β-Lapachone accelerates the recovery of burn-wound skin

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Introduction

Wound healing is a cascade of different processes that have to be closely orchestrated in order to regain normal functional restoration of the damaged tissue (Ulrich et al., 2007). Thermal injury remains a significant health problem over the world, and the proper healing of the burn-wound site is critical for patient’s successful recovery (Brigham and McLoughlin, 1996).

Wound healing has been classically organized in a progression of overlapping processes: hemostasis, inflammation, fibroplasia/proliferation, and wound remodeling. In this schema, inflammation can be characterized as a circumscribed stage of wound healing that would suggest its effects end as the next stage begins (Henry and Garner, 2003). The latest understanding indicates, however, that inflammation persists throughout all wound healing phases, stimulating and coordinating the essential functions of wound repair. In the early stages of inflammation, the recruited neutrophils quickly become the dominant leukocyte in the wound. One day after wounding, monocytes start to gather and transform into macrophages. They rapidly increase in number, replace neutrophils, and become the predominant leukocyte by day 3 after wound (Henry and Garner, 2003).

Macrophages are essential to successful wound healing, as they produce large amounts of cytokines, chemokines, and growth factors such as vascular endothelial growth factor (VEGF) and epidermal growth factors (EGF), which initiate the formation of granulation tissue. Macrophage derived growth factors are central to the initiation and propagation of new tissue growth in the wound bed. Studies have shown that macrophage depletion leads to impaired wound debridement and fibroplasia, and isolated wound...
macrophages induce angiogenesis and increase collagen synthesis and wound breaking strength. Thus, macrophages appear to play a pivotal role in the transition from the inflammatory to the proliferative phase of wound healing (Park and Barbul, 2004).

Tumor necrosis factor (TNF-α) is a pleiotropic cytokine that is mainly produced by cells of the monocyte/macrophage lineage. It is identified as an endogenous factor, induced in response to inflammatory stimuli (Tang et al., 2003). Studies regarding to the skin indicate that the significant inflammatory response to burn injury is preferentially localized to the injury site and was associated with increased levels of TNF-α (Schwacha et al., 2010).

ß-lapachone is a natural o-naphthoquinone compound obtained from the bark of the lapacho tree (Tabebuia avellanedae). Its inner bark is often used as an analgesic, anti-inflammatory, anti-neoplastic, antimicrobial, and diuretic drug in the northeastern part of Brazil (De Almeida, 1990; Ueda et al., 1994). ß-lapachone has a good antitumor effect on several carcinoma cells (Li et al., 1999), including hepatoma (Lai et al., 1998), osteosarcoma (Liu et al., 2002), breast cancer (Lin et al., 2004), prostate cancer (Don et al., 2001), and human leukemia (Chau et al., 1998; Shiah et al., 1999). Our previous findings demonstrated that punched skin wounds on mice healed much faster with ß-lapachone treatment (Kung et al., 2008). However, whether ß-lapachone can facilitate the process of burn wound healing and has a beneficial effect on the secretion of growth factor by macrophages remains to be explored.

Petroleum jelly is primarily used as an ointment for scrapes, burns, and cuts. In the present study, we compare the effect of petroleum jelly (control ointment) and ß-lapachone-containing petroleum jelly on burn wound healing in mice skin in vivo. Moreover, we demonstrated that ß-lapachone not only promoted the proliferation of macrophages both in vitro and in vivo, but also had the ability to enhance EGF and VEGF secretion, suggesting a potential application of this compound as an accelerator of burn wound repair.

Materials and methods

Chemicals

ß-lapachone, prepared as described by Schaffner-Sabba et al. (1984), was dissolved as a 20mM stock solution in DMSO and stored at -20°C. White Petrolatum Jelly was purchased from Tien Chen Pharmaceutical Co., Ltd. (Taipei, Taiwan).

Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), lysis buffer, and Triton X-100 were purchased from Sigma (St. Louis, MO). Pentobarbital were purchased from Somnotol (MTC Pharmaceuticals, Cambridge, Ontario, Canada). Hematoxylin and Eosin were purchased from Anatomical Pathology (Pittsburgh, PA). Permount was purchased from Fisher Scientific (New Jersey, USA). TNF-α, murine EGF ELISA Development Kit and murine VEGF ELISA Development Kit were purchased from Peprotech Inc. (New Jersey, USA).

2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was purchased from Sigma (St. Louis, MO). L-glutamine was purchased from Biowest (Denver, CO). Antibiotics were purchased from Biological Industries (Kibbutz Bet Haemek, Israel).

Fetal bovine serum was purchased from GIBCO-BRL (Rockville, MD). VEGF rabbit-anti-mouse IgG polyclonal antibody was purchased from Santa Cruz (Santa Cruz, CA). EGF rabbit-anti-mouse IgG polyclonal antibody was purchased from Sigma. Macrophage marker antibody (MCA1849) was purchased from AbD Serotec (Raleigh, NC). TRITC conjugated goat-anti-rabbit IgG secondary antibody was purchased from Perkin Elmer (Boston, MA). FITC conjugated rabbit-anti-rat IgG secondary antibody was purchased from Sigma.

Animals

A total of 20 adult C57BL/6 male mice (10 wk old) purchased from the National Taiwan University Animal Center were housed in individual cages in a temperature- and humidity-controlled room (12:12-h light-dark cycle) with free access to tap water and diet. All of the animal experiments were performed according to National Institutes of Health guidelines and were approved by the Laboratory Animal Committee of the College of Medicine, National Taiwan University.

Wound biopsy and measurement of wound closure

Mice (C57BL/6) were anesthetized with sodium pentobarbital (1.5 ml/25 g bw). The back of the mouse was shaved and then sterilized by using an alcohol swab. A 100°C iron stick connecting to a thermostat was used to burn the back of the mouse below the shoulder blades for 60 seconds to create 2 wounds (6-mm diameter). The wounds placed in this area cannot be reached by the mouse and therefore prevents self-licking. Control ointment (100mg pure white petroleum jelly, Vaseline) or the ointment containing 29.8 µg ß-lapachone/g petroleum jelly was applied to the wound area and changed every 2 days. Wounds from individual mice were digitally photographed daily after wounding. For all measurements, the wound area was quantified by software Scion Image 4.03 (http://en.bio-soft.net/draw/ScionImage.html).

Histological examination

During the process of wound closure, skin samples (approximately 1x1 cm2) containing the wound areas were collected at 1, 3, 7, 14, or 21 days post-wounding and fixed in 4% formaldehyde for histological study.
The samples were frozen and transversely cut into 8-µm-thick sections and all the sections were stained with hematoxylin and eosin before examination in a Zeiss Axiphot light microscope.

**Macroage assay**

At 1, 3, 7, 14, or 21 days post-wounding, mice were euthanized with an overdose of sodium pentobarbital. The wound areas were removed by cutting out a square area containing the entire wound site, and the tissues were immediately placed in 4% paraformaldehyde for 1wk. Cryosections (8 µm) from the middle part of the wound areas were used for immunofluorescence for general histological observation. For immunofluorescence, the sections were blocked with 10% NGS in PBS for 2h at room temperature and then incubated overnight at 4°C with polyclonal rat-anti-mouse macroage marker MCA1849 (1:300). After several washes with PBS, the sections were incubated for 1h at room temperature with fluorescence-conjugated secondary antibody, washed, and mounted with 50% glycerol in PBS. The number of macroages in the wound area was counted, and the sections were photographed with a Leica fluorescence microscope (DM 2500) equipped with a DFC310 FX 1.4-megapixel digital color camera.

**Cell culture**

Mouse macroage RAW264.7 cells were cultured in 5% CO₂ at 37°C in DMEM containing 10% fetal bovine serum, 2mM glutamine, and 100 µg/ml each of penicillin/streptomycin (all from GIBCO-BRL, Rockville, MD).

