

## *Lycium barbarum* glycoconjugates: effect on human skin and cultured dermal fibroblasts

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### Abstract

*Lycium barbarum* L. (Solanaceae) glycoconjugates (LbGp) display an interesting array of anti-apoptotic and antioxidant properties, which may be beneficial for human skin. We therefore set out to determine the effects of LbGp in full-thickness human skin, and in dermal fibroblasts. It was found that LbGp decreased the level of MMP (matrix metalloproteinase)-1 significantly, but not that of MMP-3 or -13, in the whole human skin system, without compromising the viability of the skin. Consistently, LbGp inhibited skin expansion under mechanical stress, which in this model depends on the activity of MMP-1. We found that one of *L. barbarum* glycoconjugates, the LbGp5, promoted the survival of human fibroblasts cultured in suboptimal conditions. Furthermore, in the presence of LbGp5, these cultures also contained higher levels of the MMP-1 substrate—collagen type I. Together these results suggest that *L. barbarum* glycoconjugates in general, and LbGp5 in particular, may have important skin-protective properties.

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**Keywords:** *Lycium barbarum*; Glycoconjugates; Skin and cultured dermal fibroblasts

### Introduction

*Lycium barbarum* L. (Solanaceae) is a plant highly valued in traditional Chinese medicine for its broad health benefits (for a review, see Huang, 1999). *L. barbarum* glycoconjugates (LbGp) extracted from its fruits (Fructus Lycii) are believed to be the main active therapeutic components of this plant, due mostly to their impressive antioxidant effects (Zhang, 1993; Zhao et al., 2001; Wang et al., 2002). LbGp are

peptidoglycans with a short peptide backbone and a complex, branched glycan moiety.

Recently, we have shown that standardized LbGp not only inhibit lipid peroxidation and cytochrome C oxidation, but also protect seminiferous epithelium from structural damage and apoptosis, in a testicular tissue culture system (Wang et al., 2002). This observation prompted us to investigate whether LbGp have beneficial effects on yet another important type of epithelium and its underlying tissues—the skin. The skin is the organ with the most exposure to external insults, such as free radicals, which can cause premature ageing and neoplasms, in part through the upregulation of matrix metalloproteinases (MMPs; Wlaschek et al., 1995; Kregel et al., 2002). Given the antioxidant and

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anticancer (Lu and Cheng, 1991; Cao et al., 1994) properties of LbGp, we hypothesized that LbGp may have a protective effect on the skin. We probed this hypothesis with a model system designed to induce MMP expression in full-thickness human skin through the application of a cyclic mechanical force to the skin. Furthermore, we used human dermal fibroblasts to test the activity of one purified component of LbGp—the LbGp5, which was shown previously to have the strongest antioxidant activity among *L. barbarum* glycoconjugates (Huang et al., 2001).

## Materials and methods

### Reagents

All reagents were purchased from Fisher Scientific (Philadelphia, PA) except where indicated.

### Isolation of LbGp

LbGp were purified from dried fruits of *L. barbarum* L. (Solanaceae; harvested in Ningxia province of People's Republic of China) by hot water extraction, ultrafiltration, ethanol precipitation and DEAE chromatography as described elsewhere (Wang et al., 2002). The yield was 8 mg LbGp/g of dry fruit. LbGp5 was further purified by filtration through a 30 kD cut off centriprep centrifugal device (Millipore, Bedford, MA). Flow-through was collected, precipitated with one volume of cold ethanol, lyophilized, redissolved in water and filtered through a 10 kD cut off centriprep centrifugal device. The upper chamber material was collected, the volume was adjusted to 10 ml with ultrapure water and refiltered through the 10 kD cut off centriprep. The upper chamber material was collected and lyophilized (yield: 0.45 mg LbGp5/g of dry fruit). Glycoconjugate concentrations were estimated by a sulfuric acid—phenol method according to Dubois et al. (1956). LbGp5 was visualized on 18% SDS-polyacrylamide gel (BioRad, Hercules, CA) by silver staining according to Shevchenko et al. (1996). Briefly, after electrophoresis, gels were incubated in fixation solution (methanol 50%: acetic acid 5%: water 45% v/v) for 20–30 min, washed in water for 60 min, and incubated in 0.02% sodium thiosulfate for 2 min. Gels were then rinsed, incubated in cold 0.1% silver nitrate at 4 °C for 30 min, rinsed again and developed using 0.04% formaldehyde in 2% sodium carbonate.

