BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 21(3), 246-250 (2013)

Chitin from the Extract of Cuttlebone Induces Acute Inflammation and Enhances MMP1 Expression

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Abstract

We previously reported that the extract from cuttlebone (CB) has wound healing effect in burned lesion of rat. In present study, the main component of CB extract was analyzed and its wound healing activity was evaluated by using *in vitro* acute inflammation model. The extract of CB stimulated macrophages to increase the production of TNF-α. The extract also enhanced the production of TGF-β and VEGF, which were involved in angiogenesis and fibroblast activation. The treatment with CB extract enhanced proliferation of murine fibroblast. CB extract also induced the activation of fibroblast to increase the secretion of matrix metalloproteases 1 (MMP1). The constituent of CB extract which has wound healing activity was identified as chitin by HPLC analysis. The mechanism that the CB extract helps to promote healing of burned lesion is associated with that chitin in CB extracts stimulated wound skins to induce acute inflammation and to promoted cell proliferation and MMP expression in fibroblast. Our results suggest that CB or chitin can be a new candidate material for the treatment of skin wound such as ulcer and burn.

Key Words: Cuttlebone, Chitin, Fibroblast, Matrix Metalloprotease, Cytokine

INTRODUCTION

The wound healing consists of acute inflammation, angiogenesis, and re-epithelialization and a cellular, physiologic, and biochemical reaction initiated after the stimulus of injury to tissue (Singer and Clark, 1999). When a dermal wound is filled by a clot, inflammatory cells migrate to skin lesion and secret growth factors, such as transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF), which stimulate fibroblasts from the adjacent dermis to migrate to the wounded site and involve in angiogenesis.

MMPs (matrix metalloproteases) are enzymes pivotal to the remodeling of the extracellular matrix (ECM) in a variety of physiological processes. MMPs are also involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling (Sternlicht and Werb, 2001). Among the MMPs, MMP-1 is the main collagenase secreted by fibroblasts and degrades the native collagens initiating tissue remodeling (Birkedal-Hansen *et al.*, 1993).

Cuttlebone (CB), which is known as cuttlefish bone, has been traditionally used as a medicine for treating sore skin. Recent studies showed antibacterial activity (Shanmugam *et al.*, 2008) and bone healing properties (García-Enriquez *et al.*, 2010) of CB. We, previously, demonstrated that the extract of CB showed the wound healing activity on skin ulcer lesion in burned rat (Jang *et al.*, 2013). However, the component showing wound healing has not been elucidated yet. In this study, CB extract were evaluated for their wound healing activity by using *in vitro* acute inflammation model and its component was analyzed by HPLC. In addition, MMP-1 activity and mRNA expression in cells stimulated with the extract were investigated.

MATERIALS AND METHODS

Preparation of cuttlebone (CB) extract

CB extract was prepared following the procedures outlined by previous reports (Hackman, 1954; Hackman and Goldberg, 1965) with some modifications (Jang *et al.*, 2013). In brief, CB powder was treated with 6N HCl for de-mineralization at room temperature. Additional de-mineralization was performed by treating with 2N HCl. CB was washed with distilled water (DW)

Open Access **http://dx.doi.org/10.4062/biomolther.2013.036**

Received Apr 24, 2013 **Revised** May 10, 2013 **Accepted** May 14, 2013

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and precipitated with 4% NaOH solution. And then sample was washed with ethanol and dried (Fig. 1A). The resulting extract was used for this study.

HPLC analysis

CB extract was analyzed using the HPLC system consisted of a temperature-controlled autosampler, column oven, and a binary pump. A 20 μl volume of sample solution or standard was directly injected on a Gemini C18 column (150×4.6 mm; Phenomenex) using a gradient acetonitrile-water solvent system. The step gradient elution was as follows: 10% acetonitrile for the first 5 min, 10% to 80% acetonitrile for a further 25 min, and then, 80% to 90% for the next 5 min, followed by maintaining the condition for another 5 min. A conditioning phase was subsequently used to return the column to its initial state. The flow rate was 0.7 ml/min, and the column temperature was set at 35°C. The solution was detected at 254 nm using a DAD detector. Chitin (Sigma-Aldrich Korea) was used as a standard compound.

