds to the site in *COL1A1* and *COL1A2* promoters, leading to inhibition of transcriptional activity [20,21]. Furthermore, activated NF- κ B suppresses the TGF- β /Smad pathway which promotes fibrosis by inducing inhibitory Smad7 protein [22,23]. On the contrary, it has been found that IL-4-mediated activation

of the *COL1A2* promoter needs cooperation with NF- κ B in human embryonic lung fibroblasts [24], and *COL1A2* promoter activation by TGF- β also requires NF- κ B binding to its consensus site [25]. These facts imply that NF- κ B has dual role in type I collagen regulation, presumably depending on cell/tissue circumstances. However, the main role of activated NF- κ B in keloid fibroblasts seems to be the positive regulation of type I collagen accumulation, thus its inhibition by DHMEQ resulted in decreased accumulation of type I collagen (Fig. 6). Our result suggests another contribution of NF- κ B pathway to keloid pathogenesis.

A variety of anti-inflammatory agents are known to affect NF- κ B activity. For example, steroids, the most widely used drugs in keloid treatment, increase the expression of I κ B α , resulting in the cytoplasmic retention of NF- κ B [26,27]. In parallel, direct protein-protein interaction between the activated glucocorticoid receptor and NF- κ B seems to suppress the activation of its pathway [28]. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and sodium salicylate also suppress NF- κ B pathway by inhibiting ATP binding to IKK β [29]. Recently, Zhu *et al.* have reported that aspirin successfully attenuated the cell proliferation and enhanced

TNF- α -mediated apoptosis in keloid fibroblasts [30]. However, it is evident that steroids and NSAIDs are not specific for IKK nor NF- κ B. To the best of our knowledge, DHMEQ is the most specific inhibitor that exclusively suppresses NF- κ B translocation from the cytoplasm to the nucleus. Together, these facts and our results suggest that NF- κ B-targeted therapy by DHMEQ may be a very attractive approach to the management of keloids.

One concern about inhibiting the NF- κ B pathway in skin tumors including keloids is that NF- κ B seems to have distinct role in epidermal keratinocytes. It has been reported that NF- κ B activation is required for normal differentiation of keratinocytes [31], and inhibition of the NF- κ B pathway in murine epidermis resulted in hyperproliferation of keratinocytes and spontaneous development of squamous cell carcinomas [32-34]. However, in keloids, not only fibroblasts but also keratinocytes seem to be abnormal and contribute to keloid pathogenesis [35,36]. Consistent with this, our results of immunohistochemical analysis suggested that NF- κ B is activated in overlying epidermis of keloid lesion as well (Fig. 2B). Thus, topical treatment with NF- κ B inhibitor might be ideally suited to keloid, although further assessment of

NF- κ B function in other type of cells e.g. keratinocytes would be necessary.

In conclusion, our results provide a new insight into keloid pathogenesis and therapeutic strategy. DHMEQ effectively suppressed the growth of keloid fibroblasts and type I collagen accumulation through the inhibition of NF- κ B activity, suggesting that the inhibition of NF- κ B by DHMEQ may be an attractive treatment modality for keloids.

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Figure legends

Fig. 1 Basal NF- κ B DNA-binding activity of normal and keloid fibroblasts. p65

DNA-binding activity was measured using nuclear extracts isolated from primary normal fibroblasts (NF1-4) and keloid fibroblasts (KF1-4) as described in Materials and methods. Results are presented as the mean \pm SD of the data collected in triplicate.

Fig. 2 Representative immunostaining for p65 in normal and keloid tissues. (A, C)

Normal skin specimen. (B, D) Keloid tissue specimen. (A, B) Original magnification: x100; Bars: 100 μ m. (C, D) Original magnification: x400; Bars: 50 μ m. Similar results were obtained in all cases. (E) The number of positive cells in each high power field (hpf) was counted under microscope at magnification of x400. Data are presented as the mean \pm SD of six different fields. *P<0.05 vs normal tissue.

