

Chitin from Cuttlebone Activates Inflammatory Cells to Enhance the Cell Migration

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Abstract

Our previous report showed that the extract from cuttlebone (CB) had wound healing effect in burned lesion of rat and the extract was identified as chitin by HPLS analysis. We herein investigated the morphology in CB extract using scanning electron microscope (SEM). Chitin was used as a control. There is no difference in morphology between CB extract and chitin. We also assessed the role of CB extract on the production of inflammatory mediators using murine macrophages and the migration of inflammatory cells. The extract induced the production of nitric oxide (NO) in macrophages. While the extract of CB itself stimulated macrophages to increase the expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6, CB extract suppressed the production of those cytokines by LPS. CB extract also induced the production of mouse IL-8 which is related to the cell migration, and treatment with CB enhanced fibroblast migration and invasion. Therefore, our results suggest that CB activates inflammatory cells to enhance the cell migration.

Key Words: Cuttlebone, Macrophages, Inflammatory mediator, Fibroblast, Migration

INTRODUCTION

The wound healing includes a complicated process that requires the combination of the biological and molecular events of process that consists of inflammatory response, cell migration and proliferation as well as matrix deposition, angiogenesis, and remodeling (Falanga, 2005). This process is driven by a complex signaling network that consists of a variety of cytokines, chemokines and growth factors. These are very important for the orchestrated sequence of diverse cell types during the healing process and can make cutaneous wound healing possible (Radek *et al.*, 2009; Nizamutdinova *et al.*, 2009).

During an inflammatory response, activation of resident and infiltrating inflammatory cells leads to the production of several cytokines and chemokines. One of the main cytokines produced during this response is interleukin-1 (IL-1) (Dinarello, 2009). IL-1 stimulation results in the production of several cytokines, including the chemokine IL-8 (Eckmann *et al.*, 1993; Gross *et al.*, 1995). IL-8 or CXCL8, which is also known as *neutrophil chemotactic factor*, is a chemokine produced by

macrophages and other cell types such as epithelial cells, airway smooth muscle cells, causing them to migrate toward the injury site. In target cells, IL-8 induces a series of physiological responses required for migration (Hedges *et al.*, 2000; Zimmerman *et al.*, 2008).

In the process of skin wound healing, macrophages are signaled to gather around the wound by carriers secreted by inflammatory cells. The macrophages begin to secrete cytokines and the fibroblasts move to the wound and proliferate therein forming a matrix in the dermis of the wound, thereby healing the wound.

We, previously, demonstrated that the extract of CB showed the wound healing activity on skin ulcer lesion in burned rat (Jang *et al.*, 2013) and the constituent of CB extract which has wound healing activity was identified as chitin by HPLC analysis (Lee *et al.*, 2013). We herein examined the role of CB extract on the production of inflammatory mediators in macrophages and the migration of fibroblasts.

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MATERIALS AND METHODS

Preparation of cuttlebone (CB) extract and scanning electron microscope (SEM) analysis

CB extract was prepared according to our previous reports (Jang *et al.*, 2013, Lee *et al.*, 2013). Chitin was purchased from Sigma-Aldrich (Gyeonggi-do, Korea). The CB extract and chitin were fixed by 2.5% (v/v) paraformaldehyde-glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 4°C) for 1 h. The fixed samples were washed by sodium cacodylate buffer (pH 7.4, 4°C), which containing sodium cacodylate, calcium chloride, 0.2 N hydrochloric acid, for 15 min, and postfixed by 1% osmium tetroxide (OsO₄) for 1 h. The samples were washed by sodium cacodylate buffer (pH 7.4, 4°C) for 15 min. And then, these were dehydrated by ascending grades of ethanol and brought to critical point drying for 30 min. The samples were affixed to a metal SEM stub and sputter coated in gold using an SEM coating unit. The coated specimens were viewed under SEM (JEOL, JSM-6510, Japan) at an accelerating voltage of 15 kV.

