

# Effect of xyloglucan (tamarind seed polysaccharide) on conjunctival cell adhesion to laminin and on corneal epithelium wound healing

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**PURPOSE.** To explore the role of a natural polysaccharide extracted from tamarind seed (xyloglucan, or tamarind seed polysaccharide, TSP) on the integrin-substrate recognition system and on repair of corneal wounds.

**METHODS.** a) Cultured human conjunctival cells were labeled by addition of a tritiated amino acid mixture. Their adhesion to laminin-coated culture wells in the absence or presence of TSP was checked by radioactivity count. b) The corneal epithelium of albino rabbits was damaged by applying a paper disc soaked with *n*-heptanol. The eyes were then treated with TSP, with a hyaluronate reference formulation and with normal saline solution (controls). The diameter of corneal wounds was measured daily, after fluorescein staining.

**RESULTS.** Compared to hyaluronate, TSP slightly but significantly increased the wound healing rate. TSP 1.0% exerted a positive influence on cell adhesion to laminin, up to a certain laminin concentration.

**CONCLUSIONS.** The ability of the polysaccharide to promote corneal wound healing might depend on its influence on the integrin recognition system. (*Eur J Ophthalmol* 2000; 10: 71-6)

**KEY WORDS:** Human conjunctival cells, Cell adhesion, Laminin, Rabbits, Rate of corneal repair, Xyloglucan

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## INTRODUCTION

Various insults to the corneal and conjunctival epithelium are common in clinical ophthalmology. They may occur after trauma, ocular surgery, ocular surface diseases or as a result of environmental factors (1). Corneal/conjunctival surfaces can also be damaged by prolonged use of ophthalmic preparations: irritant effects, corneal lesions and alterations of conjunctival layer integrity produced by drugs and/or by common ingredients of eyedrops are well documented (2-5).

Corneal or conjunctival wounds are commonly treated pharmacologically with the aim of promoting

formation of an intact epithelial layer as rapidly as possible and then re-establishing the correct flow of biological information between corneal and conjunctival cells. Hyaluronic acid is reported to have a good effect on healing of clinical and experimental alkali wounds of the corneal epithelium, stroma, and endothelium (6-8). The epidermal growth factor (9), collagen lenses (10) and mucin extracted from bovine submaxillary glands (11) are also active in different models of corneal erosion.

One of the first events in tissue repair is cell attachment and adhesion to specific substrates of the extracellular matrix, such as the glycoproteins laminin -

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the main glycoprotein of corneal basement membrane (12) – and fibronectin. Both glycoproteins are highly glycosylated, and exert their biological activity through recognition of specific membrane receptors called integrins (13). Since integrin recognition can be affected by natural or synthetic polysaccharides (14), it has been suggested that polysaccharides and glycosaminoglycans may influence cell adhesion and the wound-healing process. The effect of sulphated polysaccharides, i.e. heparin and fucoidans (15, 16), and of hyaluronic acid on cell adhesion and proliferation has been reported (17).

The continuous search for compounds favouring cell adhesion and promoting ocular wound healing prompted us to investigate the utility of a natural polysaccharide from tamarind seed (xyloglucan, or tamarind seed polysaccharide, TSP) (18, 19) in the repair of corneal wounds and in the integrin-substrate recognition system. Previous work in our laboratories on cultured conjunctival cells has shown that TSP is well tolerated, and exerts a protective action against toxic effects induced by timolol, merthiolate and fluoroquinolones, possibly on account of its mucin-like structure (20). An experimental TSP formulation tested in a rabbit dry eye model protected the eyes against the appearance of dry spots (21).

We therefore studied the effect of TSP on adhesion of human conjunctival cells to laminin. We also investigated the effect of TSP on the rate of corneal repair in rabbits after heptanol-induced lesions in an attempt to clarify the mechanism of action.

## MATERIALS AND METHODS

### Materials

Human conjunctival cells were obtained from the European Cell Culture Collection (ECACC, Salisbury, UK). Laminin from EHS tumor (LM), bovine serum albumin (BSA), M199-HBSS medium, glutamine, trypsin, n-heptanol, antibiotic mixtures and other cell culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and sodium dodecylsulphate (SDS) were obtained from Boehringer Mannheim (Mannheim, Germany). The tritiated amino acid mixture (56 mCi/mmoles) was obtained from Amersham Italia srl (Milano, Italy). Drop-

star® TG (NaHA, 0.4% sodium hyaluronate) was kindly supplied by Farmigea S.p.A. (Pisa, Italy); tamarind gum from the endosperm of *Tamarindus Indica L.* seeds (Glyloid® 3S) was purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan); all other chemicals or solvents were of reagent grade.