Fig. 3 Inhibition of NF- κ B DNA-binding activity by DHMEQ. Primary normal

fibroblasts (NF) or keloid fibroblasts (KF) were treated with DHMEQ for 1 h. Nuclear extracts were used for binding assays as described in Materials and methods. Results are

shown as percentage relative to the normal skin control. Data are presented as the mean \pm SD of four different lines (NF1-4 and KF1-4). *P<0.05 vs control.

Figure 4 Induction of cell death by DHMEQ. (A) Viability of normal and keloid fibroblasts after treatment with various concentrations of DHMEQ for 24 h was estimated by WST-based assay. Each point indicates mean \pm SD of the data collected from 6 wells. (B) Cells were exposed to various concentrations of DHMEQ for 24 h, and Western blotting for PARP was performed using total cell lysates. Densitometric analysis was done and the level of PARP cleavage was expressed as a ratio of cleaved band to uncleaved band of untreated cells. Data are presented as mean \pm SD of four different lines (NF1-4 and KF1-4). Similar results were obtained in three independent experiments.

Figure 5 Effect of DHMEQ on cell growth. (A) Cells were treated with 5 or 10 μ g/ml of DHMEQ and cultured for up to 4 d. Each point indicates the mean \pm SD of the data collected from 6 wells. (B) Relative cell number at day 4. Data are presented as

mean \pm SD of four different lines (NF1-4 and KF1-4). *P<0.05 vs normal fibroblasts.

Similar results were obtained in three independent experiments.

Figure 6 Effect of DHMEQ on type I collagen accumulation. (A) Representative blot images. Indicated cells were treated with 5 or 10 μ g/ml of DHMEQ for 24 h. Cultured media (extracellular type I collagen) or total cell lysates (intracellular type I collagen and HSP47) were analyzed by Western blotting. β -actin was used as a loading control. (B) Results of densitometric analysis of detected bands. Results are expressed as relative levels compared to those of untreated cells. Data are presented as mean \pm SD of four different lines (NF1-4 and KF1-4). *P<0.05 vs untreated cells. Similar results were obtained in three independent experiments.

Table 1

	Sex	Age (years)	Biopsy site	Duration of the lesion (years)
<hr/>				
Normal				
<hr/>				
NF1	Female	20	Back	-
NF2	Female	77	Thigh	-
NF3	Male	22	Thigh	-
NF4	Male	64	Back	-
<hr/>				
Keloid				
<hr/>				
KF1	Female	29	Ear	3
KF2	Male	38	Chest	6
KF3	Male	64	Chest	8
KF4	Female	28	Shoulder	2
<hr/>				

