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Wound Healing Activity of Gamma-Aminobutyric Acid (GABA) in Rats

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Abstract Gamma-aminobutyric acid (GABA) is a non-protein amino acid. It is well known for its role as an inhibitory neurotransmitter of developing and operating nervous systems in brains. In this study, a novel function of GABA in the healing process of cutaneous wounds was presented regarding anti-inflammation and fibroblast cell proliferation. The cell proliferation activity of GABA was verified through an MTT assay using murine fibroblast NIH3T3 cells. It was observed that GABA significantly inhibited the mRNA expression of iNOS, IL-1β, and TNF-α in LPS-stimulated RAW 264.7 cells. To evaluate *in vivo* activity of GABA in wound healing, excisional open wounds were made on the dorsal sides of Sprague-Dawley rats under anesthesia, and the healing of the wounds was apparently assessed. The molecular aspects of the healing process were also investigated by hematoxylin-eosin staining of the healed skin, displaying the degrees of re-epithelialization and linear alignment of the granulation tissue, and immunostaining and RT-PCR analyses of fibroblast growth factor and platelet-derived growth factor, implying extracellular matrix synthesis and remodeling of the skin. The GABA treatment was effective to accelerate the healing process by suppressing inflammation and stimulating re-epithelialization, compared with the epidermal growth factor treatment. The healing effect of GABA was remarkable at the early stage of wound healing, which resulted in significant reduction of the whole healing period.

Keywords: Gamma-aminobutyric acid (GABA), skin, wound healing, rat

Skin is the largest organ in the body in terms of weight and surface area, and it normally separates and protects the internal structures from the external environment. The functions of the skin are divided into five general classes: protective, sensory, respiratory, heat regulatory, and secretory. As a protective covering, it guards against the damaging effects of the sun's rays, the loss of body fluids, the entrance of toxic substances into the body, and mechanical violence. Skin is composed of several layers. The lowest layer is called the dermis, which is composed of connective tissue, blood vessels, nerve endings, hair follicles, and sweat and oil glands. The outer skin, the epidermis, is a tightly bound, stratified, and squamous epithelium, covered by keratin [30]. If the skin is injured, irrespective of the size and severity of the injury, it should repair rapidly and perfectly. The healing response by the body's self-treatment system starts immediately at the moment an injury occurs [21].

Wound healing is a complex biological process, including inflammation, cell migration, angiogenesis, extracellular matrix synthesis, collagen deposition, re-epithelialization etc. In the first half phase of the wound healing process, there are two major stages. The first is the inflammatory stage and the second is new tissue generation. At the inflammatory stage, infiltrating neutrophils cleanses foreign agents in the area, accelerating wound cleaning [31]. During new tissue formation, the local accumulation of collagen strongly correlates with the accretion of tensile strength; hence, measuring the content and concentration of collagen at a repair site permits an estimate of the healing rate [4].

Insights into controlling the wound repair process have focused on signal molecules such as cytokines and growth factors [16]. It is well known that these factors bind to specific receptors on the membrane of fibroblasts, which initiates a signal cascade, leading to specific cellular functions such as cell locomotion or new gene expression [10]. It has been previously reported that the fibroblast growth factor (FGF) family regulates proliferation, differentiation, migration, and/or survival of a variety of different target cells [3, 25]. Platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) also promote the fibroblast proliferation and collagen deposition, respectively [22]. Interleukins-1 and -8, which are known to be released

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early in the repair process, have direct effects upon fibroblast physiology.

Until now, there have been few pharmacological agents identified to enhance the wound healing process with certainty. In order to promote accelerating skin repair, many clinicians have been focused on searching for novel pharmacological agents, especially from natural herbal extracts, since a newly developed agent should possess both aspects of maximum efficiency of wound closure and minimum cytotoxicity.

Gamma-aminobutyric acid (GABA) is a non-protein amino acid component that is present in a large range of organisms including bacteria, yeasts, plants, and animals. In animals, GABA acts as an important cell signal during embryonic and adult neurogenesis, as well as a principal excitatory neurotransmitter in the developing brain. It usually acts as an epigenetic factor to control the processes including cell proliferation [11], neuroblast migration, and dendritic maturation [20]. In plants, the levels of GABA are quite low and highly changeable, responding to biotic and abiotic stresses, including acidic environments and mechanical damage, even though their biological roles are still obscure. It has been reported that the GABA (A) receptor existed in the epidermal keratinocyte and induced chloride ion flux and inhibited the depolarization [28]. In other studies, GABA was known to improve the skin barrier homeostasis by the regulation of the GABA (A) receptor [9].

The aim of this study is to report a novel function of GABA, a wound healing activity, in rats. In this study, to verify the wound healing activity of GABA, the effects of anti-inflammation and fibroblast cell proliferation were experimentally exploited using *in vitro* analysis of RT-PCR and MTT assay. The macroscopic appearance, histological observations, and immunohistochemical analysis were subsequently examined using a rat model with a full-thickness cutaneous wound on the animal's back.