### Enzymatic digestion

LbGp5 (100 µg/ml, pH 5.5) was incubated with 10 µg/ml of  $\beta$ -glucosidase from almonds (Sigma, St. Luis, MO)

for 15 min at 37 °C. The reaction was terminated by heating at 90 °C for 5 min. Heating of mock-digested LbGp5 was performed in parallel and did not affect the activity of LbGp5.

### *In vitro* tissue expansion assay

Full-thickness human skin was obtained under informed consent from patients undergoing abdominoplasty surgery. The skin was transported in oxygenated transport medium (Dublecco's Modified Eagles Medium (DMEM) (high glucose) JRH Bioscience, Lenexa, KS)+15 mM HEPES (Sigma, St. Louis, MO)+10,000 U Penicillin/Streptomycin (JRH Bioscience) at 4 °C ( $\pm 1$  °C), immediately defatted and 1-in diameter pieces punched from the whole tissue. The full-thickness skin punch was placed in growth medium (high glucose DMEM (JRH)+.015 M HEPES (Sigma)+2 mM Glutamine (BioWittaker, Walkersville, MD)+10,000 U Penicillin/Streptomycin (JRH Bioscience)+4.4 µM Insulin (Boehringer Mannheim, Germany)+0.28 µM Hydrocortisone (Calbiochem, San Diego, CA)+0.3 µM ascorbic acid (Calbiochem) in a custom designed bioreactor. The bioreactor consisted of a sterile growth chamber containing ports for medium addition and removal and gas exchange, nylon cord actuators, and a stepper motor controlled by custom software running under the Windows operating system. The skin was attached radially to 10 actuators using sterile garment tags, which do little damage to the skin. Tension on the skin was set at 100 g per attachment, the device assembled and the skin cycled using a sine wave at 10 cycles/min under an atmosphere of 40% O<sub>2</sub>, 5% CO<sub>2</sub> and 50% N<sub>2</sub>. Tissue surface area was measured at time 0 and then every 24 h using a digital camera photographed through the clear bioreactor top. Changes in surface area were calculated as a percent change in surface area using a customized digitizing program. To test the effects of LbGp on full-thickness human skin *in vitro*, varying concentrations from 0 to 1% (w/v) of lyophilized LbGp were dissolved in culture medium prior to addition to the bioreactor. Growth medium and LbGp were replaced daily.

### Quantitation of tissue levels of MMP-1 by ELISA

Concentrations of matrix metalloproteinase (MMP)-1 protein were measured in culture medium of whole skin in a quantitative sandwich immunoassay. The assay was performed using an MMP-1 ELISA kit from Calbiochem (# QIA 55) and following the manufacturers instructions. Briefly, 5 ml of media removed from the bioreactor after 48 h incubation with various concentrations of LbGp were centrifuged to remove particulate material, concentrated and assayed immediately. Standards were prepared at the same time as the samples and

assayed in duplicate. One hundred microliters of sample or standard was placed into each well of a 96-well microtiter plate and incubated at room temperature for 2 h. The wells were washed  $5 \times$  with  $1 \times$  wash buffer (sodium phosphate, pH 7.0). One hundred microliters of MMP-1 conjugate was added to each well and incubated for 1 h at room temperature, and washed as above prior to adding 100  $\mu$ l of color reagent (tetra-methylbenzidine) to each well. After a 30-min incubation in the dark, the reaction was stopped with 2.5 N sulfuric acid. Absorbance was measured in a spectrophotometric plate reader at 490 nm. The concentration of MMP-1 protein in the samples was determined by interpolation from the standard curve. Samples were standardized to total protein in the medium as measured by the Bradford Protein Assay (BioRad, Hercules, CA). Assays for MMP-3 and -13 were performed as described above using kits from Calbiochem (#QIA 73) and Amersham (#RPN2621), respectively.