**Cell treatment and cell viability assay**

RAW264.7 cells (1x10⁴) in 100µl medium were seeded for 24h at 37°C in a 96-well culture plate in a humidified 5% CO₂ atmosphere. For MTT assay, various concentrations of ß-lapachone were added to the medium 24h before the cell viability assay. And the best concentration of ß-lapachone for cell growth was chosen to perform the time-dependent cell growth assay. In brief, 10 µl MTT (0.5 mg/ml) were added to each well and the plates were incubated at 37°C for 4h. The formazan product was then dissolved in 100 µl DMSO at 37°C for 30 min, and absorbance at 570 nm was measured with a microplate reader.

**Measurement of VEGF and EGF**

1x10⁴ macroages were seeded in 96-well plates and different treatments were applied (10 ng/ml TNF-α, 0.5 µM ß-lapachone, 10ng/ml TNF-α + 0.5 µM ß-lapachone, or 5 min pre-treatment of 10ng/ml TNF-α alone before 10 ng/ml TNF-α + 0.5µM ß-lapachone) for 0, 5, 15, 30, 60, 180, 1080 min, respectively. The medium was collected, and the levels of VEGF and EGF were measured with the Murine EGF/VEGF ELISA Development Kits and ABTS is used as a chromogen. The level of VEGF or EGF was determined by the intensity of the absorbance/reference absorbance at 405 nm/ 650 nm on an ELISA reader (BioTek, Winooski, VT).

**Statistical analysis**

All data are presented as mean ± SD, and differences between groups were analyzed using Student’s t-test. P<0.05 was considered statistically significant.

**Results**

**Effects of ß-lapachone on burn-wound healing in vivo**

To determine whether ß-lapachone had a therapeutic effect on burn-wound healing, control ointment or ß-lapachone-containing ointment (29.8 µg/g, equivalent to 100 µM) were applied to a wound on the back of C57BL/6 mice for 22 days. Based on daily observations, the area of the ß-lapachone-treated wounds was significantly reduced than that of the mice treated with control ointment. On day 7, scar tissue was thick in both groups. On day 10, the wound treated with ß-lapachone started to shrink and was completely sealed on day 21, while the wound treated with control ointment still had about the size of 4 mm² (Fig. 1). The wound area was measured daily and analyzed quantitatively.

Our statistical results showed that the recovery process of wound healing by ß-lapachone treatment was faster than that of the control. From day 3 after wounding, wound area treated with ß-lapachone-containing ointment became markedly smaller than that of mice treated with control ointment and continued to shrink at a tremendous pace (Fig. 2).

Histological observations showed that on day 1 post-wounding, tissues in burn-wound areas from both control and ß-lapachone-treated groups were sparse and contained many small cavities. That’s because cells of the epidermis, dermis, and hypodermis were destructed by heat. On day 3, cells treated with ß-lapachone started to line up in order. In contrast, tissue treated with control ointment appeared as a big hollow because the cells were no longer alive and were easily washed away during the staining process. Moreover, the skin started to recover on day 7 and hair follicles can be seen on day 14 in the wound treated with ß-lapachone, while the dermis still appeared disorderly in the wound treated with control ointment (Fig. 3A,B).

We also used micrographs of H&E stain to measure the diameter of the wounds (If the wound appeared in an oval shape, the average of long and short axes was measured). The diameters of the ß-lapachone-treated wounds were significantly shorter than those treated...
with control ointment from 3 to 21 days post-wounding (Fig. 3C).

Data from immunostaining with MCA1849, a macrophage marker, showed that the number of macrophages appearing in the wound area after β-lapachone treatment was significantly greater than that treated with ointment without β-lapachone at day 3, 7, and 14 (Fig. 4).

Effects of β-lapachone on RAW264.7 macrophage cell line

Since β-lapachone can increase the number of macrophages in the healing process of the burn-wounds as we have seen in the cut-wound (Kung et al., 2008), we used the MTT assay to examine the effect of 24h treatment with different concentrations of β-lapachone on a macrophage cell line, RAW264.7.