Cell culture and treatment

The mouse macrophage cell line, RAW 264.7 cells and murine embryonic fibroblast, MEF were cultivated in DMEM (Hyclone, Logan, UT. USA) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (Sigma-Aldrich Korea) in 24-well cell culture plate in 5% CO_2 at 37°C overnight. The cells were treated with the CB extract at dose dependent manner for 24 h. Cell culture supernatants and cell lysates were used for measurement of cytokines and for western blot, respectively. Total RNA was also used for RT-PCR.

Cell viability assay

MTT (methythiazolyldiphenyl-tetrazolium bromide, Sigma-Aldrich Korea) assay was used to measure the cytotoxicity of CB extract on the cells. Cells were plated in 96-well plates and incubated for 24 h (approximately 80 to 90% confluency), followed by treatment with CB extract. After incubation, the medium was removed and culture media containing MTT was added and cultured for 4 h. Absorbance of samples was measured at 550 nm with a microplate reader (Molecular Devices, Menlo Park, CA, USA). Trypan blue exclusion method was also used to determine viable and dead cells to test cytotoxicity of CB extract. This assay was repeated three times with triplicate samples for each measurement.

Measurement of cytokines

Cell culture supernatants were assayed for mouse tumor necrosis factor (TNF)-α, TGF-β, and VEGF using DuoSet ELISA kit (R&D system, Woongbe MeDiTech, Inc. Seongnam, Kyunggi-Do, Korea) according to the manufacturer's instruction.

Measurement of MMP-1 activity

MMP-1 expression and activity were determined by RT-PCR and western blot, respectively. Secreted protein from cell culture supernatants and cell lysates were mixed with 5× sample buffer [10% SDS, 12.5% β-mercaptoethanol, 300 mM Tris (pH 6.8), 0.05% bromophenol blue, and 50% glycerol] and heated at 95°C for 3 min before electrophoresis on SDS-PAGE gels. Proteins were transferred onto polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% nonfat milk in PBS containing 0.2% Tween® 20 (Sigma) and incubated with anti-MMP-1 antibody (Abcam). Reactive proteins were visualized by HRP conjugated secondary antibodies (Zymed) and developed by chemiluminescence using ECL-plus (Amersham Biosciences). β-actin was used as a control.

RT-PCR

Total RNA was extracted with Trizol reagent from the cells. Reverse transcription was performed using 5 μg of total RNA and cDNA was synthesized using Transcriptor First Strand cDNA synthesis kit (Roche Applied Science). RT-PCR was performed for 35 cycles of denaturation (94°C, 30 s), anneal-

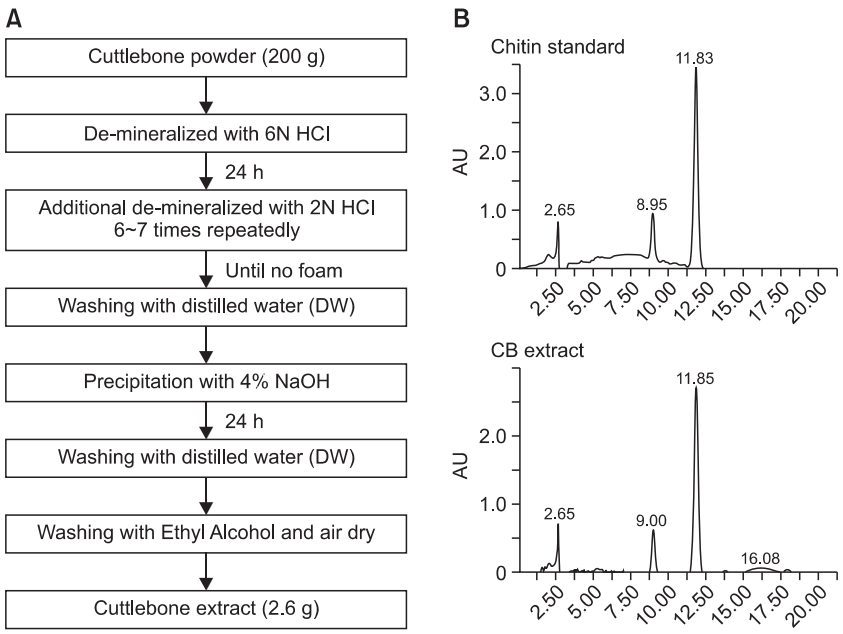

Fig. 1. (A) The scheme for the preparation of cuttlebone extract. (B) HPLC chromatogram of chitin standard and CB extract.

ing (58°C 45 s), and extension (72°C, 45 s) with a final extension at 72°C for 10 min. PCR products were visualized by electrophoresis on 2% agarose gel with ethidium bromide. The sequences of the primers were as follows: GAPDH; forward primer, 5'-aca gtc ttc tga gtg gca gtg a-3' and reverse primer, 5'-gtg ctg agt atg tcg tgg agt ct-3', MMP-1; forward primer, 5'-tgg acc tgg agg aaa tct tgc-3' and reverse primer 5'-aga gtc caa gag aat ggc cga-3'.

