Induction of Metalloproteinase 9 Secretion from Human Keratinocytes by Pleuran (β-Glucan from *Pleurotus ostreatus*)

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Z. Naturforsch. **64c**, 597–600 (2009); received February 25/March 16, 2009

Glucan preparations, primarily modified water-soluble glucans, are involved in the activation of the body's natural defense mechanisms and in the acceleration of the skin's wound-healing processes. Pleuran, an insoluble β-D-glucan in hydrogel form, offers a natural alternative to more common chemically derivated soluble β-D-glucans. Pleuran was applied to human keratinocyte primary cultures, and after 24 h of incubation the release of matrix metalloproteinase 9 (MMP-9) and metalloproteinase 2 (MMP-2) by stimulated keratinocytes was detected using gelatine zymography. There was a concentration-dependent increase in pro-MMP-9 release after treatment with pleuran over the concentration range of 2 to 200 μg/ml, but pro-MMP-2 was detected at a constant level. Moreover, the active forms of both MMPs were not detectable, indicating that *in vitro* autoactivation of these zymogens did not occur. The results indicate that pleuran is a potent keratinocyte stimulator of pro-MMP-9 release, which implies its application in dermatological therapies.

Key words: MMP-9, Glucan, Keratinocytes

Introduction

Pleuran is an unique biopolymer, which has been isolated from the fruiting bodies of the edible mushroom *Pleurotus ostreatus* and has been in commercial production for many years. The primary structure of pleuran is similar to that of β-D-glucans commonly found in other Basidiomycetes and Ascomycetes (Karacsonyi and Kuniak, 1994). Nevertheless, the solubility properties of pleuran appeared to be distinctly different in contrast to water- and alkali-soluble β-D-glucans; pleuran was found to be insoluble in alkali (Karacsonyi and Kuniak, 1994). It has been shown that pleuran exhibits high antitumour activity (Yoshioka *et al.*, 1975, 1972), enhances the antioxidant defense of the colonic wall against inflammatory attack (Bobek *et al.,* 2001), and stimulates delayed-type hypersensitivity response and phagocytic ability of blood leucocytes in mice (Paulík *et al.,* 1996).

Pleuran in a hydrogel form is much more refined in order to have smaller particle sizes for easier use in cosmetics. It was documented that carboxymethyl-glucan (CM-glucan) takes part in accelerating the skin's wound-healing process and improves the skin function (Zulli *et al.*, 1998). The critical factor in optimal healing of skin wounds is re-epithelialization, which involves migration and proliferation of keratinocytes to cover the denuded dermal surface. Keratinocytes provide a barrier between the host and the environment. Immature keratinocytes produce matrix metalloproteinases (MMPs) and plasmin to dissociate from the basement membrane and facilitate their migration. Matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) are zinc-dependent metalloenzymes and have gelatine-degrading activity. Both MMPs are known to be secreted by many types of cells and play important roles in several biological events including tissue re-modeling and wound healing.

The aim of the present study was to characterize the effect of fungal β-D-glucan hydrogel (pleuran) on selected MMPs release from human keratinocytes.

0939–5075/2009/0700–0597 $ 06.00

Material and Methods

Preparation of pleuran hydrogel

β-D-Glucan was isolated from fruiting bodies of *Pleurotus ostreatus* according to the Slovak patent No. 282870 (Pleuran s.r.o., 2004).

A method to prepare a fungal glucan hydrogel by alkaline deproteination and subsequent elimination of water-soluble components consists of subsequent hydration of the obtained insoluble glucan by wet grinding at a rotational speed of 3000 to 9000 rpm for 10 to 20 min to get a swelling volume in water of 50 to 500 ml/g. Then it is adjusted by heat-sterilization at a temperature of 90 to 110 °C for 20 to 30 min, what results in a gel which is formed by a fungal polysaccharide with a β-(1,3)-D-bond in the principal chain, with a content of 0.5 to 3% by weight. In the present study 2% (w/v) pleuran was employed, and treatments were carried out with pleuran over the concentration range 2 to 200 μg/ml in human keratinocyte cultures.

Isolation of keratinocytes

Full thickness skin obtained from healthy children undergoing plastic surgery procedures (otoplasty) following approval from the Ruzinov Hospital Ethical Committee was used for the isolation of keratinocytes. Skin tissue was cut into small pieces and incubated overnight with 2 U/ml of dispase (Invitrogen, Paisley, UK) at 4 °C. The epidermis was separated from the dermis with fine forceps and placed in 3 ml of 0.05% trypsin/0.02% EDTA solution (Invitrogen) for 15 min. An equal volume of soybean trypsin inhibitor (0.25 mg/ml; Sigma-Aldrich, Poole, UK) was employed to stop the trypsin activity. The cells were passed through an 100-μm sterile gauze, washed and cultured in serum-free keratinocyte-specific growth medium (Invitrogen) according to the manufacturer's instruction. Cell purity was evaluated by immunocytochemical detection of cytokeratin, and cells were employed in experiments within the first or second passage. Standard 24-well plates were used to subculture human keratinocytes at a seeding density of 50,000 cells per well.

Gelatine zymography

The keratinocyte cell supernatant was subjected to gelatine zymography in 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAGE) incorporating 1 mg/ml gelatine according to the method of Kleiner and Stetler-Stevenson (1994). Gels were incubated overnight in MMP activation buffer (50 mM Tris/HCl, pH 7.8, 50 mM $CaCl_2$, and 0.5 M NaCl) and stained with Coomassie Blue R-250. After destaining, the extent of gelatine digestion was examined.