MATERIALS AND METHODS

Cell Culture

Mouse macrophage RAW 264.7 cells and murine fibroblast NIH3T3 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were routinely kept in a Dulbeco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum and penicillin G (100 U/ml)/ streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2-95% air incubator under standard conditions.

Cell Proliferation Assay

Cell proliferation activity of GABA on mouse fibroblast was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated at a density of 1×10^4 cells/well on 96-well flat bottom plates (Nunc, Roskilde, Denmark) and incubated at 37°C in 100 μl of a Dulbeco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS). After 12-h incubation (37°C, 5% CO_2), adherent cells were washed two times with the culture medium. Subsequently, adherent cells were incubated in the presence of different concentrations (1, 10, 100, 1,000, 10,000, and 50,000 μM) of GABA (Sigma-Aldrich Co., MO, U.S.A.). Forty-eight h later, the 10 μl MTT solutions (Sigma-Aldrich Co., MO, U.S.A.) (5 mg/ml in PBS) were added and incubated for 4 h at 37°C in a 5% CO_2 incubator. MTT acts as a substrate for dehydrogenase enzyme and is converted to a colored formazan product by living cells. After 4 h, 100 μl of 0.04 N hydrochloric acid/isopropanol was added into each well. The culture plates were surged for 10 min at room temperature to dissolve MTT crystals. Absorbance, which was directly proportional to the cellular metabolism, was measured at 570 nm in an automated ELISA reader (Molecular Devices Co., CA, U.S.A.).

LPS-induced Inflammation and RT-PCR Analysis

RAW 264.7 cells were inoculated in 6-well tissue culture plates (1.5×10^6 cells/well) and incubated in a Dulbeco's Modified Eagle's Medium, supplemented with penicillin G (100 U/ml)/streptomycin (100 μg/ml) containing 10, 100, 1,000, and 10,000 μM of the GABA (Sigma-Aldrich Co., MO, U.S.A.) for 2 h at 37°C. After 2 h, the cells were stimulated with 5 μg/ml LPS for inducing inflammation. The cells were harvested and lysed 8 h after stimulation. Total RNA was extracted from RAW 264.7 cell preparations with TRIzol reagent following the supplier's instructions. In brief, 5×10^5 cells were lysed in 1 ml of TRIzol® and then transferred into Eppendorf tubes. Following addition of 200 μl of chloroform, the suspension was centrifuged for 15 min at 12,000 ×*g* at 4°C. The upper hydrophilic layer was recovered, mixed with 500 μl of isopropyl alcohol, again centrifuged, and the supernatant discarded. The pellet was washed twice with 75% ethanol, dissolved in DEPC-H_2O, adjusted to 1 mg RNA/ml, and stored at 80°C. The chromosomal DNA was removed using RQ1 RNase-free DNase (Promega Co., Madison, WI, U.S.A.). One μg of total RNA was reverse-transcribed with 200 U Moloney murine leukemia virus (MoMLV) reverse transcriptase (Gibco BRL, Gaithersburgh, MD, U.S.A.) in a 25 μl reaction mixture containing random hexamers (Bioneer Co., Deageon, Korea). The cDNA was amplified in a 20 μl final volume of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 M each primer, and 0.5 U *Taq* polymerase (TaKaRa Co., Shiga, Japan) in a PTC-100 programmable thermal controller (MJ Research, Waltham, MA, U.S.A.). All primers were designed from the published cDNA sequences using primer selection software offered through a Web site, Primer 3 (The

Whitehead Institute for Biomedical Research, Cambridge, MA, U.S.A.; http://www.genome.wi.mit.edu). The following primer sequences, annealing temperatures, cycle numbers, and GenBank accession numbers were used: for GAPDH (579 bp) (58°C for hybridization step, 30 cycles), (forward) 5'-ATC CCA TCA CCA TCT TCC AG-3' and (reverse) 5'-CCT GCT TCA CCA CCT TCT TG-3'; for iNOS (401 bp) (58, 25 cycles, NM_010927.1), (forward) 5'-ATG TCC GAA GCA AAC ATC AC-3' and (reverse) 5'-TAA TGT CCA GGA AGT AGG TG-3'; for IL1-β (387 bp) (58, 25 cycles, NM_008361.2), (forward) 5'-TGC AGA GTT CCC CAA CTG GTA CAT C-3' and (reverse) 5'-GTG CTG CCT AAT GTC CCC TTG AAT C-3'; and for TNF-α (374 bp) (58, 20 cycles, NM_013693.1), (forward) 5'-CCT GTA GCC CAC GTC GTA GC-3' and (reverse) 5'-TTG ACC TCA GCG CTG AGT TG-3'. The PCR products were separated on 1.2% agarose gels, stained with ethidium bromide, and photographed. The intensities of the bands were measured by using an ImageMaster VDS gel documentation system (Amersham Pharmacia Biotech, Uppsala, Sweden) with an image analysis software, ImageMaster TotalLab (Amersham Pharmacia Biotech, Uppsala, Sweden). The amounts of target PCR product were normalized against GAPDH PCR products.