Statistical analysis

For statistical evaluation, one-way ANOVA was used. When significant differences were found, Newman-Keuls test was used as a post-hoc test. All data were expressed as the mean ± SEM. Significance was set at $p<0.05$.

RESULTS

HPLC analysis of CB extract

A high performance liquid chromatographic method was applied to compare the chemical of CB extract, which showed the wound healing effect on skin burned lesion in rat (Jang *et al.*, 2013). Our result showed that the constituent of CB extract which has inflammatory activity was identified as chitin by HPLC analysis (Fig. 1B).

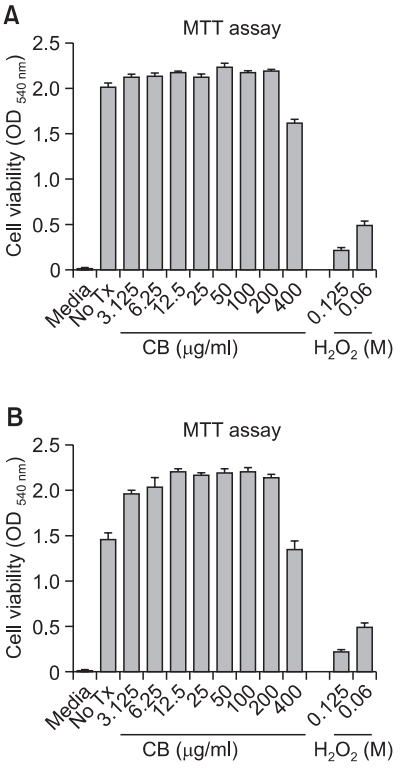

Fig. 2. The cytotoxicity of CB extract in (A) RAW 264.7 cell and (B) fibroblast. The cells were treated with the extract at a dose dependent manner for 24 h, and media containing MTT were added for measurement of cell viability. Data are representative of at least three independent experiments, each done in triplicate. *$p<0.05$, **$p<0.01$ compared to non-treated cells.

The extract of CB increased production of TNF-α, TGF-β and VEGF by macrophages

The cytotoxicity the CB extract used in this study was evaluated in RAW 264.7 cells and fibroblasts using MTT assay and trypan blue exclusion method, and there is no effect on cell viability at the concentrations used and at even higher dose, 200 μg/ml (Fig. 2).

The mechanisms underlying wound healing processes involve the acute inflammatory mediators. The new formation of blood vessels (angiogenesis) is necessary and a vital component in wound healing. The migrating fibroblasts fill the wounded site and stimulate the formation of granulation tissue (Peppa *et al.*, 2003; Folkman, 2007). TNF-α has a pivotal role in the activation of vascular endothelial cell, the induction of angiogenesis, and proliferation of fibroblast. TGF-β and VEGF are involved in fibroblast migration and angiogenesis, respectively. Therefore we first examined the *in vitro* mechanism of CB extract on wound healing and accessed whether