Cell culture and treatment

The murine macrophage cell line, RAW264.7 cells were cultivated in DMEM (Hyclone, Logan, UT, USA) with 10% fetal bovine serum (Hyclone) and antibiotics (Sigma-Aldrich, Korea). The cells were treated with the CB extract at dose dependent manner in presence or absence of LPS. Cell culture supernatants and total RNA from cell lysates were used for measurement of cytokines and for RT-PCR.

Nitric oxide determination

Nitric oxide (NO) levels were measured with cell culture supernatants by Griess reaction as described previously (Kang *et al.*, 2011). 50 µl of sample were incubated with 50 µl of 1% sulfanilamide (Sigma-Aldrich, Korea) solution and 50 µl of 0.1% *N*-1-naphthylethylenediamine dihydrochloride (Sigma-Aldrich, Korea) solution at room temperature for 10 min. The data was recorded and analyzed using SOFTmax version 4.6 software (Molecular Devices, Menlo Park, CA, USA).

Measurement of cytokines

Cell culture supernatants were assayed for mouse tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 (KC) using DuoSet ELISA kit (R&D system, Woongbe MeDiTech, Inc. Seongnam, Kyunggi-Do, Korea) according to the manufacturer's instruction.

RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously (Lee *et al.*, 2013). Total RNA was extracted with Trizol reagent from the cells. Reverse transcript was performed using 5 µg of total RNA and cDNA was synthesized using Transcriptor First Strand cDNA synthesis kit (Roche Applied Science). RT-PCR contained in 10 pM primer set (Table 1), reaction buffer, 10 mM dNTP, 20 mM MgCl₂ and Taq polymerase carried out for 35 cycles of denaturation, (95°C, 30 seconds), annealing (each temperature, 45 seconds) and extension (72°C, 1 minutes) with a final extension at 72°C for 5 minutes. The PCR products were visualized by electrophoresis on 2% agarose gel with ethidium bromide and under ultraviolet (UV) light.

Table 1. Sequence of the primers used in this study

Target	Primer
TNF-α	Sense 5'-TTG ACC TCA GCG CTG AGT TG-3'
	Anti-sense 5'-CCT GTA GCC CAC GTC GTA GC-3'
IL-1β	Sense 5'-GAG GAT GAG GAC ATG AGC ACC-3'
	Anti-sense 5'-CTC TGC AGA CTC AAA CTC CAC-3'
IL-6	Sense 5'-GTA CTC CAG AAG ACC AGA GG-3'
	Anti-sense 5'-TGC TGG TGA CAA CCA CGG CC-3'
GAPDH	Sense 5'-GAG TCT ACT GGC GTC TTC AC-3'
	Anti-sense 5'-CCA TCC ACA GTC TTC TGA GT-3'

Cell migration assay and invasion assay

Cell migration assay (Hulkower and Herber, 2011) was performed using modified Boyden chambers (transwell inserts) (8.0 µm pore size, BD Falcon). Murine embryonic fibroblasts (MEFs) were used for migration and invasion assay. The cells were harvested with trypsin/EDTA (Sigma-Aldrich, Korea), re-suspended to 5×10⁴ cells using serum free DMEM medium and added to the upper transwell chamber. DMEM containing 10% FBS and each sample were added into the lower chamber. The cells were allowed to migrate at 37°C in 5% CO₂ incubator. Matrigel invasion assay was performed to assess the effect of CB on invasive property of the MEF cells. Transwell inserts (8.0 µm pore size, BD Falcon) were coated with 200 µl of the Matrigel (1.0 mg/ml in ice-cold serum-free medium) solution (BD Biosciences, San Jose, CA, USA) and allowed to dry at 37°C. The control and treated cells were detached using trypsin/EDTA and 200 µl of cell suspension (5×10⁴ cells) with serum free DMEM medium were added to the upper chamber. DMEM containing 10% FBS and each sample were added into the lower chamber. After incubation for 24 hrs, cells were fixed by 3.7% formaldehyde and permeabilized by 100% methanol and stained with Giemsa solution (Daejung Chem Co, Korea). The cells of non-migrating or non-invasive cells on the upper surface of the membrane were removed with a cotton swab. The cells on the bottom surface of each membrane were photographed by microscope and counted in 6 random fields. Each migration or invasion assay was done on at least three independent occasions.