Low concentrations of β-lapachone promote cell proliferation

After incubation with low concentrations (up to 0.5 µM) of β-lapachone, the cell proliferation rate increased in a dose-dependent manner and peaked at 0.5 µM at 133%. At higher concentrations, β-lapachone was cytotoxic to cells and cell survival rate dropped to 13-71%. Because 0.5 µM β-lapachone promotes cell proliferation the most, we used this concentration of β-lapachone to do rest of the experiments (Fig. 5A).

We next examined the effect of 0.5 µM β-lapachone on RAW264.7 cells with different treatment time. From MTT data, it showed that cells treated with β-lapachone proliferated faster than cells treated with control medium

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**Fig. 1.** β-lapachone is effective in the in vivo burn wound healing. A-D. Control ointment or ointment containing β-lapachone (100µM) was applied to a wound on the back of C57BL/6 mice for 22 days, and photographs were taken every day from wounding day (day 0) to day 21 postwounding. The wound area after treatment with ointment containing β-lapachone (B, D) is smaller than that of on mice treated with control ointment (A, C).
and reached significant statistical difference at 18 and 24 hr (Fig. 5B,C).

*Treatments of TNF-α and β-lapachone stimulate macrophage to secrete EGF*

Except of cleaning the debris of the wounds, macrophages can also secrete many growth factors, such as VEGF and EGF, to accelerate the growth of endothelial cells, epithelial cells, and those contributed to the healing of wounds. We used TNF-α to mimic the inflammation status appeared in the healing process and to see whether β-lapachone can increase the release of growth factors from macrophages which may explain the reason why macrophage appears a lot in the wounded area treated with β-lapachone in mice. We set up 5 different conditions to investigate the effects of β-lapachone on macrophage growth factor release: (1) DMEM with no serum (control), (2) 10 ng/ml TNF-α, (3) 0.5 µM β-lapachone, (4) 10 ng/ml TNF-α + 0.5 mM β-lapachone, and (5) 5 min pretreatment of 10 ng/ml TNF-α alone before adding 10 ng/ml TNF-α + 0.5 µM

**Fig. 2.** Histogram showing the recovery of burn wounds in the in vivo experiment that the area of the β-lapachone-treated wounds is significantly smaller than that of wounds treated with control ointment. Note that at day 3 post-wounding, the wound area of both control and β-lapachone-treated wounds were larger than those at day 0. After day 10, wound healing was significantly progressed when treated with β-lapachone. *Significant difference (P < 0.05) as analyzed by Student’s t-test.

**Fig. 3.** Histological examinations of the wound area in the in vivo experiment. A-B. Hematoxylin and eosin stained sections at day 1, 3, 7, 14, or 21 postwounding. The recovery of epidermis (cuticle and scar tissue) and dermis (connective tissue and hair follicle) in the wounds treated with β-lapachone (B) was faster than that treated with control ointment (A). C. The greatest diameters of the burned wounds. Wounds treated with β-lapachone healed faster than wounds treated with control ointment. The greatest diameters of β-lapachone-treated wounds were shorter than the control group and reached a significant difference on day 3, 7, 14, and 21. *Significant difference (P < 0.05) as analyzed by Student’s t-test.
β-lapachone. The effects of TNF-α and β-lapachone on the secretion of macrophages EGF were analyzed by ELISA. Medium was collected after 5, 15, 30 min and 1, 3, 18 hr of treatments. Results showed that after 5 min of treatment in these five groups, the amount of EGF apparently raised and the total amount of EGF increased 4 folds within 30 min and then decreased in a time-dependent manner in the group that were treated with “5

![Immunostaining of macrophages in the wound area with macrophage marker (MCA1849) on day 3 postwounding showed that β-lapachone enhances macrophage recruitment. The density of macrophages in wounds treated with β-lapachone (C, D) was greater than that treated with control ointment (A, B). E. Histogram shows the significant quantitative increase (P < 0.1) in the ratio of the number of macrophages in the β-lapachone treated wounds as compared with vehicle treatment at day 7, and 14 after wounding.](image-url)
min of 10 ng/ml TNF-α alone before adding 10ng/ml TNF-α + 0.5 µM β-lapachone”. The amount of EGF had also increased to almost 300% within 30min and then decreased slowly in TNF-α + β-lapachone treatment. The other two groups didn’t show any significant change as compared with control (Fig. 6A).