Animals

Adult male Sprague-Dawley (SD) rats weighing from 250 to 270 g were used for this study. All experimental animals were obtained from Samtako Co. Ltd. (Kyunggi-do, Korea). The animals were maintained in individual wire-bottom cages on a 12 h light/dark cycle at 22°C with water and food *ad libitum*. The experimental procedures were carried out according to the animal care guidelines of the Kyung Hee University Institutional Animal Care and Use Committee.

Experimental Design

Experimental animals were divided into five groups: (1) non-treated group (Control, n=6); (2) epidermal growth factor-treated group (0.5 μg, EGF, n=6); (3) gamma-aminobutyric acid-treated group (0.1 mmol, GABA 0.1, n=6); (4) gamma-aminobutyric acid-treated group (5 mmol, GABA 5, n=6); and (5) gamma-aminobutyric acid-treated group (10 mmol, GABA 10, n=6). Gamma-aminobutyric acid for *in vivo* and *in vitro* studies was purchased from Sigma-Aldrich Co. (MO, U.S.A.). Epidermal growth factor was purchased from Millipore Co. (Carlsbad, CA, U.S.A.). The 100 μl of the EGF solution and each concentration of GABA were topically applied to the wounds once a day for 10 days.

Wound Generation

On the day before wound generation, the hair on the back of the rats was carefully clipped using a clipper and an electric shaver under anesthetization by halothane inhalation. A template was used to mark a square (2×2 cm) on the mid-back, and a full-thickness wound was made by excising the skin corresponding to the mark. After the wound generation, the incisions were closed with a hydrophilic form dressing. The rats were allowed to recover from anesthesia and each rat was then returned to its cage.

Wound Rank Scoring System

The macroscopic differences in healing were quantified using a wound rank scoring system, [26] which assessed a wound closure by measuring wound length and gape, inflammation, redness, and swelling. Measurements of wound gape and length at each time point were divided into four categories according to the best and worst reading, and on this basis, the wound was given a score of 0–3 for each measurement. Redness and swelling were also individually scored on a scale of 0–3 (no, slight, significant, or extensive redness/swelling). The four scores were then summed. The minimum score of 0 indicates the best possible condition of a wound, and the maximum score of 12 indicates the worst. The macroscopic appearance of the wound was recorded daily with a Camedia digital camera (c-2040zoom, Olympus Co., Hamburg, Germany).

Histology

Wound tissue excision for histological analysis was performed on the 5th day post-wounding. Under anesthetization by halothane inhalation, the entire wound region, with liberal margins of surrounding skin, was excised in depth to include underlying connective tissue above the external fascia of dorsal muscles. These biopsy specimens were fixed in 4% paraformaldehyde overnight, dehydrated through the graded ethanols, embedded in paraffin, sectioned at 10 μm thickness, and mounted onto the slides. The serial sections were de-paraffinized and stained with hematoxylin-eosin. The specimens were then observed under a light microscope.

RT-PCR Analysis of Skin Tissue

Wound tissue excision for RT-PCR analysis was performed on the 5th day post-wounding. The newly formed (regenerated) tissues in the wounded area were excised using a blade to avoid contamination from surrounding tissue. The excised skin samples were stored at −80°C under liquid nitrogen. Total RNA was isolated from the skin tissue using the same protocol of total RNA extraction from a cell line. The following primer sequences, annealing temperatures, cycle numbers, and GenBank accession numbers were used: for GAPDH (579 bp) (58°C for hybridization step, 30 cycles), (forward) 5'-ATC CCA TCA CCA TCT TCC AG-3' and (reverse) 5'- CCT GCT TCA CCA CCT TCT TG -3'; for FGF (300 bp) (57°C, 35 cycles, X07285.1), (forward) 5'-

GGA GAA GAG CGA CCC ACA-3' and (reverse) 5'-GCA GAC ATT GGA AGA AAC AGT ATG-3' and for PDGF-α (58°C, 35 cycles, NM_012801.1), (forward) 5'-ATG CCT TGG AGA CAA ACC TG-3' and (reverse) 5'-CCT CAC CTG GAC CTC TTT CA-3'.