## Histology

Skin samples were removed from the bioreactor, washed in PBS and placed in 10% formalin. The tissue was dehydrated, embedded in paraffin, sectioned (5  $\mu$ m) and stained with Hematoxylin and Eosin (H&E). Slides were examined using a Zeiss axioplan 2 microscope equipped with a Hamamatsu color digital camera.

## Lactate/glucose determination

Lactate and glucose were determined using a YSI 2700 Select dual channel biochemistry analyzer (YSI, Yellow Springs, OH). Briefly, tissue was removed from the bioreactor, washed in DMEM and placed into 5 ml of DMEM (low glucose) medium at 37 °C. Sixty microliters samples of medium were removed at set intervals over a 3-h period. Samples were either analyzed for glucose and lactate immediately or stored at 4 °C for up to 24 h prior to analysis.

## Fibroblast culture

Human neonatal dermal fibroblasts (passage 3–25; Cambrex, Walkersville, MD) were cultured at 5% CO<sub>2</sub> in complete FBM medium (FBM medium with FGM2 singlequot supplement, containing insulin, basic fibroblast growth factor and 2% fetal bovine serum; Cambrex). For suboptimal culture conditions this medium was replaced with DMEM without serum (Hyclone, Logan, UT).

For the measurement of type-I collagen and cell proliferation, fibroblasts were seeded in 96-well plates (Corning brand) at 6000 cells/well, and were grown for 3 days to late subconfluence. Cells were rinsed subsequently and incubated in suboptimal conditions with

2% calf serum for three additional days, in the absence or presence of LbGp5. Cultures were observed with a Nikon Eclipse TS100 inverted microscope equipped with a Canon camera. At the end of the experiment, cell media were collected for type I collagen quantification, and cells were counted by the colorimetric method based on sulforhodamine B staining and optical density measurement at 575 nm in a microplate reader 3550-UV (BioRad, Hercules, CA), by standard method (Skehan et al., 1990).

## Type I collagen quantization

Type-I collagen content in the fibroblast culture media was measured by the sandwich enzyme-linked immunoabsorbant assay (ELISA), as reported before (Dobak et al., 1994). Briefly, 96-well plates (Immulon 4HBX from Dynex, Chantilly, VA) were coated with goat anti-type I collagen antibody (Southern Biotechnology Associates, Birmingham, AL) overnight at 4 °C, and incubated with conditioned media for 1 h. After rinsing, sandwich was completed by adding biotinylated anti-type I collagen antibody for 1 h, followed by horseradish peroxidase-labeled streptavidin (Southern Biotechnology, Birmingham, AL) for 30 min. The assay was developed with peroxidase substrate 2, 2'-Azino-bis-3-ethylbenziazoline-6-sulfonic acid (ABTS; Rockland Immunochemicals, Gilbertsville, PA) and read at 405 nm. Statistical differences between the means were assessed using Student's *t*-test analysis.

## Results

### Effect of LbGp on matrix metalloproteinases in whole skin

Because skin cancer and aging are associated with the upregulation of metalloproteinases, we tested the effect of LbGp on the expression of these matrix-digesting enzymes. Whole human skin was cycled at 10 cycles/min under 100 g of force per skin attachment and exposed to increasing concentrations of LbGp in the bioreactor for 48 h; then MMP (matrix metalloproteinases) 1, 3 and 13 in the conditioned medium were quantified by ELISA. Fig. 1A shows that in the presence of LbGp, skin samples contained 3–4 times less MMP-1 than the control samples. In contrast, these glycoconjugates had no effect on the expression of MMP-3 and MMP-13 (Fig. 1B, C). Importantly, this decrease in MMP-1 was not due to an overall decrease of skin viability, as determined by the ratios of lactate/glucose in treated and control skin samples. These ratios were comparable regardless of the presence of LbGp, indicating a constant level of metabolic activity in the

tissue (Fig. 1D). LbGp did not interfere directly with the ELISA and lac/glu assays. Skin viability was confirmed by comparative observations of H&E stained paraffin sections of control skin samples and those incubated with LbGp. There was no observable effect on the epidermis, dermis or adnexal structures (results not shown).