Figure 1

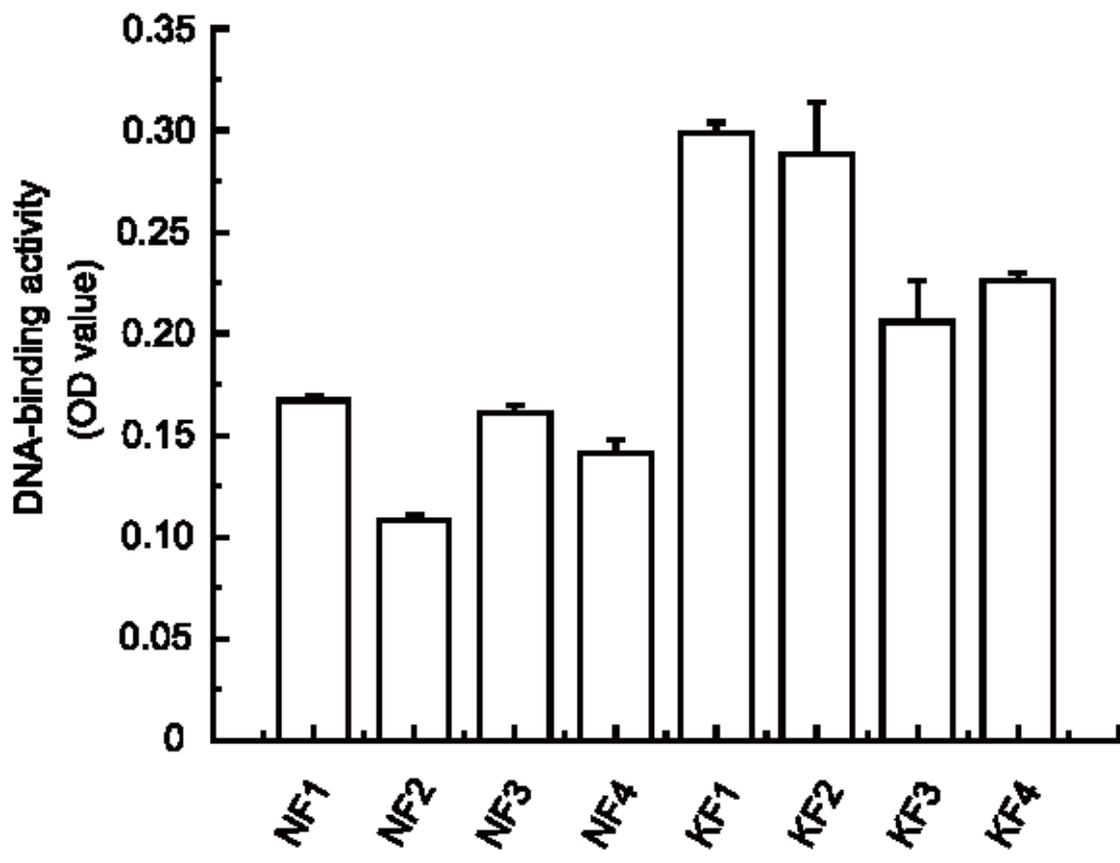


Figure 2

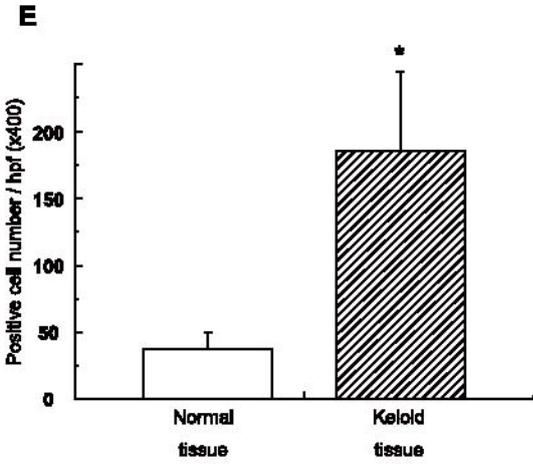
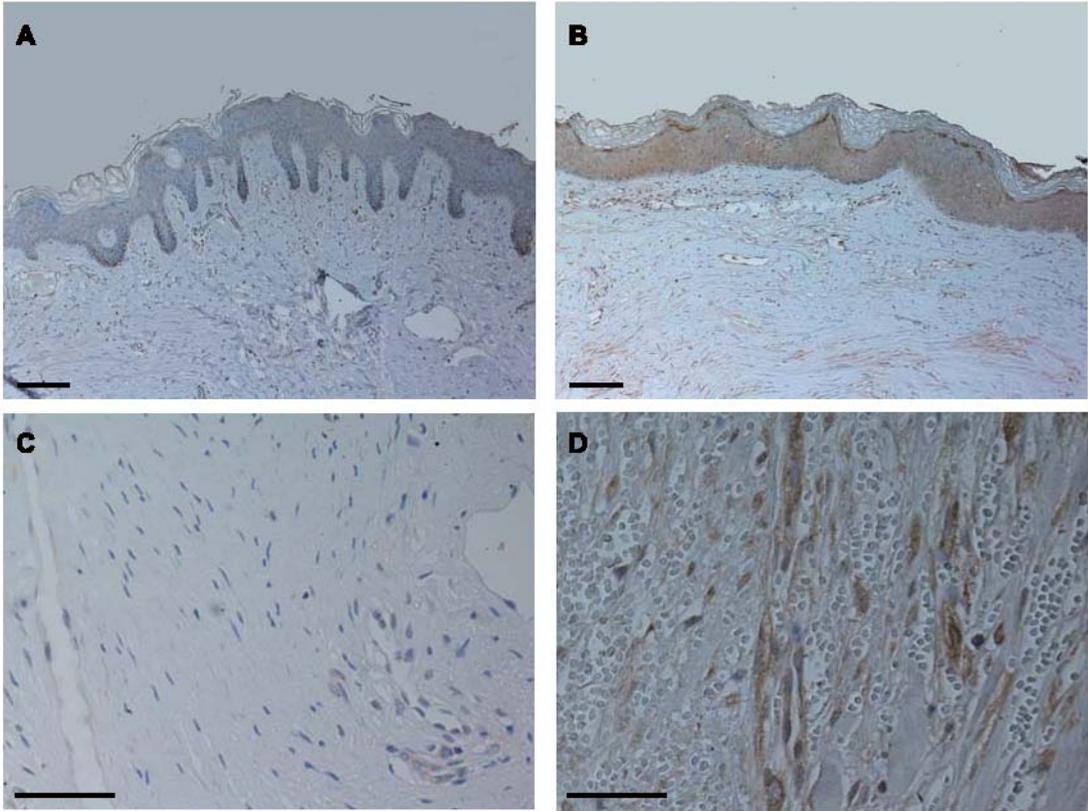