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 ± SD. Significance was set at *p*<0.05.

RESULTS

The morphology of CB extract by scanning electron microscopy (SEM)

We previously demonstrated that the constituent of CB extract we used was identified as chitin by HPLC analysis (Lee *et al.* 2013). We herein investigated the morphological characteristic in CB extract by using SEM and chitin was used as a control. There is no difference in morphology between CB extract and chitin. Although two groups had similar forms, a variety of sizes was observed (Fig. 1).

The effect of CB extract on nitric oxide production

The mechanisms underlying wound healing processes involve the acute inflammatory mediators. The first step for wound healing is to induce acute inflammation (Singer and Clark, 1999). Nitric oxide (NO) is a well known inflammatory mediator (Vane *et al.*, 1994). Therefore, we first studied the effect of CB extract on NO production in macrophages. To assess the effect of CB extract on NO production in RAW 264.7 macrophage cells, the cells were treated with or without CB

extract for 18 h. Cell culture supernatants were collected and NO levels were determined using the Griess reaction. CB extract induced NO production more than the non-treatment control group in a dose-dependent manner (Fig. 2).

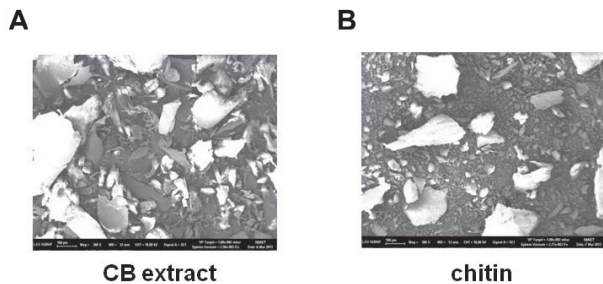


Fig. 1. SEM photos of (A) extract from cuttlebone and (B) chitin ($\times 10$ k).

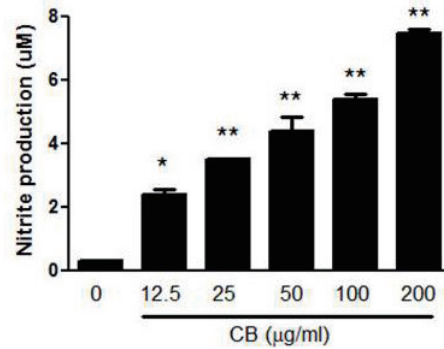


Fig. 2. The effect of CB extract on nitric oxide production by macrophages. RAW264.7 cells were treated with the CB extract at a dose dependent manner for 18 h, and then cell culture supernatants were collected for Griess reaction. Data are representative of at least three independent experiments, each done in triplicate; * $p < 0.05$, ** $p < 0.01$ compared to non-treated cells.