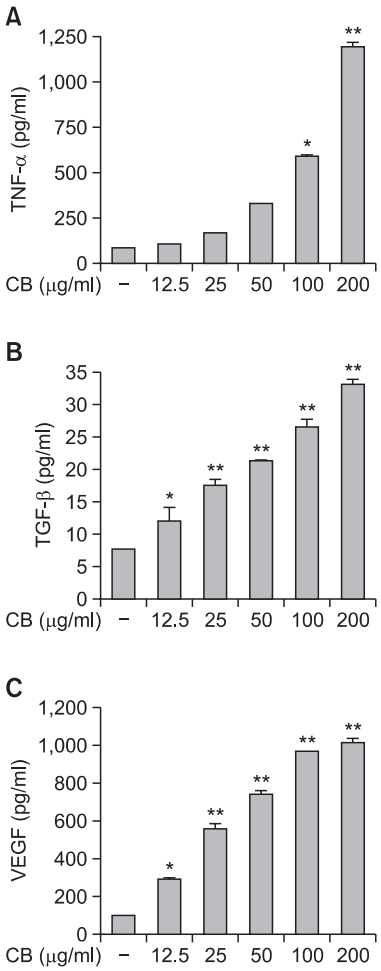

Fig. 3. The effect of CB extract on the production of cytokines by macrophages. RAW264.7 cells were treated with the CB extract at a dose dependent manner for 24 h, and cell culture supernatants were collected for (A) TNF-α, (B) TGF-β, and (C) VEGF assay. Data are representative of at least three independent experiments, each done in triplicate. *$p<0.05$, **$p<0.01$ compared to non-treated cells.

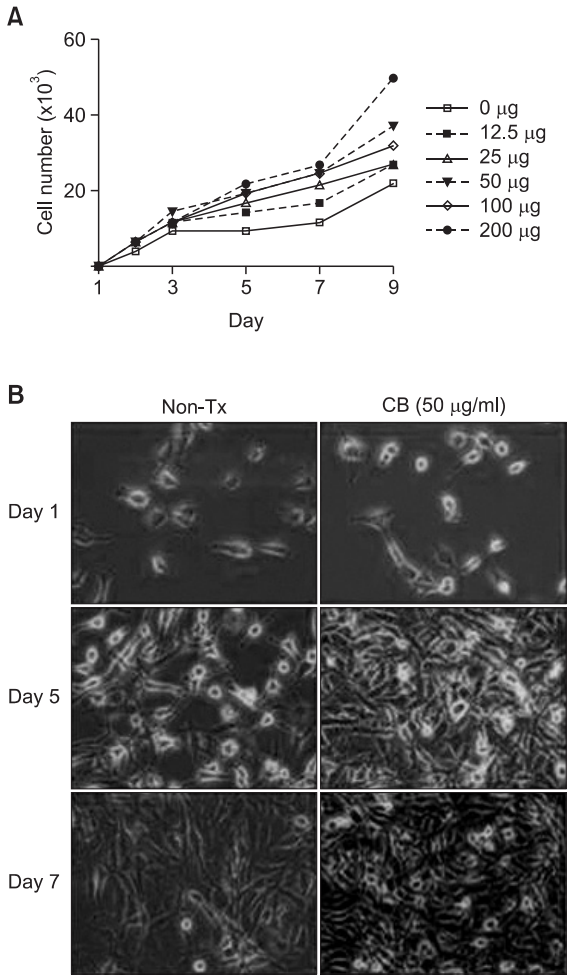

Fig. 4. Effect of CB on fibroblast proliferation. The cells were treated with the extract at a dose dependent manner for indicated times, and cell numbers were measured using (A) the trypan blue exclusion method and (B) microscopic observation. Data are representative of at least three independent experiments, each done in triplicate.

the wound healing effect of CB extract is due to the activation of macrophages to produce cytokines, TGF-β and VEGF. RAW 264.7 cells were treated with CB extract and the levels of TNF-α, TGF-β and VEGF were measured with the cell culture supernatant. The treatment with CB extract induced the activation of macrophages and increased the production of TNF-α, TGF-β and VEGF in macrophages at dose dependent manner (Fig. 3). It seems that CB extract activates macrophages to induce acute inflammation for wound healing.

The extract of CB enhanced the proliferation of fibroblast

We, next, investigated the effect of CB extract on fibroblast cell proliferation because CB increased the production of TNF-α, which induce the proliferation of fibroblast. To assess cell proliferation, we performed the trypan blue exclusion. Microscopic observation was used for cell proliferation. As shown in Fig. 4, the crude extract of cuttlebone prompted a significant proliferation of fibroblasts at a dose dependent manner.