Commercial tamarind gum was purified as follows: a mixture of gum (2.5% w/v), powdered nitrocellulose (2.0% w/v) and diatomaceous earth (4.0% w/v) was dispersed in water at 60°C. After stirring overnight, all undissolved solids were removed by filtration (glass fiber filters with 2.4 and 1.2 µm pore size, Whatman International Ltd., Maidstone, UK). Tamarind seed polysaccharide (TSP) was precipitated by adding acetone and was recovered by filtration.

The experimental formulation based on TSP (TSP eyedrops) contained 1.0% w/w polysaccharide and 5.0% w/w mannitol for isotonicity; its pH was adjusted to 7.0 with NaOH. The formulation was sterilized by autoclaving and did not contain preservatives since it was used immediately after opening the vial.

### Laminin (LM) well coating

Solutions (50 µl) containing increasing concentrations of LM were added to 96-multiwell plates and incubated for at least 1 h at 37 °C in an atmosphere of 5% CO<sub>2</sub>. The wells were washed twice with phosphate buffer solution (PBS), 50 µl of 0.1% heat-denatured BSA was added, and they were incubated under the same conditions for another hour. The plates were washed twice with PBS before cell plating. Plates treated only with BSA were used as control.

### Cell adhesion test

Confluent conjunctival cells were labeled for 3 h at 37 °C in an atmosphere of 5% CO<sub>2</sub>, by addition of tritiated amino acid mixture (0.5 µCi/ml) in serum-free culture medium. The cells were then washed, trypsinised, centrifuged at 1000 rpm for 5 min, resuspended in medium containing 10% heat-inactivated FBS and incubated at 37°C for 15 min in order to allow recovery after trypsinisation. The cells were then centrifuged, resuspended in serum-free culture medium in the absence or presence of TSP (1.0 or 2.0% w/v), and plated on laminin-coated multiwell plates. Density was maintained between 1000 and

1500 cells/well. Adhesion on the plates incubated at 37°C was followed by microscopic observation.

After 90 min the medium was removed and its radioactivity was counted. Adherent cells were thoroughly washed with PBS and resuspended by addition of 0.2% SDS in 0.1 N NaOH. The radioactivity present in the medium and in suspended cells was counted in a  $\beta$ -counter. Adhesion was expressed as the ratio of radioactivity in cells to total radioactivity recovered (cells+medium)  $\times$  100. Results are the means  $\pm$  S.E. of at least four experiments run in triplicate. Statistical analysis was done using Student's t-test.

#### *Experimental corneal lesion in rabbits*

Male New Zealand albino rabbits, weighing 2.8-3.0 kg, were obtained from Pampaloni Rabbitry (Fauglia, Italy). They were treated as prescribed in the publication "Guide for the care and use of laboratory animals" (NIH Publication No. 92-93, revised 1985). All experiments were carried out under veterinary supervision, and the protocols were approved by the university ethical-scientific committee. The animals were housed singly in standard cages, in a light-controlled room at  $19 \pm 1^\circ\text{C}$  and  $50 \pm 5\%$  rel-hum, with no restriction of food or water.

The animals were divided into three groups, each consisting of at least eight rabbits. The corneal surface was preliminarily examined by the slit-lamp to verify the integrity of the corneal epithelium. All animals were anesthetized by i.m. injection of  $30 \text{ mg kg}^{-1}$  ketamine (Inoketam 1000 solution, Virbac s.r.l., France) and  $5 \text{ mg kg}^{-1}$  xylazine (Rompum 2% solution, Bayer AG, Leverkusen, Germany). The right eye was then kept open by a blepharostat and the corneal epithelium was removed by applying a paper disc (diameter 6 mm; Whatman No. 50 filter paper) shaped like a small contact lens and soaked with  $10 \mu\text{l}$  of n-heptanol for 1 min (22). The eyes were then carefully rinsed with normal saline (50 ml) and only the lesioned eyes of each group of eight animals were treated three times daily with: 1st group,  $50 \mu\text{l}$  of TSP eye-drops (experimental formulation); 2nd group,  $50 \mu\text{l}$  of NaHA (Dropstar® TG, reference formulation); 3rd group,  $50 \mu\text{l}$  of normal saline solution (controls).

Immediately after producing the epithelial damage and before each daily treatment, the right eye was stained with fluorescein strips (Fluorets®, Smith & Nephew

Pharmaceuticals Ltd., Romford, UK) to visualize the damaged area. This staining method was preferred because topically applied fluorescein has been shown not to adversely affect corneal epithelial healing (23).

The diameter of corneal wounds was measured using a slit-lamp biomicroscope fitted with a blue filter and a micrometer. Statistical analysis was done using the Fisher PLSD test.