Immunohistochemical Analysis

For histological examination, tissue samples were obtained on the 5th day. The section was examined by immunofluorescence staining with fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) antibodies. Sections were de-paraffinized and rehydrated. After washing in a Tris-buffered saline with Tween-20 (TBST), the endogenous peroxidases in the sections were blocked with TBST containing 0.3% H_2O_2 for 30 min at room temperature. In order to block a nonspecific binding, the sections were incubated with 10% goat serum and 1% bovine serum albumin (BSA) in TBST for 30 min at room temperature. The sections were then incubated with rabbit anti-FGF polyclonal antibody (1:100 dilution; ab16828) (Abcam Inc., Cambridge, MA, U.S.A.) and rabbit anti-PDGF BB polyclonal antibody (1:100 dilution; ab15499) (Abcam Inc., Cambridge, MA, U.S.A.) in a humidified chamber overnight at 4°C. After washing with TBST, the sections were labeled with a secondary antibody, Alexa Fluor 488 goat anti-rabbit immunoglobulin G (1:200 dilution; a11034) (Molecular Probes Co., Carlsbad, CA, U.S.A.). The sections were rehydrated and mounted with buffered glycerol. The slides were photographed using a Zeiss Axoplan fluorescence microscope (Carl Zeiss MicroImaging GmbH, Hamburg, Germany).

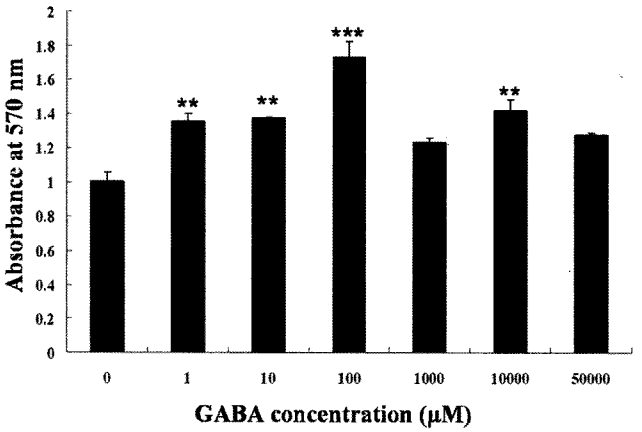

Fig. 1. Cell proliferation activity of GABA in mouse fibroblast cells.

The NIH/3T3 cell proliferation was analyzed by an MTT assay on GABA-treated and untreated cells after 48 h cultivation at 5% CO_2 and 37°C. Data (n=3) are presented as mean values of the absorbance reading of optical density at 570 nm. Values are means with SEM from at least three independent experiments. Significance with Tukey's HSD post-hoc test following a one-way ANOVA is indicated as ***P<0.001 and **P<0.01 *vs.* non-treated control cells.

Data Analysis

The data were presented as mean±SEM. The experimental data were analyzed by one-way and repeated ANOVA using a SPSS program (version 8.0). Statistical differences among groups were further analyzed using Tukey's post-hoc test. P values <0.05 were considered statistically significant.

RESULTS

Effect of GABA on Cell Proliferation of Fibroblast NIH3T3 Cells

The proliferation activity of GABA was studied using mouse fibroblast NIH3T3 cells. The addition of GABA promoted the growth of NIH3T3 cells in a dosage-dependent manner in the concentrations of 100, 10, and 1 μM (P<0.001, P=0.04, P=0.013, respectively), and then slightly decreased at the concentrations of 1,000, 10,000, and 50,000 μM (Fig. 1). It means that there was an optimal concentration of GABA, which led to the maximum

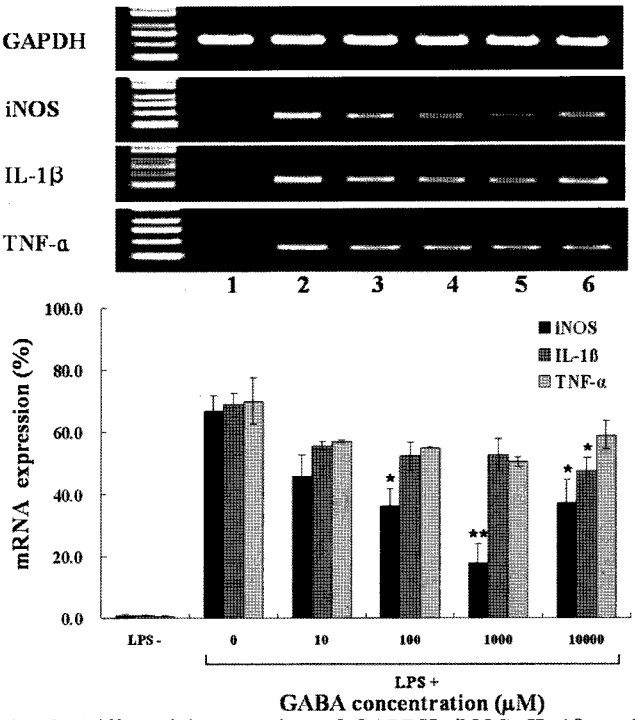

Fig. 2. Differential expression of GAPDH, iNOS, IL-1β, and TNF-α mRNAs in RAW 264.7 cells.