Figure 3

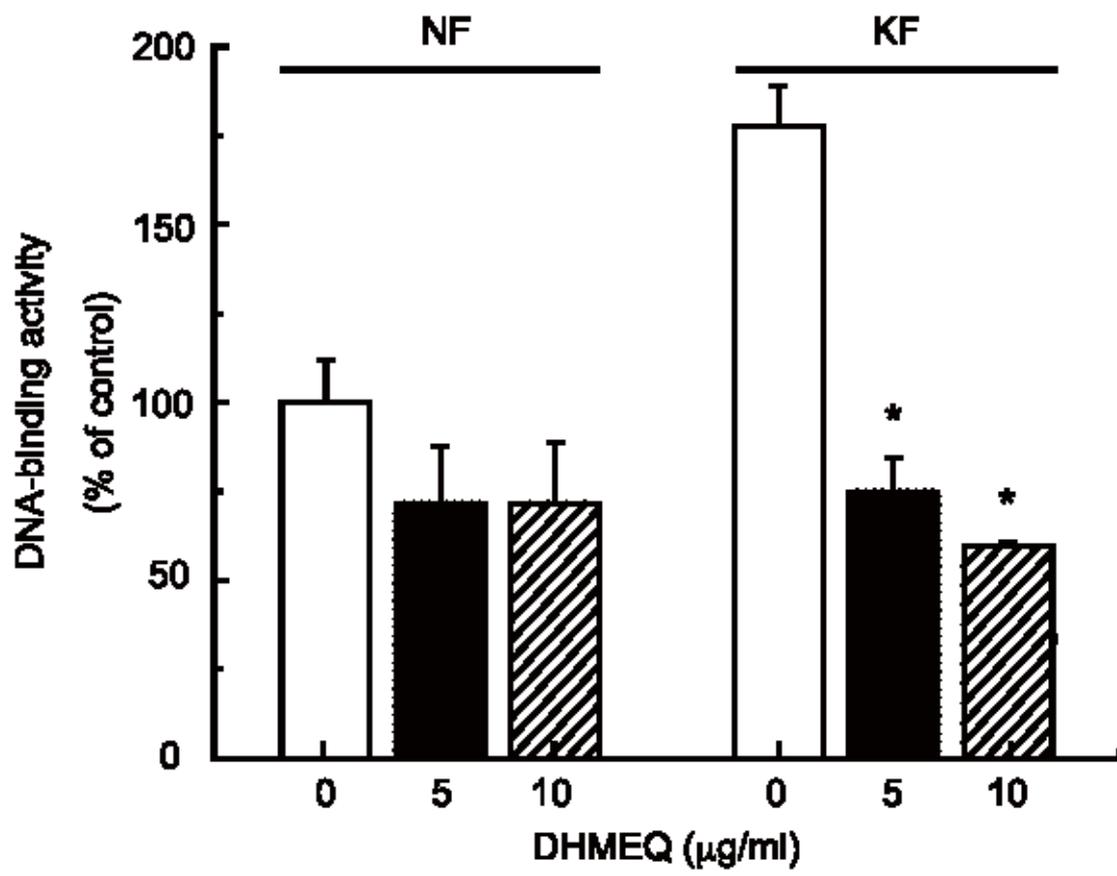


Figure 4