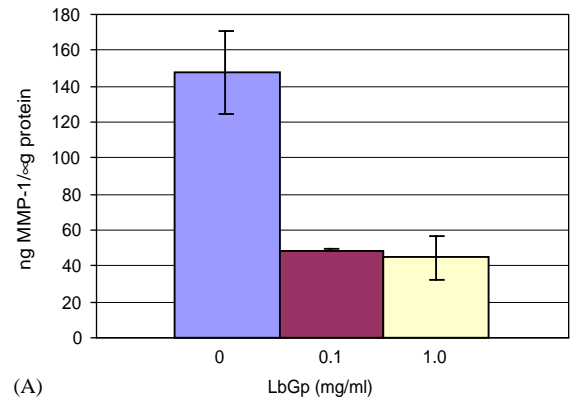
Subsequently, we investigated whether this LbGp-induced selective MMP-1 inhibition has physiological relevance. For this we cycled skin and determined the percent change in surface area over time as described in Materials and Methods. This skin expansion model has been shown to be associated with an increase of MMP-1 expression (Estelles et al., 2000). Fig. 2 shows that LbGp inhibits skin expansion in a dose-dependent manner, by over 60%. This inhibition cannot be explained by a general suppression of skin viability (Fig. 1D), and is consistent with the selective MMP-1-blocking effect of these glycoconjugates (Fig. 1A).

**Effect of LbGp5 on dermal fibroblasts at suboptimal culture conditions**

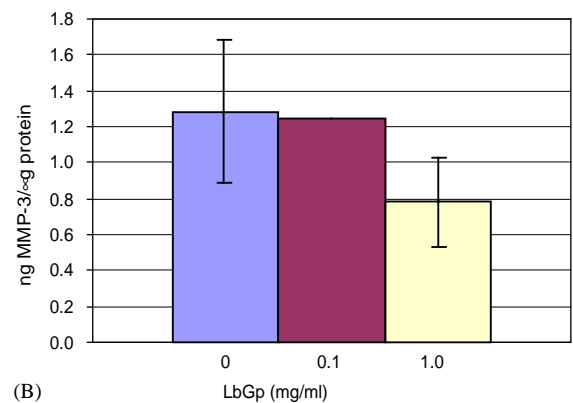
LbGp fraction is composed of several glycoconjugates of distinct molecular weights: LbGp2 (68.2kD; Peng and Tian, 2001); LbGp3 (92.5kD; Huang et al., 1998), LbGp4 (215kD; Huang et al., 1999) and LbGp5 (23.7kD; Peng et al., 2001). Of particular interest is LbGp5, because of its relatively low molecular weight and especially strong antioxidant effect *in vitro* (Huang et al., 2001). Therefore, we decided to determine whether LbGp5 has an effect on human dermal fibroblasts.

It was found that LbGp5 had no effect on fibroblast growth and morphology at optimal conditions (complete FBM medium; not shown). When cells were grown in suboptimal conditions, however, (DMEM without serum), addition of LbGp5 (10–100 µg/ml) resulted in better overall cell morphology (i.e., less rounded cells) as compared to fibroblasts cultured in the absence of this glycoconjugate (compare Fig. 3B and C). Similarly, suboptimal cultures with LbGp5 had significantly higher

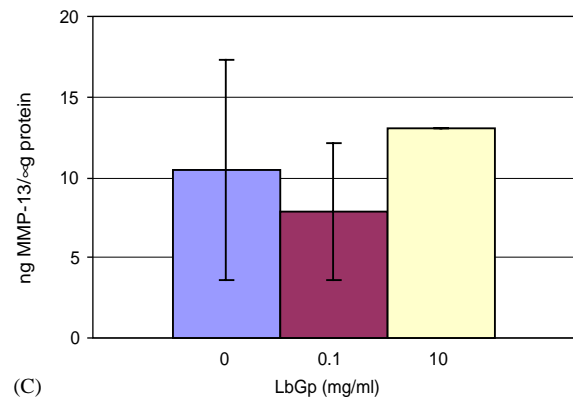
cell counts than the same cultures without LbGp (compare Fig. 3E’s first two “LbGp” bars and the “ctr (DMEM)” bar). The morphology and cell numbers of