Appearance of non-treated group (LPS-), lane 1; LPS-treated group (0), lane 2; LPS+10 μM GABA-treated group (10), lane 3; LPS+100 μM GABA-treated group (100), lane 4; LPS+1,000 μM GABA-treated group (1,000), lane 5; and LPS+10,000 μM GABA-treated group (10,000), lane 6; in an LPS-stimulated RAW 264.7 macrophage cell line. RAW 264.7 cells were incubated in the presence of LPS with or without GABA for 8 h. Values are means with SEM from at least three independent experiments. Significance with Tukey's HSD post-hoc test following a one-way ANOVA is indicated as **P<0.01 and *P<0.05 *vs.* LPS+group.

growth yield. The stimulation effect of cell growth was not observed in the high concentration range (1,000, 10,000, 50,000 μM).

Antiinflammatory Activities of GABA by RT-PCR Analysis

The proinflammatory mediators such as TNF-α, IL-1β, and iNOS are differentially expressed in response to various inflammatory stimuli such as lipopolysaccharide (LPS) and mitogen. The RT-PCR analysis was performed to determine whether the inhibitory effects of GABA on the proinflammatory mediators NO is related to the modulation of the expression of iNOS. In LPS-stimulated RAW 264.7 cells, iNOS was strongly expressed and GABA significantly inhibited iNOS expression in a dose-dependent manner (Fig. 2). However, in the case of 10,000 μM GABA, iNOS and TNF-α expressions were slightly increased, implying that the inhibition of inflammatory response by GABA has an optimum in relation to the concentration. We further investigated the effect of GABA on the LPS-induced expression of TNF-α and IL-1β mRNAs using RT-PCR analysis. The TNF-α and IL-1β mRNA expression levels, highly increased in LPS-stimulated RAW 264.7 cells, were significantly decreased in a concentration-dependent manner by the treatment with GABA (Fig. 2).

Wound Healing Activities of GABA on Macroscopic Appearance

Changes in macroscopic appearance, including the size variation, of the cutaneous open wounds were monitored daily by capturing digital images of each animal at each time point of the 2nd, 5th, 7th, or 10th day post wounding (Fig. 3) Until the 5th day, the GABA treatment dose-dependently improved the healing rates and macroscopic appearance of the cutaneous open wounds. The wounds in the GABA-treated groups appeared to be less moist and inflamed and exhibited reduced wound areas, as compared with those in the non-treated control group. On the 7th day, the control rats demonstrated little contraction of wound surface and significant inflammation. As compared with the wounds of control rats, the GABA-treated wounds were smaller and more contracted. Contrary to the control rats, the GABA-treated groups did not show skin blistering, which signifies the separation of the outer layer (epidermis) of the skin from the fiber layer (dermis). On the 10th day, the EGF- and GABA-treated rats exhibited almost complete wound closure. However, the wounds of the control rats were in the progress of wound contraction and were still inflamed. With regard to the macroscopic appearance in wound healing, the treatment efficiency of the 10 mmol GABA-treated rats on the 5th day appeared to be similar to that of the control rats on the 10th day.

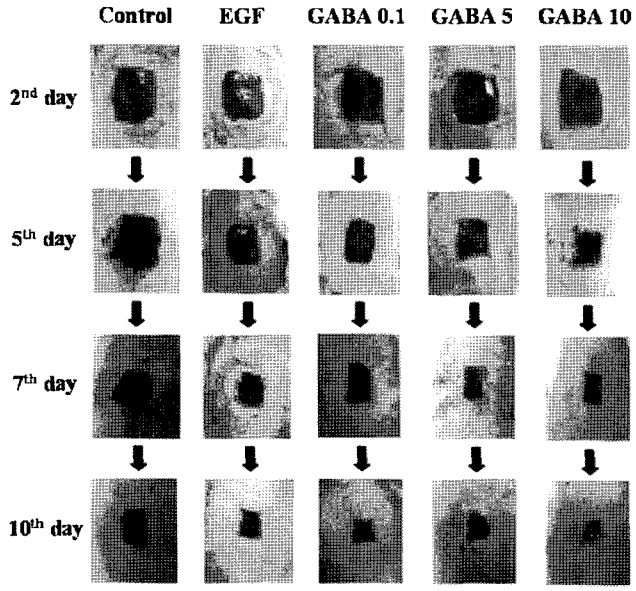

Fig. 3. Macroscopic appearance of wounds on control, EGF, GABA 0.1, GABA 5, and GABA 10 treated-rats on the 2nd, 5th, 7th, and 10th days post-wounding.

Wound Healing Activities of GABA on Wound Rank Scoring System

The macroscopic appearance of wounds in control and GABA-treatment groups was blindly assessed *via* a wound rank scoring system that scores the degree of wound closure (wound length and gape) and inflammation (redness and swelling). The EGF-treated group was used as a positive control. The wound rank scores were highly improved in the GABA-treated groups and dose-dependently increased at the concentration of 0.1, 5, and 10 mmol. The statistical analysis indicated a marked improvement of wound healing at the 5th and 7th days ($P<0.001$) (Fig. 4).