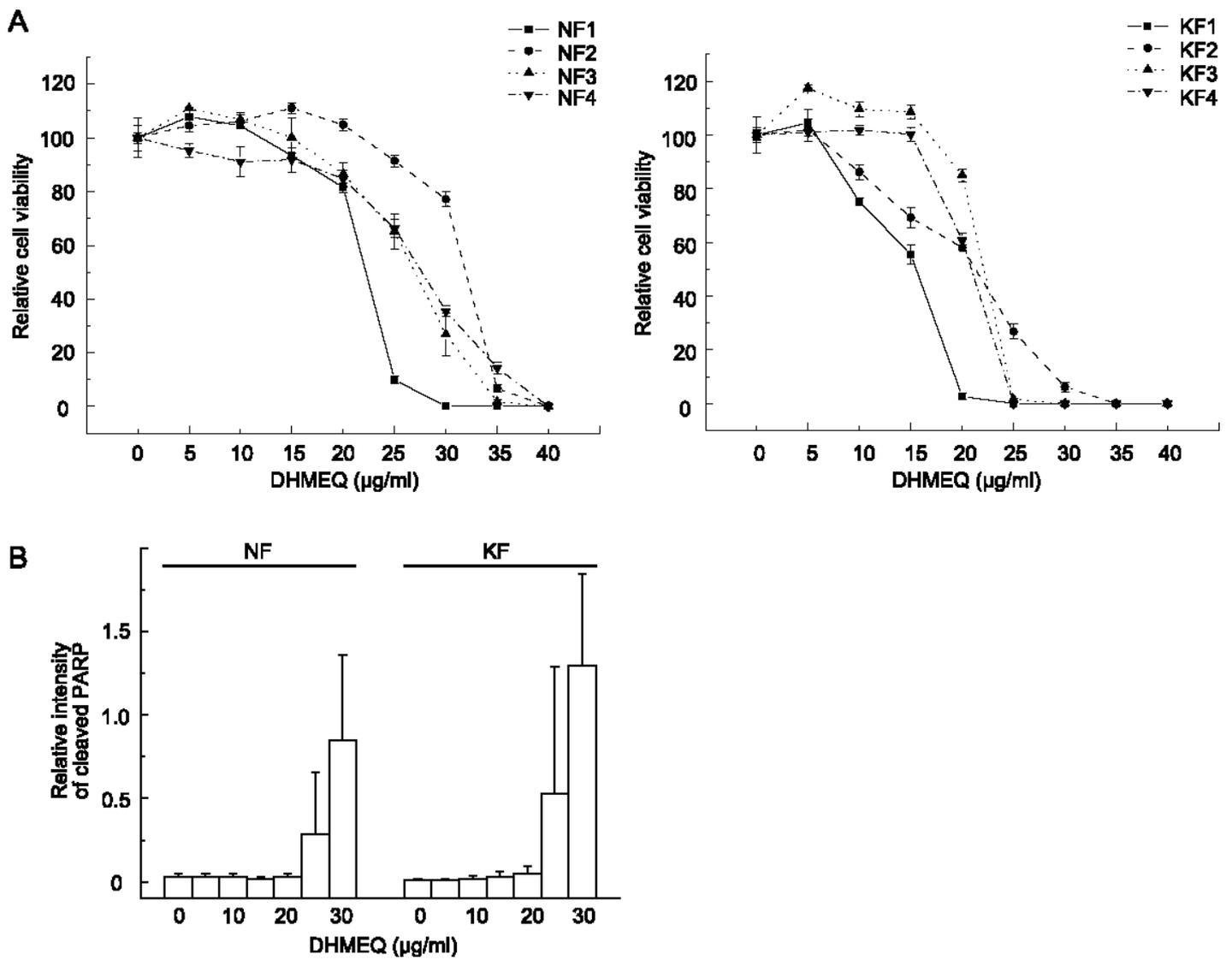
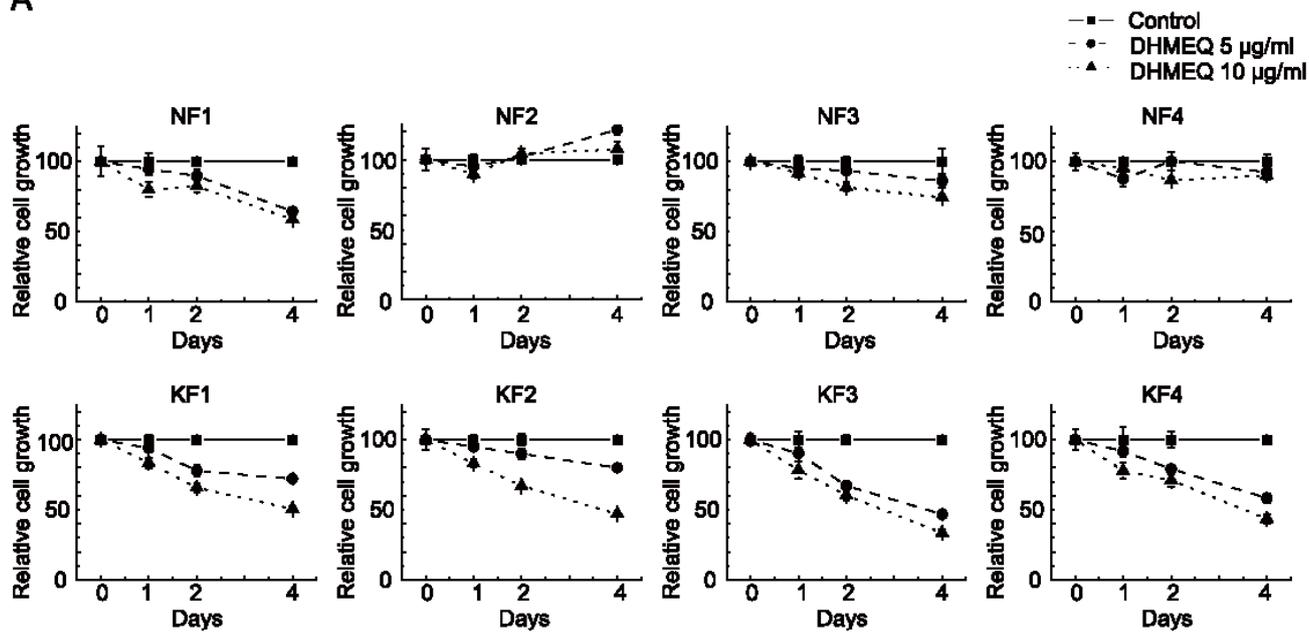