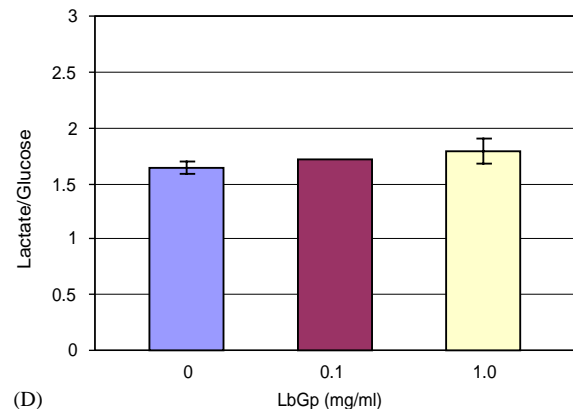
(A)



(B)



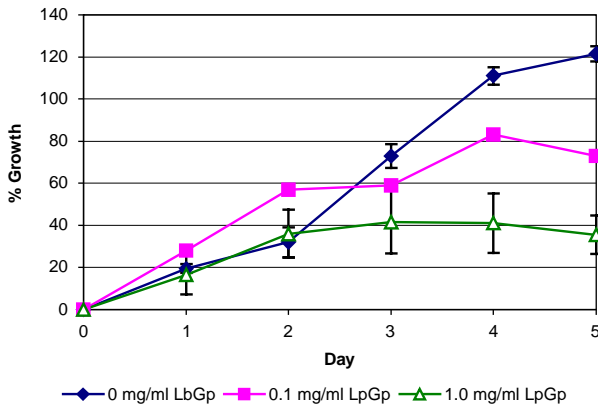
(C)



(D)

**Fig. 1.** Effect of LbGp on the expression of matrix metalloproteinases in human skin. Full-thickness skin punches (surface area ~6.5 cm<sup>2</sup>) from abdominoplasty were incubated in a bioreactor with increasing concentrations of LbGp while subjected to cyclic force as described in Materials and Methods. LbGp induce a decrease of MMP-1 level (A), but not of other tested matrix metalloproteinases (MMP-3; Panel B, and MMP-13; Panel C) in full thickness human skin conditioned medium as measured by ELISA (the decrease of MMP-3 at LbGp 1 mg/ml is not statistically relevant). (D): skin viability, as measured by lactate to glucose ratio is not affected by LbGp. Error bars represent standard deviation. Number of tests (n) = 3.

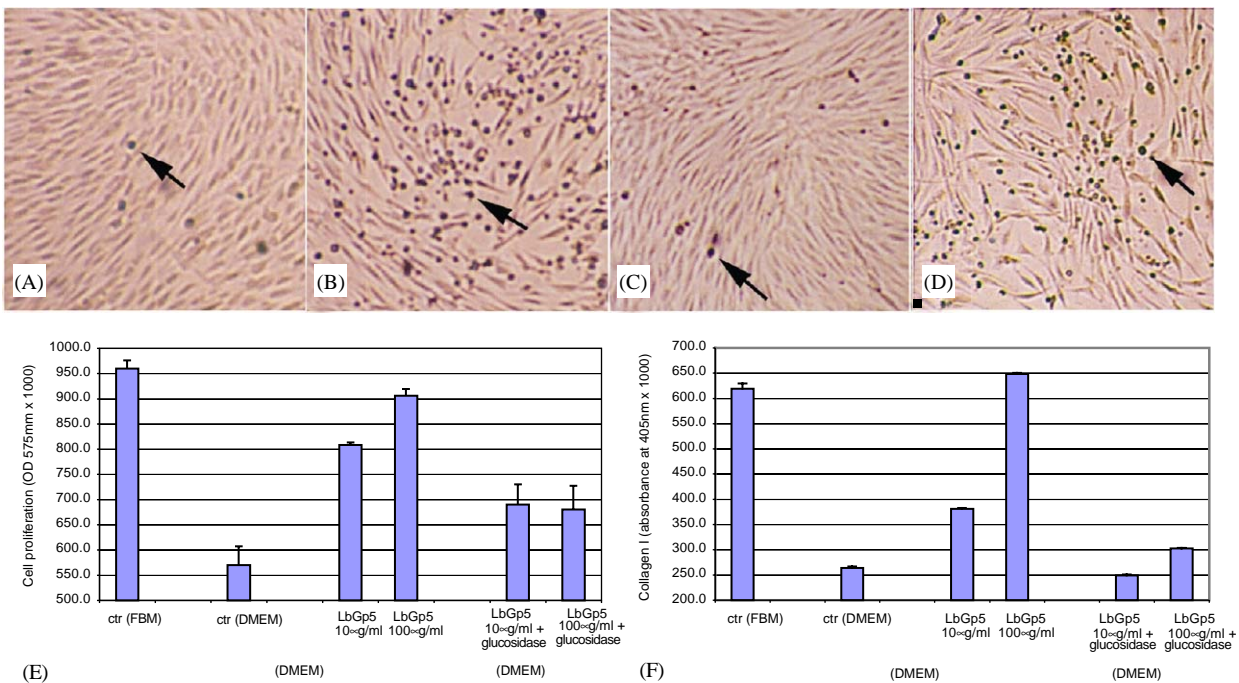
cultures with LbGp were comparable to cultures maintained continuously at optimal conditions (compare Fig. 3A and C, and Fig. 3E's first two "LbGp" bars and the "ctr (FBM)" bar).