## RESULTS

At the end of the 90-min incubation period, in the absence of TSP the conjunctival cells adhering to LM increased with the LM concentration, and spread onto the substrate. The effect of 1.0% and 2.0% w/v TSP (present in M199 serum-free medium from the beginning of incubation) on cell adhesion to LM is shown in Figure 1: TSP influenced cell adhesion depending on both the LM and polysaccharide concentrations. At low LM concentrations (from 1 to  $10 \mu\text{g/ml}$ ) 1.0% TSP increased cell adhesion, whereas at higher LM concentrations TSP inhibited cell adhesion to the glycoprotein. TSP 2.0% had a positive effect on cell adhesion only at  $1 \mu\text{g/ml}$  LM. Above this concentration, the polysaccharide appeared to inhibit cell adhesion.

In the rabbit model of corneal lesion, the original wounds were very regular in shape and had a constant area of  $28.27 \text{ mm}^2$ . This was possibly because of the small amount of n-heptanol ( $10 \mu\text{l}$ ) used to soak the paper disc: a larger amount would spread onto the cornea, producing irregularly shaped lesions. Examination of the mean defect areas for each time point (Tab. I) showed that all lesions were completely healed within 48 hours.

The mean epithelial defect areas for each treatment group are illustrated in Figure 2 as percentages of the initially damaged area (mean $\pm$ S.E.) vs time. For greater clarity, the figure only reports the data for the last three observation times (24, 27 and 31 h). The NaHA and TSP eyedrops groups produced a small decrease in the damaged area 24 h after the first treatment, with respect to the control group. However, as shown by statistical analysis (Tab. I), only TSP achieved significantly different results from the controls at the last three time points ( $p < 0.05$ ), and

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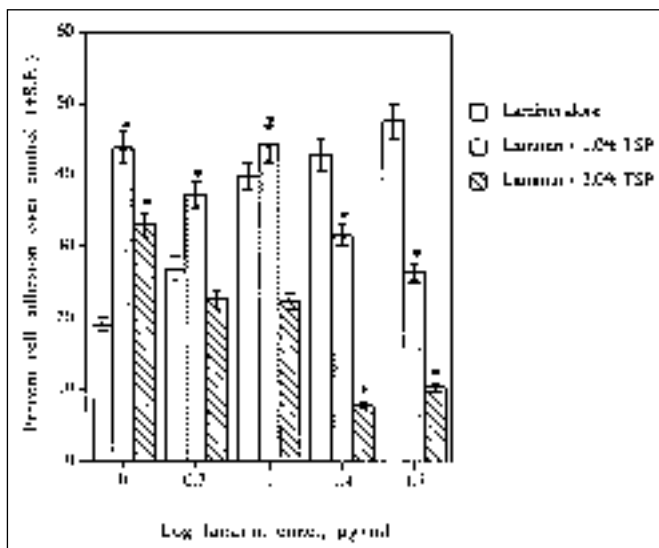


Fig. 1 - Effect of TSP on conjunctival cell adhesion to laminin. \*Significantly different from laminin alone ( $p < 0.05$ , Student's *t*-test).

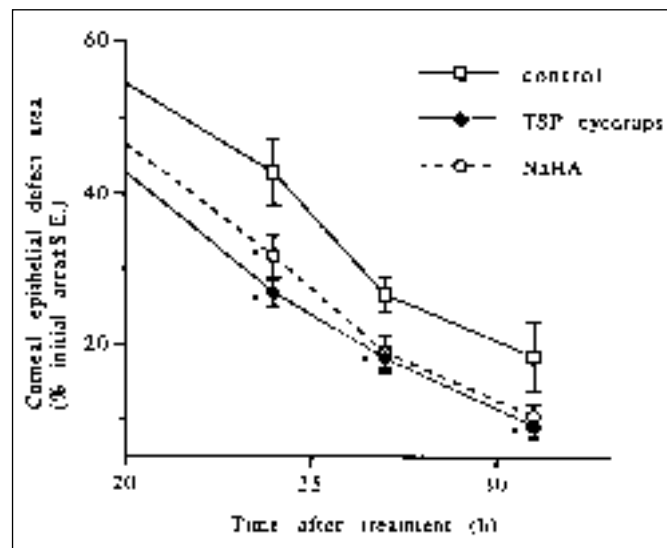


Fig. 2 - Last stages (24, 27 and 31 h) of the shrinkage of the corneal defect area in rabbits. \*Significantly different from control ( $p < 0.05$ , Fisher PLSD test).

NaHA was significantly different only at 24 h.

For each group, the best-fit straight line was determined by least-squares regression analysis, using the defect area measured from 7 to 31 hours after the first treatment. The slope of the line was taken

as the healing rate ( $\text{mm}^2/\text{h}$ ) and its x-axis intercept as the healing time (h). The results are summarized in Table II. TSP and NaHA both accelerated wound healing, with a shorter healing time than in the control group.