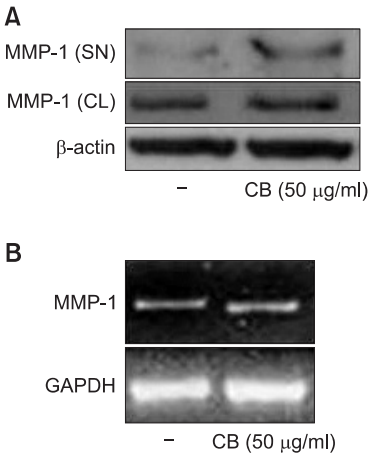

Fig. 5. The effect of CB extract on expression of MMP-1 in fibroblast. The cells were stimulated with CB extract at 50 μg/ml. (A) Cell culture supernatants were harvested and the activity of secreted MMP-1 was determined by western blot. β-actin was used as a control. SN: supernatant, CL: cell lysate. (B) Cells were harvested with Trizol and mRNA was isolated. The expression of MMP-1 mRNA was analysed by RT-PCR method and GAPDH was used as a control.

MMP-1 activity and expression

MMP-1 is secreted from fibroblast during inflammation for cell migration and wound healing. We, thus, examined whether CB extract induced MMP-1 activation in fibroblast. The MMP-1 activity was increased after treatment of the CB compared to non-treated group (Fig. 5A). We then investigated whether the increased MMP-1 activity is caused by the induction of MMP-1 mRNA. The mRNA level of MMP-1 were determined by RT-PCR and increased slightly by treatment of the extract (Fig. 5B).

DISCUSSION

Our study has a new finding that may have potential therapeutic implications. During research for novel topical drug for burn injury from natural products, we demonstrated that the CB extract has wound healing effect in burned animal model (Jang *et al.*, 2013). However, it is unknown which constituents of CB extract show the healing in burn wounds. This study showed that the constituent of CB extract showing wound healing activity is identified as chitin by HPLC analysis (Fig. 1B). In addition, we found that CB extract induced acute inflammation. Our results suggest that the increased production of TNF-α , TGF-β, and VEGF in the macrophages treated with CB extract (Fig. 4), through an unknown mechanism, is associated with wound healing effect. It is possible that the enhanced healing of wounds in burned animal by CB extract is a result of its inflammatory activity and its capacity to stimulate wound healing.

The important relationship of wound healing has been found to exist between macrophages and fibroblast (Raghow, 1994). TNF-α induced the activation of vascular endothelial cell and proliferation of fibroblast. We found that the CB extract increased the production of TNF-β in a dose-dependent manner. Furthermore, our results also demonstrated that the

production of VEGF and TGF-β also increased in macrophages treated with CB extract, suggesting that VEGF and TGF-β accelerate wound healing (Fig. 4). VEGF is one of the most potent angiogenesis stimulating growth factors and functions as an inducer of vascular permeability and an endothelial cell mitogen (Ferrara, 1999; Yamagishi *et al.*, 1999; Gavard and Gutkind, 2006). Several reports showed that VEGF increase re-epithelialization in wounded sites (Michaels *et al.*, 2005; Saaristo *et al.*, 2006; Li *et al.*, 2007).

Wound healing involving a number of processes is an orderly but complex phenomenon. An essential feature of wound healing is re-epithelialization which depends on two basic functions of keratinocytes, proliferation and migration (Broughton *et al.*, 2006). Re-epithelialization process is influenced by a combination of growth factors and cytokines including VEGF and TGF-β (Amendt *et al.*, 2002). The initial immune response to burn injury is largely pro-inflammatory.

MMP-1 is one of the most abundant enzymes in the MMP family and tight regulation of MMP-1 secretion and activity is important for tissue development and homeostasis (Sternlicht and Werb, 2001). In this study we investigated the role of CB in the regulation of MMP-1 expression in fibroblasts. CB induced the expression of MMP1 gene and the secretion of MMP1 protein, which may play an important role in the regulation of acute inflammatory reaction in pathologic status such as burn.

Chitin activates macrophage by interacting with cell surface receptors such as mannose receptor and toll-like receptor-2 (Lee, 2009). Chitin-activated macrophage enhances the formation of tissue in the wound and the production of endothelial growth factor, consistent with our results (Ueno *et al.*, 2001). Moreover, chitin is known to play an essential role in hemostasis (Valentine *et al.*, 2010).

Our results suggest that CB or chitin can be a new candidate material for the treatment of skin wound such as ulcer and burn. Identifying the relation between each step involved in wound healing and CB was pivotal for better understanding of the molecular mechanism underlying the cellular response to CB treatment during wound healing. In future studies, we will investigate the role of CB or chitin in normal functions of the connective tissue as well as in angiogenesis.

ACKNOWLEDGMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education Science and Technology (2010-0003810).

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