**Fig. 2.** Dose-dependent inhibition of skin expansion by LbGp. Full thickness human skin punches (~10 cm<sup>2</sup>) from abdominoplasty were incubated in a bioreactor and subjected to cyclical force as described in Materials and Methods in the presence of increasing concentrations of LbGp over a 5 day period. Skin expansion (% growth) was determined by measuring the surface area once daily for 5 days and expressed as percentage of the surface of the untreated control on day 0. Error bars represent standard deviation. Number of tests (n) = 3.

We examined also the effect of LbGp5 on type-I collagen output in dermal fibroblast cultures. Type-I collagen is a major substrate of MMP-1 in dermal fibroblasts (Welgus et al., 1981). It is also an important metabolic marker (Weber et al., 1995). In cultures incubated during the last 3 days under suboptimal conditions, the type-I collagen level was 60% lower, as compared with cultures maintained at optimal conditions during the full 6-day period. In contrast, in suboptimal cultures incubated with LbGp5 (100 μg/ml), the type-I collagen level was similar to the optimal culture control (Fig. 3F). The effect of LbGp5 on collagen output by dermal fibroblasts under these conditions was dose dependent (LbGp5 in the medium did not interfere with the ELISA assay; data not shown).

In order to confirm that LbGp5 is indeed responsible for these effects, we subjected it to enzymatic digestion. LbGp5 contains β-linked residues, which makes it sensitive to hydrolysis by β-glucosidase. As shown on Fig. 3(D)–(F), β-glucosidase-digested LbGp5 lost most of its cell-protective and collagen-stimulatory potency. Fig. 4 confirms that β-glucosidase treatment resulted in the degradation of LbGp5.



**Fig. 3.** Protective effect of LbGp5 on subconfluent cultures of human neonatal dermal fibroblasts. A–D: Morphology of cultures incubated at (A) optimal conditions (complete FBM medium) for 6 days; (B) 3 days at optimal conditions following 3 days at suboptimal conditions (DMEM without serum) in the absence of LbGp5; (C) 3 days at optimal conditions following 3 days at suboptimal conditions in the presence of LbGp5 (100 μg/ml); (D) 3 days at optimal conditions following 3 days at suboptimal conditions in the presence of LbGp5 (100 μg/ml) pretreated with β-glucosidase, as described in Materials and Methods (magnification × 40). Arrows indicate rounded cells. E: Quantification of cell numbers at conditions depicted on panels A–D. F: Quantification of type I collagen at conditions depicted on panels A–D. P < 0.05.



**Fig. 4.** Digestion of LbGp5 by  $\beta$ -glucosidase visualized by silver stain. Two  $\mu$ g of LbGp5 (left line) and of LbGp5 digested by  $\beta$ -glucosidase (right line) were electrophoresed on 18% SDS-polyacrylamide gel and stained with silver nitrate according to the procedure of Shevchenko and collaborators (1996). Using BioRad molecular weight standards run in parallel, LbGp5 size was determined to be 24kD, which is in accordance with the previous report (Peng et al., 2001). The LbGp5 sample preincubated with  $\beta$ -glucosidase appeared completely digested.