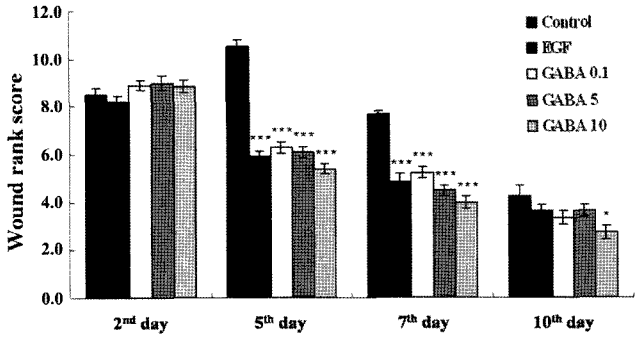

Fig. 4. Histogram showing wound rank scores of control, EGF, GABA 0.1, GABA 5, and GABA 10-treated rats with full-thickness wounds.

Each bar represents a mean wound rank score in a full-thickness wound healing rat model (n=6 in all groups). Significance with Tukey's HSD post-hoc test following a one-way ANOVA is indicated as ***$P<0.001$ and *$P<0.05$ *vs.* non-treated control group on the same day.

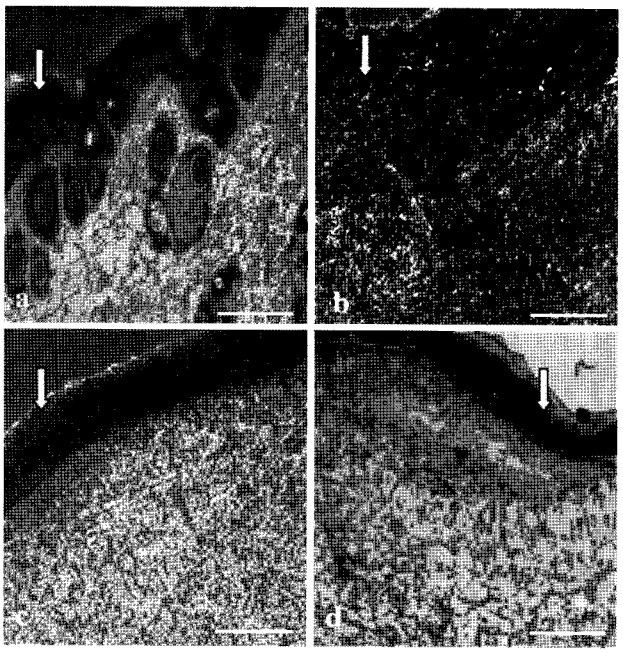

Fig. 5. Histological micrographs of wound regions on the 5th day post-wounding.
The arrows indicate the epidermis. Normal skin (panel a), and non-treated (panel b), EGF-treated (panel c), and GABA 10-treated (panel d) wound tissues were harvested from rats on the 5th day post-wounding. Magnification: scale bar=50 μm.

Wound Healing Activities of GABA by Histological Analysis

Fig. 5 shows the histology of healed skin tissue on the 5th day. In the EGF- and 10 mmol GABA-treated wound tissues (panels c and d), the collagenous fibers in the dermis tissue were more regularly organized and parallel with complete epithelialization, as compared with those of non-treated skin tissue (panel b). Fibroblasts, reformed in the dermis layer, also appeared to be more mature, with a shape of linear alignment. The histological micrograph of non-treated wound tissue exhibited an incomplete epithelialization, the irregular arrangements of newborn fibroblasts, and less dense collagenous fibers in the dermis.

Wound Healing Activities of GABA by RT-PCR and Immunohistochemical Analyses

The excision sampling of wounded tissue was conducted on the 5th day post-wounding for RT-PCR and immunohistochemical analyses. Fig. 6 and 7 show the RT-PCR data (A) and immunostaining photographs (B) of FGF and PDGF, respectively, in the epidermis and dermis of the wounded skin area. For RT-PCR analysis, total RNA was extracted from the tissues of entire wounded areas including newly filled tissue above the external fascia of dorsal muscles. As shown in Figs. 6A and 7A, the mRNA expressions of FGF and PDGF were significantly stimulated by the treatment of 10 mmol GABA, compared