Figure 5

A



B

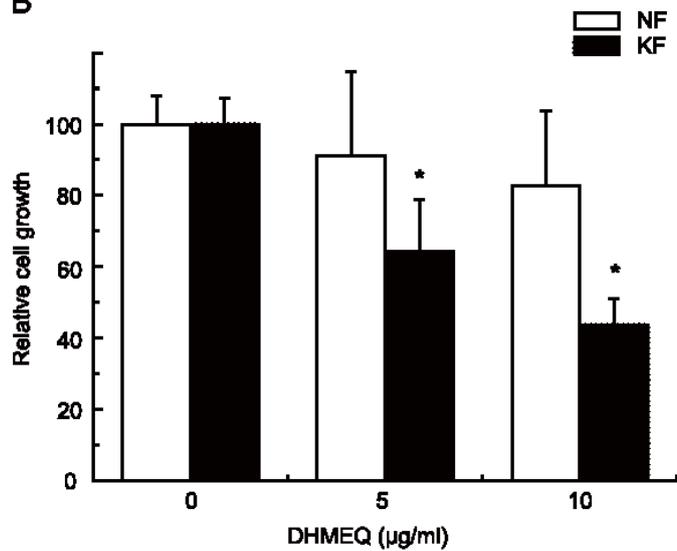


Figure 6