**β-lapachone induces macrophage to secrete VEGF**

The effects of TNF-α and β-lapachone on the secretion of VEGF by macrophages under five different treatments described above were also analyzed by ELISA. The amount of VEGF in the 0.5 µM β-lapachone-treated group apparently raised within 5min and reached the climax of 213% at 30min. Significant difference appeared up to 60min after β-lapachone administration as compared with control. On the other hand, macrophages treated with 5 min of 10ng/ml TNF-α alone before 10 ng/ml TNF-α and 0.5 µM β-lapachone secreted least VEGF, and significant difference appeared in 30 to 180min when compared with control (Fig. 6B).

Immunostaining of macrophages shows that 30 min after “5 min pre-treatment of TNF-α alone before TNF-α + 0.5 µM β-lapachone” treatment (Fig. 6D), the secretion of EGF was greater than those treated with control vehicle (DMEM without serum) (Fig. 6C). Administration of β-lapachone induced macrophages releasing more VEGF (Fig. 6F) than those with vehicle treatment did (Fig. 6E).

**Discussion**

Recent studies have shown that β-lapachone at high concentrations (>2 µM) has a cytotoxic effect and is a novel antitumor agent with specific anticancer activity against human lung, prostate, and breast tumors (Ough et al., 2005). It has also been shown to have anti-inflammatory effects because it can decrease inducible nitric oxide synthase (NOS) expression, NO production (Liu et al., 1999), and NF-κB activation (Moon et al., 2007) in LPS (lipopolysaccharide)-stimulated macrophages, thus protecting against LPS-induced lung edema and decreasing mortality in LPS-mediated sepsis (Tzeng et al., 2003). Previous studies have reported that high doses (>2 µM) of β-lapachone induce either apoptotic or necrotic cell death in a variety of human carcinoma cells, but not in normal cells. On the contrary, we reported that low doses of β-lapachone (0.1 µM) have the ability to enhance the proliferation of various cells, including different keratinocytes, fibroblasts, and endothelial cells, and may play an important role in promoting wound healing process (Kung et al., 2008). However, very little is known about the biological activity of low-dose β-lapachone on macrophages which appears to be of critical importance in the recovery of burn-wounded skin. This leads us to investigate the effect of β-lapachone on the macrophage cells line RAW264.7.

In the present study, we reported that β-lapachone
Fig. 6. *In vitro* assay showing the effects of β-lapachone and TNF-α on the secretion of EGF and VEGF from macrophages. A. Pre-treatment of TNF-α alone 5 min before TNF-α + 0.5 μM β-lapachone enhanced the greatest release of EGF. B. β-lapachone only (0.5 μM) treatment induced macrophage to secrete VEGF, while 5 min pre-treatment of TNF-α alone before TNF-α + 0.5 μM β-lapachone restricted the release of VEGF following 30 min-3 hr. *Significant difference (P<0.05) in EGF or VEGF secretion after various treatments (for 5, 15, 30 min and 1, 3, 18 hr) as compared with vehicle treatment (DMEM without serum). (n=5 respectively). C-F. EGF and VEGF secretion under 30 min of various treatments. Immunostaining of macrophages shows that after "5 min pre-treatment of TNF-α alone before TNF-α + 0.5 μM β-lapachone" treatment (D), the secretion of EGF was greater than those treated with control vehicle (DMEM without serum) (C). Administration of β-lapachone induced macrophages releasing more VEGF (F) than those with vehicle treatment did (E).
\textbf{β-lapachone in burn-wound skin}