TABLE I - DEFECT AREAS ( $\text{mm}^2 \pm \text{S.E.}$ )

| Time after wound (h) | Control          | TSP eye drops    | NaHA             |
|----------------------|------------------|------------------|------------------|
| 0                    | 28.27            | 28.27            | 28.27            |
| 3                    | 27.51 $\pm$ 0.51 | 27.14 $\pm$ 0.59 | 27.14 $\pm$ 0.59 |
| 7                    | 26.03 $\pm$ 0.86 | 26.78 $\pm$ 0.82 | 25.74 $\pm$ 1.29 |
| 24                   | 12.08 $\pm$ 1.25 | 7.56 $\pm$ 0.49* | 8.94 $\pm$ 0.77* |
| 27                   | 7.51 $\pm$ 0.67  | 5.15 $\pm$ 0.38* | 5.33 $\pm$ 0.67  |
| 31                   | 5.18 $\pm$ 1.31  | 2.58 $\pm$ 0.40* | 2.94 $\pm$ 0.49  |
| 48                   | 0.00             | 0.00             | 0.00             |

\*significantly different from control ( $p < 0.05$ , Fisher PLSD test)

TABLE II - HEALING PARAMETERS OF THE DAMAGED CORNEAS

| Group         | Healing rate ( $\text{mm}^2/\text{h} \pm \text{S.E.}$ ) | Healing time (h) | Regression coefficient (r) |
|---------------|---|------------------|----------------------------|
| Control       | 0.881 $\pm$ 0.058                                       | 36.7             | 0.914                      |
| TSP eye drops | 1.040 $\pm$ 0.033                                       | 32.2             | 0.978                      |
| NaHA          | 0.973 $\pm$ 0.045                                       | 33.3             | 0.973                      |

## DISCUSSION

The extracellular matrix is considered important both for expression of cell phenotype and for tissue remodeling and repair (24, 25). Epithelial cells are associated with connective tissue elements, and the extracellular matrix components of these cells may play dynamic roles in tissue remodeling and repair. Corneal and conjunctival cells are contiguous, specialised epithelia of the eye, which act synergistically in maintaining eye cell function. In cases of corneal epithelium destruction, conjunctival cells may repopulate the corneal tissue by becoming corneal-like cells (26, 27). Moreover, a role of corneal extracellular matrix in inducing conjunctival differentiation has recently been demonstrated (28), the cell matrix inducing conjunctiva phenotype expression depending on the presence of specific substrates produced by corneal cells. These effects require transduction of signals from the basement membrane to the overlying epithelium, which could be mediated by integrin, as suggested in a recent report (25). The interaction between the substrate and its specific integrin might involve exchanges of information between different cell layers vital to tissue homeostasis. During re-epithelialization, epithelial cells adhere to components of the extracellular matrix, then migrate toward the wound area and begin proliferating to restore the damage. Each of these events is mediated by integrins and their ligands.

Corneal and conjunctival cells recognise different components of the matrices (29-31): integrins mediate several behavioural aspects of these cells, including differentiation (32). This suggests that topical addition of matrix components might serve as a reasonable pharmacological approach to wound healing of both epithelia.

Corneal wounds show very complex healing modes depending on their size and depth. The heptanol wound model used in this study (22) consists only in removal of the surface epithelium, and does not damage ei-

ther the basement membrane or the extracellular stromal components; it heals spontaneously in a short time (<48 h). Evaluation of the corneal epithelium healing of heptanol-treated rabbits showed small but significant shrinkage of the defect area of TSP-treated corneas compared to the control group.

The results of the LM adhesion study of conjunctival cells in the presence of TSP casts some light on the possible mechanism of action of the polysaccharides. The cells showed good adhesion to LM and the presence of TSP appeared to influence adhesion, thus confirming that the polysaccharides can interact with the integrin recognition system. In particular, the effect of the lower TSP concentration (1.0% w/v) on the low LM concentration suggests the polysaccharide may possibly be involved in promotion of cell adhesion *in vivo*. In these conditions, TSP might facilitate cell adhesion by increasing the cell affinity for LM, as occurs for other structures on the plasma membrane (33). This synergism between polysaccharide and integrins seems to be lost at LM concentrations exceeding 1.0 µg/ml. At higher LM and TSP concentrations adhesion was clearly inhibited, suggesting there may be an optimal ratio between substrate and receptor (1:10000 and 1:1000, LM, TSP), which would govern the effect on adhesion.

These findings indicate the concentration-dependency of the effect of TSP, and suggest that 1.0% w/v TSP might have a beneficial effect on conjunctival cell adhesion, a primary event before cell differentiation. These results suggest TSP may be involved in corneal healing *in vivo*.

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