Statistical analysis

Results are presented as the mean ± standard error (SEM). All data were statistically analyzed by the parametric paired *t*-test to determine whether there were differences between control and cultures treated with pleuran. *P* values smaller than 0.05 were considered to be significant. Analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Results and Discussion

β-Glucans have been used for a long time for cosmetic and pharmaceutical purposes. They are held to have revitalizing properties, to act as anti-inflammatory agents, to protect against UV radiation, and to have soothing, immunostimulating, antiageing, antiwrinkle and antiacne effects (Pillai *et al.*, 2005). In addition, β-glucans have been shown to enhance wound repair (possibly by triggering the collagen production in fibroblasts) (Chen and Seviour, 2007). The recognition and response to β-glucans is mediated primarily by cell surface receptors. To date, four receptors have been identified, including scavenger receptors, complement receptor 3, lactosylceramide, and, more recently, dectin-1. However, dectin-1 is sufficient for many cellular responses to β-glucans; other responses, such as the production of inflammatory cytokines, require or are enhanced by cooperative signaling from toll-like receptors (TLRs) (Brown *et al.,* 2003; Gantner *et al.,* 2003).

In a very recent study it was documented that the expression of dectin-1 in normal human keratinocytes was elevated by β-glucan. Furthermore, β-glucan, a ligand for TLR2 and dectin-1, promoted the production of IL-1α and IL-8 cytokines at an optimal concentration of 10 μg/ml (Kobayashi *et al.,* 2008).

Until now, there has been no study demonstrating the effect of β-glucans on MMPs production. The family of MMPs is central to tissue homeostasis and remodeling. The activity of MMPs was

shown to be regulated at the level of transcription and the activation of the zymogen form. Only active forms of MMPs could degrade extracellular matrix components *in vivo*. In addition, these enzymes may also be required for cell motility in the process of wound healing.

In the present study, isolated human keratinocytes were cultured and monitored in tissue culture flasks until they reached ~60% confluence and cells were subcultured in 24-well plates and treated with appropriate concentrations of pleuran. The supernatants from the human keratinocyte culture were collected after 24 h of incubation and loaded on the gelatine gel. Gelatine zymography of the culture supernatants from keratinocytes revealed the presence of 92-kDa and 72-kDa bands corresponding to pro-MMP-9 and pro-MMP-2, respectively (Fig. 1).

There was a concentration-dependent increase in pro-MMP-9 release following a 24-h treatment with pleuran over the range 2 to 200 μg/ml, but pro-MMP-2 was detected at a constant level. The high molecular weight complex of MMP-9 and its native inhibitor TIMP1 had also been observed in gelatine gel. The active forms of both MMPs were not detectable, indicating that *in vitro* autoactivation of these proenyzmes did not occur. Induction of MMP-9 secretion from keratinocytes by pleuran may involve both dectin-1 and TLR2 receptors, which were found to be up-regulated in human keratinocytes (Kobayashi *et al.,* 2008). Interesting results have been observed employing hyaluronan (polymer of disaccharides) on human keratinocyte MMPs release (Isnard *et al.,* 2001). In the presence of 1 mg/ml hyaluronan there was an increase in the MMP expression and also an activation of latent MMPs by keratinocytes. In contrast to our data, the active forms of MMPs were not detected in the culture medium after stimulation with pleuran.

A variety of oligo- and polysaccharides from medicinal herbs have been characterized and tested on human keratinocytes *in vitro*. It has been found that purified β-glucan from reed mace seeds improved the keratinocytes proliferation. Cell viability and proliferation of keratinocytes

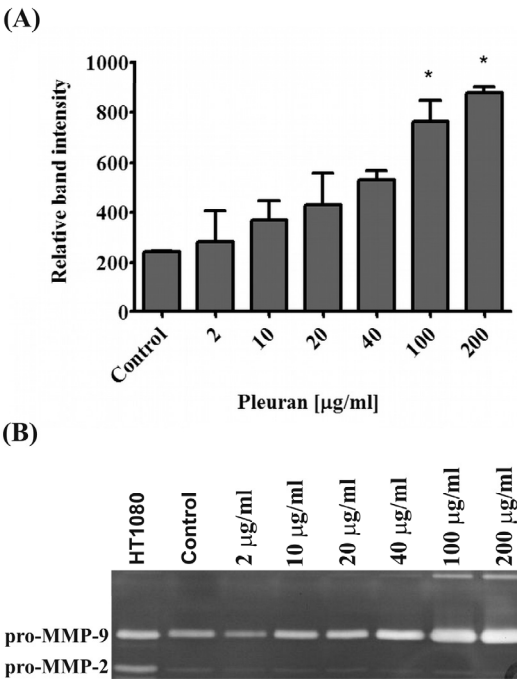

Fig. 1. Increased pro-MMP-9 secretion from human primary keratinocytes. (A) Gelatine zymography of supernatants of human primary keratinocytes incubated with various concentrations of pleuran. The supernatant from the fibrosarcoma line HT1080 was used as positive control. (B) Densitometric analysis of bands for pro-MMP-9 by zymography. Data represent mean ± SEM of at least 3 independent experiments. * $P \leq 0.01$ was calculated vs. control.

were also enhanced by an arabinogalactan of kaki fruits (Deters *et al.,* 2005). In contrast, chitin and less deacetylated chitosans showed no effect on keratinocytes proliferation. Moreover, highly deacetylated chitosan significantly inhibited the proliferation. These results indicate that the effect of chitosan on keratinocytes appears to be dependent on the degree of deacetylation (Howling *et al.,* 2001).

The documented ability of pleuran to significantly stimulate the pro-MMP-9 release from culture human keratinocytes opens new possibilities for the study of the mechanism of keratinocytes activation and the wound-healing process.

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