## Discussion

LbGp have been reported to have interesting protective properties on seminiferous epithelium, such as preservation of stratified structure of the cultured seminiferous tubules and inhibition of apoptosis (Wang et al., 2002). These results prompted us to test LbGp for protective effects in the dermis, using the whole human skin expansion model. This unique model was developed by Reconstructive Technologies, Inc. (Palo Alto, CA) to generate skin for burn patients, and has been adapted to study MMP expression. It allows for long-term experiments by maintaining the viability of whole human skin for over a week's time. In this system, LbGp inhibited the expression of MMP-1 selectively, without affecting other tested metalloproteinases. This inhibition was strong and physiologically relevant, because at the same concentrations LbGp blocked skin expansion, that is associated with the increase of MMP-1 expression (Estelles et al., 2000). Thus, LbGp could be of interest in the processes involving excessive expression of MMP-1 in the skin, such as skin aging (Varani et al., 2000; Lahmann et al., 2001) or cancers (Tsukifujii et al., 1999; Ye et al., 2001).

The immunostimulatory (Li et al., 1984; Huang et al., 1998), hepatoprotective (He et al., 1993), anti-ageing (Zhisong, 2000), anticancer (Lu and Cheng, 1991) and antioxidant (Zhang, 1993; Huang et al., 2001) effects of LbGp have been studied extensively. Recent physico-chemical analysis showed that LbGp contains five distinct peptidoglycans labeled LbGp1-5 (Zhao et al., 2001). Out of these glycoconjugates, LbGp5 is particularly interesting due to its relatively small molecular weight and particularly strong antioxidant activity (Huang et al., 2001). We therefore tested LbGp5 on human dermal fibroblasts. Subconfluent to confluent dermal fibroblast cultures can be incubated in optimal (complete FBM) medium conditions for many days, without apparent cell loss. If the same cultures are

placed in simple DMEM medium without serum, however, cells tend to round up and detach. LbGp5 had no effect on subconfluent cultures incubated in complete FBM medium, but it protected cells from rounding up and detaching when these cultures were exposed to suboptimal conditions (DMEM without serum). In fact, the morphology of fibroblasts incubated in suboptimal conditions in the presence of LbGp5 was very similar to the morphology of the same cells cultured in optimal conditions. This result is consistent with our previous findings in the cultured seminiferous tubule system, where LbGp showed an antiapoptotic and morphology-protective effect (Wang et al., 2002). The underlying mechanism of action remains to be determined, but it seems that LbGp5 could exert its activity through interaction with a growth factor or adhesion receptor on the cell surface. For example, LbGp5 could facilitate binding of fibroblast growth factor (FGF) to its receptor, as does another proteoglycan, perlecan, (Aviezer et al., 1994). Alternatively, LbGp5 could act as a growth factor on its own, interacting with a sugar-binding site on a receptor. Such sites exist, for example, on the FGF receptor, and their binding by proteoglycan heparin results in signal transduction even in the absence of FGF (Gao and Goldfarb, 1995). LbGp5 could also act, by structural analogy, as an agonist of a glycosylated growth or survival factor, whose active site is composed of both sugar and amino-acid moieties.

Finally, because LbGp5 is such an efficient antioxidant, it may exercise its effect, at least partially, through protecting cells from free radical damage. Such protection has been reported for various glycoconjugates composing the extracellular matrix. In this respect, it is interesting to note that the aging of certain tissues such as cornea and endothelium correlates with the decrease of the most efficient anti-oxidant components of their extracellular matrices (Albertini et al., 2000).

The fact that fibroblasts grown in suboptimal conditions in the presence of LbGp5 secrete normal amounts of type-I collagen, shows that LbGp5 does not merely inhibit cell detachment, but allows cells to maintain their regular metabolic functions. This effect, as well as the protective effect of LbGp5 on cells, can be abrogated by pretreating this glycoconjugate with  $\beta$ -glucosidase. The degradation of LbGp5 by this  $\beta$ -linked glycan hydrolyzing enzyme, as visualized by silver staining, results in the loss of its fibroblast-protective effects, confirming the importance of the glycan moiety for the activity of this *L. barbarum* peptidoglycan.

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