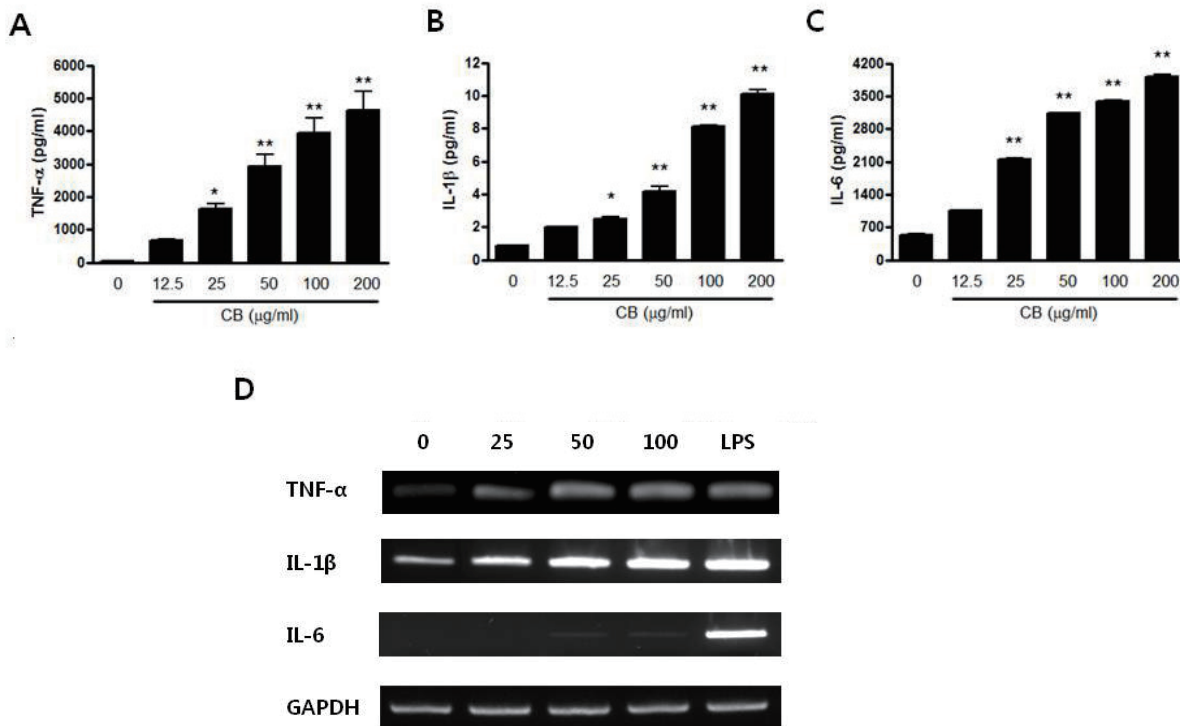


Fig. 3. The effect of CB extract on the expression of pro-inflammatory cytokines in macrophages. The cells were treated with the extract at a dose dependent manner for 18 h, and then cell culture supernatants and cell lysates were collected for (A) TNF- α , (B) IL-1 β , and (C) IL-6 ELISA assay and (D) RT-PCR, respectively. Data are representative of at least three independent experiments, each done in triplicate; * $p < 0.05$, ** $p < 0.01$ compared to non-treated cells.