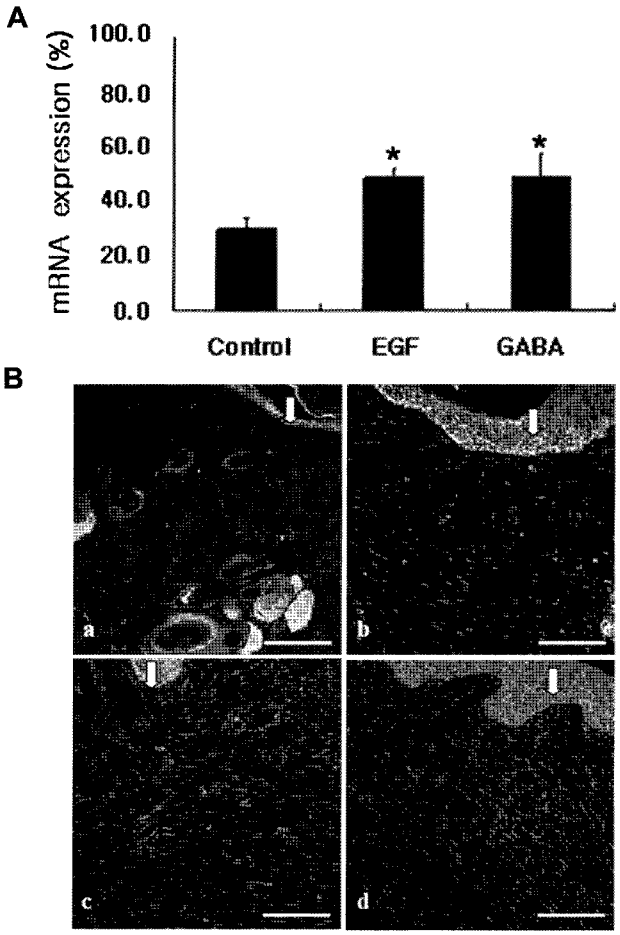

Fig. 6. Representative photographs showing FGF expression **A.** RT-PCR analysis; **B.** Immunofluorescence staining) in the healing process of wounds on the 5th day post-wounding.
The arrows indicate the epidermis. Normal skin tissue (a), non-treated wound tissue (b), EGF-treated wound tissue (c), and GABA 10-treated wound tissue (d) are indicated in **B**. Magnification: scale bar=50 μm. Values are means with SEM from at least three independent experiments. Significance with Tukey's HSD post-hoc test following a one-way ANOVA is indicated as $*P<0.05$ *vs.* non-treated control (Control) group.

with those in the non-treated control group. The increment of the expression at the transcriptional level was also highly compatible to that in the EGF-treated group. It was also confirmed that the protein expressions of FGF and PDGF were activated by GABA treatment in the immature fibroblasts of dermis and epithelial cells.

In the normal skin tissue, the immunoreactivities of FGF and PDGF were weakly detectable on the skin appendage and epidermis (panel a in Figs. 6B and 7B). In the case of the GABA-treated group, more extensive expressions of FGF and PDGF were observed in the immature fibroblasts in the dermis (panel d in Figs. 6B and 7B), as compared to the non-treated control group. In particular, the expression of PDGF was remarkable in the 10 mmol GABA-treated group (panel d in Fig. 7B), compared with the non-treated

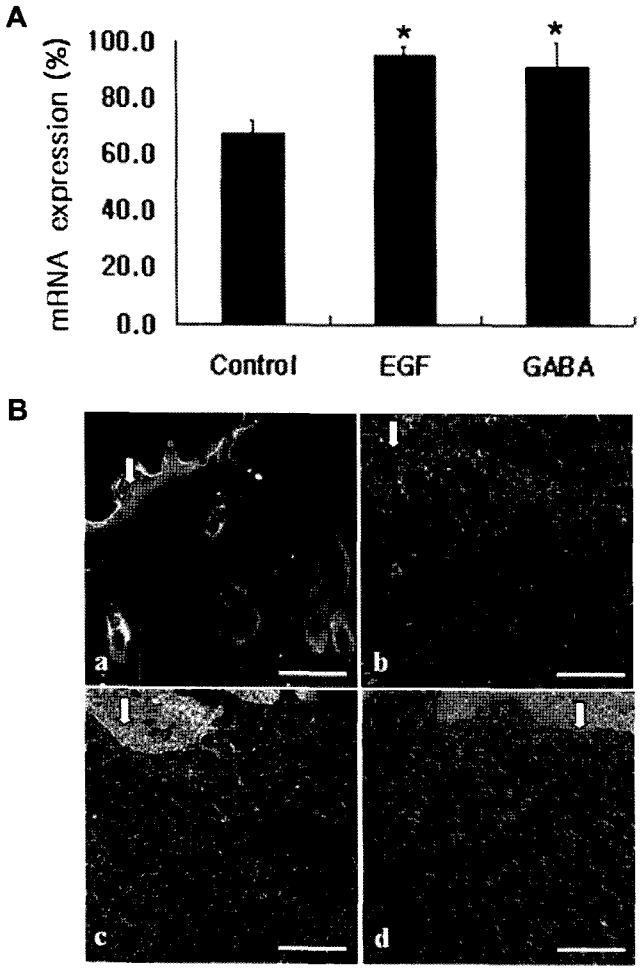

Fig. 7. Representative photographs showing PDGF expression (**A**. RT-PCR analysis; **B**. Immunofluorescence staining) in the healing process of wounds on the 5th day post-wounding.
The arrows indicate epidermis. Normal skin tissue (a), non-treated wound tissue (b), EGF-treated wound tissue (c), and GABA 10-treated wound tissue (d) are indicated in **B**. Magnification: scale bar=50 μm. Values are means with SEM from at least three independent experiments. Significance with Tukey's HSD post-hoc test following a one-way ANOVA was indicated as $*P<0.05$ *vs.* non-treated control (Control) group.

wound tissue (panel b in Fig. 7B) and EGF-treated wound tissue (panel c in Fig. 7B).