has the ability to accelerate the recovery of burn-wounded skin. Major thermal injury occurs at an alarming frequency in the United States with greater than 2 million cases per year (Schwacha, 2003) and healing of the burn wound is definitely a critical component of the burn patient’s successful recovery. Treatment with β-lapachone significantly accelerated wound healing with the wounds being made by a sterile biopsy punch. We therefore did a further study to examine whether β-lapachone facilitate the recovery of burn-wounded skin. To test the therapeutic effect of β-lapachone, a 100°C iron stick connecting to a thermostat was used to burn the skin on the back of the mouse. The concentration of β-lapachone applied to the wound was approximately 100 times of that in the in vitro experiment as described in our previous report (Kung et al., 2008). Microscopic examination showed that the recovery of the epidermis and dermis was faster in the burn wounds treated with β-lapachone-containing ointment than those treated with ointment containing no β-lapachone (Fig. 3), suggesting that β-lapachone possesses a therapeutic effect in the burned wound healing process. On the other hand, we also demonstrated that the number of macrophages found around the β-lapachone-treated wound was significantly greater than that without β-lapachone treatment (Fig. 5E), indicating that macrophages might play a pivotal role in causing this tremendous difference of healing speed between wounds treated with or without β-lapachone.

In the early stages of wound healing, there is constant and increasing trafficking of leukocytes to the wound bed. Neutrophils are the first inflammatory cells to reach the wound scene and are replaced by monocytes at day 3 after wound (Kibbey et al., 1994). Monocytes are chemoattracted to the wound by a wide variety of factors including TNF-α, which is a powerful stimulant of monocytes infiltration (Wolpe et al., 1988; Murphy, 1994; Luster and Rothenberg, 1997). Monocytes then mature into macrophages through activation by IL-2, IFN-γ, and TNF-α (Tzeng et al., 1985). These activated macrophages become the orchestrator of the wound and secrete more than 100 different substances, many of which are cytokines and growth factors that further stimulate the inflammatory process and up regulate wound repair system (Hubner et al., 1996; Leibovich and Wiseman, 1988; Lingen, 2001). Recent studies have reported that β-lapachone inhibits the LPS-induced activation of BV2 microglia (a resident macrophage-like population of brain cells) (Moon et al., 2007). In the present study, we reported that low doses of β-lapachone (0.005-1 µM) enhanced the proliferation of macrophages. Nevertheless, no other study has investigated the interactions between β-lapachone, TNF-α and EGF, VEGF secretion of macrophages. The present study is the first to demonstrate that 5min pretreatment of TNF-α alone before TNF-α + β-lapachone had the strongest effect of inducing EGF secretion while it inhibited VEGF secretion compared to control. VEGF secretion only increased when burn wound and RAW264.7 cell line were treated with β-lapachone alone. It is partially possible that the great amount of EGF secretion triggered by β-lapachone combined with TNF-α accelerates the recovery of wound by increasing the proliferation of keratinocytes first when wounds were still under inflammation. Another possibility is that by the time β-lapachone reaches its maximal effects of inducing VEGF secretion, TNF-α has already been used up and therefore promoting the angiogenesis in burned-wound area.

In summary, from in vitro assays of the present, it is clear that β-lapachone promotes the proliferation of macrophage and accelerates burned-wound healing by increasing the EGF secretion, and in the in vivo assays, β-lapachone has prominent anti-inflammatory effects. The possibility of β-lapachone possessing the ability to inhibit the inflammation effects of macrophages and simultaneously inducing EGF secretion of macrophages to facilitate the growth of other cells can not be excluded. Taking all these data together, we concluded that (1) β-lapachone has the ability to facilitate the burn wound healing and to enhance the EGF and VEGF secretion in the macrophages, and (2) β-lapachone has a potential therapeutic application for burn wound healing. Further investigations of the anti-inflammation mechanisms of β-lapachone are needed to permit full exploitation of its promise on burned wound repair.

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References


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