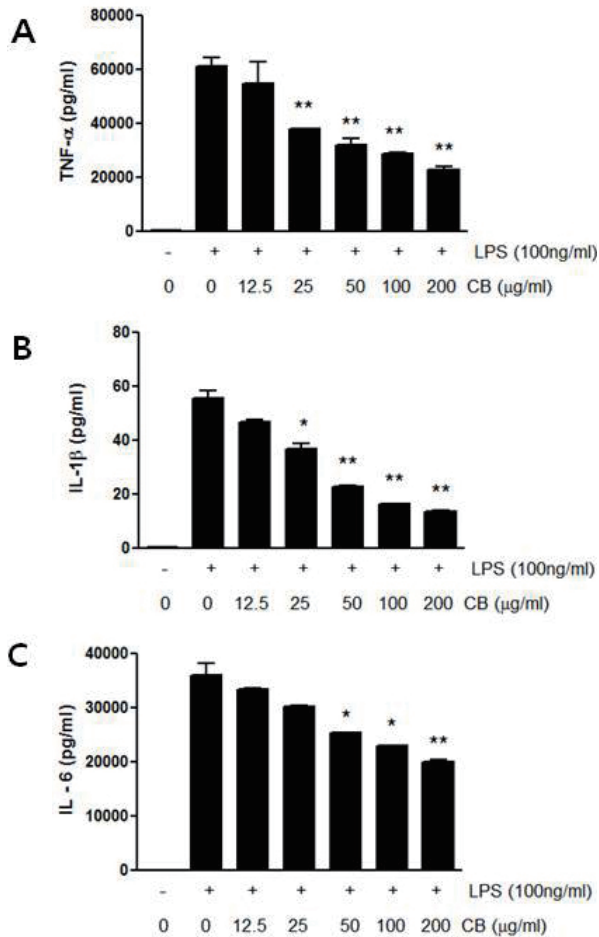


Fig. 4. The effect of CB extract on LPS-induced cytokine production in macrophages. The cells were pre-treated with LPS for 2 h and treated with the extract at a dose dependent manner for 18 h, and then cell culture supernatants were collected for (A) TNF- α , (B) IL-1 β , and (C) IL-6 ELISA assay. Data are representative of at least three independent experiments, each done in triplicate; * p <0.05, ** p <0.01 compared to LPS alone.

The role of CB extract on the production of pro-inflammatory cytokines

We, next, examined the production of pro-inflammatory cytokines, such TNF- α , IL-1 β and IL-6 and the cytokines level was measured with the cell culture supernatant. The treatment with CB extract increased the production of TNF- α , IL-1 β and IL-6 in macrophages at dose dependent manner (Fig. 3A-C). It seems that CB extract activates macrophages to induce acute inflammation for wound healing. The increase of TNF- α and IL-6 production was consistent with our previous *in vivo* data (Jang *et al.*, 2013). The mRNA expression of these cytokines by CB extract was also measured. The results showed the CB extract induced the expression of their mRNA (Fig. 3D).

Our previous study showed that CB treatment decreased the production of TNF- α and IL-6 at late time in burned rat (Jang *et al.*, 2013). Therefore we examined the effect of CB extract on the production of the cytokine in macrophages activated by LPS. Our data showed that CB extract significantly suppressed LPS-induced TNF- α , IL-1 β and IL-6 production by

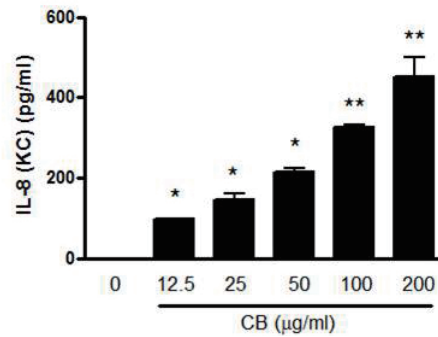


Fig. 5. The effect of CB extract on the production of IL-8 in macrophages. The cells were treated with the extract at a dose dependent manner for 18 h, and then cell culture supernatants were collected for ELISA assay. Data are representative of at least three independent experiments, each done in triplicate; * p <0.05, ** p <0.01 compared to non-treated cells.

macrophages at a dose dependent manner (Fig. 4).

The role of CB extract on the migration of inflammatory cells

Tissue formation during wound healing requires the migration of inflammatory cells such as fibroblasts and epithelial cells in particular directions to injured locations by chemotaxis (Peppas *et al.*, 2003; Folkman, 2007). IL-8 is a chemokine produced mainly by macrophages. Therefore, we examined whether CB extract induces the production of IL-8 in macrophages. CB extract increased IL-8 production in macrophages at a dose dependent manner (Fig. 5). We, next, investigated the effect of CB extract on fibroblast cell migration because CB increased the production of IL-8, which induce the migration of inflammatory cells. To assess cell migration, we performed cell migration assay using modified Boyden chambers (transwell inserts) and invasion assay (Hulkower and Herber, 2011). Microscopic observation was used for cell migration. As shown in Fig. 6, the CB extract prompted a significant migration and invasion of fibroblasts.

DISCUSSION

During research for novel bioactive material for treatment of skin injury from natural products, we demonstrated that the CB extract has wound healing effect in burned rat (Jang *et al.*, 2013) and the constituent of CB showing the wound healing effect is chitin (Lee *et al.*, 2013).