DISCUSSION

Recently, a great deal of attention has been paid to the healing of acute and chronic wounds. The primary aims of wound healing by dermatologists are in promoting rapid wound closure and preventing excess scarring at the same time. In the absence of significant infection or contamination, the inflammatory stage of wound healing is short in general [18]. This is one of the important factors by which the wound successfully proceeds to the next stage of wound healing. The re-epithelialization of the wound is made easier by the underlying contractile connective tissue, which shrinks in size to bring the wound margins toward one another [29]. In addition, growth factors and other related substances are also released, which are necessary for the initiation and propagation of granulation tissue formation. All of these factors are important in the wound healing process.

The cell proliferation activity of GABA was studied using a mouse fibroblast cell line, NIH3T3 [1, 12]. The GABA treatment significantly promoted cell proliferation and produced a dose-dependent mitogenic activity. Topical application of GABA significantly suppressed the mRNA expression of inflammatory mediators such as iNOS, IL-1β, and TNF-α, and dose-dependently stimulated the cell proliferation in an *in vitro* study.

In the following study, it was observed that GABA significantly inhibited the mRNA expression of TNF-α, IL-1β, and iNOS using an LPS-induced *in vitro* cell line. TNF-α, IL-1β, and iNOS are known to be primary inflammatory mediators in the inflammatory stage of wound healing [2, 7, 23]. Amongst them, iNOS was most strongly expressed by LPS stimulation [6, 20] and GABA significantly inhibited its expression in a dose-dependent manner, as reported in other studies [14]. Inducible NOS is instantly expressed in response to various inflammatory stimuli and catalyzes the synthesis of a large amount of NO, mainly produced by macrophages during the inflammatory process. Therefore, suppression of the production of TNF-α and iNOS by using the proper drugs might be useful for the treatments of inflammatory diseases and endotoxin shock [17].

In terms of macroscopic appearance, the dose-dependent improvements of wound size and healing rate were observed in the GABA-treated experimental group. Wound shapes of this group appeared to be less moist and inflamed, which resulted in reduced sizes of the wound areas [20]. Among the several important factors of primary care in the initial stage of wound healing, maintaining a clean environment and protecting the infections are essential for effective wound healing [19]. In this study, the macroscopic appearance was eventually assessed by a wound rank scoring system [8], which scored the degree of wound closure (wound length and gape) and the severity of inflammation (redness and swelling). These results also showed a dose-dependent activity of GABA in the improvement of wound healing.

In the histological studies of healed skin tissue, it was observed that the newly generated tissues of the GABA-treated wounds were regularly organized and parallel with a completely formed epithelial layer. Fibroblast cells in wound sites appeared to be highly matured, exhibiting a linear and systematic alignment. These histological results supported the wound healing activities of GABA. Re-

epithelialization, the formation of granulation tissue, and the faster formation of new tissues were causable for accelerating the wound healing process [13, 32]. Enhancing the cellular expression of growth factors was also identified by immunohistochemical staining. To investigate the secretion of FGF and PDGF, which are well known to accelerate wound healing [24], the paraffin-embedded sections of healed skin areas were prepared and immunostained using the antibodies of those growth factors. In the GABA-treated group, the expressions of FGF and PDGF significantly increased and their expressions were extensively observed, especially in the immature fibroblasts in the regions of the dermis and epidermis. In terms of stimulating FGF expression in the wounded area, the wound healing activity of GABA was more remarkable than the EGF treatment. In these results, GABA seems to have the ability to stimulate the early expression of growth factors. However, the action mechanism of GABA is still obscure. Future investigations will thus be focused on the growth factor signaling pathways.

Taken together, it was observed that GABA promoted wound healing by inhibition of inflammatory mediator expression, and stimulating cell proliferation, re-epithelialization, and releasing of growth factors, through *in vivo* and *in vitro* studies. In our studies, it was found that GABA exerted a compatible effect of accelerating the wound healing process, as compared with EGF in a cutaneous wound healing rat model. Epidermal growth factor is present in various body fluids and tissues, and considered the key regulator of keratinocyte proliferation at a wound edge [5]. Recently, the medicinal effect of EGF on wound healing was evaluated using an *in vivo* animal model [15].

In conclusion, through *in vivo* and *in vitro* studies, it was confirmed that GABA effectively enhanced wound healing by reducing inflammation, promoting the cell proliferation of fibroblast, and stimulating the expression of some growth factors. It is also suggested that GABA could effectively be exploited to develop a new drug for healing cutaneous open wounds.

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