In this study, the morphology of CB extract was characterized and compared to that of chitin (Fig. 1). As we expected, CB extract is similar with chitin in morphology.

Wound healing is complex phenomenon involving a number of processes. The wound healing consists of acute inflammation, angiogenesis, and re-epithelialization after the stimulus of injury to tissue (Singer and Clark, 1999). An essential feature of wound healing is re-epithelialization which depends on two basic functions of inflammatory cells, proliferation and migration (Broughton *et al.*, 2006). The initial immune response to burn injury is largely pro-inflammatory. In the absence of inflammation, wounds would never heal on the tissue. Pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 are

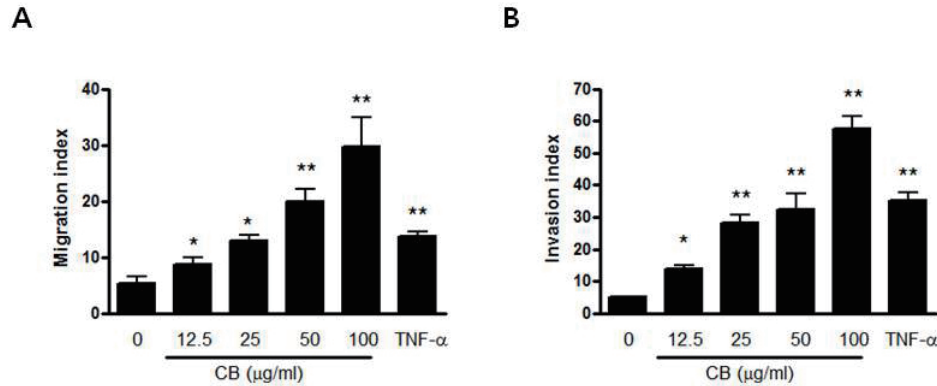


Fig. 6. The effect of CB extract on the cell migration. The assay was performed by (A) modified Boyden chamber assay and (B) cell invasion assay as described in Materials and Methods. The data were presented as the mean number of migrating cells \pm SD / microscopic field.

multifunctional cytokines that are responsible for mediating a variety of processes in the host defense response, inflammation, and the response to injury. Thus, we examined the role of CB extract on the production of the pro-inflammatory cytokine *in vitro* (Fig. 2) and CB increased the production of the cytokines in the macrophages treated with CB extract (Fig. 3) is associated with wound healing effect on damaged tissue. It is possible that the enhanced healing of wounds by CB extract in injured animal is a result of its inflammatory activity and its capacity to stimulate wound healing as we described previously (Lee *et al.*, 2013). Interestingly, the CB extract suppressed the production of pro-inflammatory cytokines in macrophages activated with LPS, suggesting that the CB may protect the cell and tissue from injury or destruction at high concentration of cytokines (Fig. 4).

The important relationship of wound healing has been found to exist between macrophages and fibroblast (Raghow, 1994). During inflammation, the migrating fibroblasts fill the wounded site and stimulate the formation of granulation tissue (Peppia *et al.*, 2003; Folkman, 2007). IL-8 induces the migration of inflammatory cells to wounded site. We found that the CB extract induced the production of IL-8 in a dose-dependent manner. In addition, our results also showed that the migration of fibroblasts was increased in CB treated group, suggesting that IL-8 accelerate wound healing via increase of cell migration (Fig. 5, 6).

It is reported that chitin activates macrophage by interacting with cell surface receptors and macrophage activated with chitin enhances the formation of tissue in the wound by migrating inflammatory cells and the production of endothelial growth factor (Ueno *et al.*, 2001; Lee, 2009), consistent with our results (Fig. 6).

Our results showed that CB extract can be a bioactive material for the treatment of skin injury. The study on the relation between each step involved in wound healing and CB was critical for understanding of the mechanism underlying the cellular response to CB treatment during wound healing.

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