Pathogens and Disease ISSN 2049-632X

SHORT COMMUNICATION

Resveratrol role in *Staphylococcus aureus*-induced corneal inflammation

Andreana Marino[1], Giuseppe Santoro[2], Francesca Spataro[1], Eugenia R. Lauriano[3], Simona Pergolizzi[3], Francesco Cimino[1], Antonio Speciale[1], Antonia Nostro[1], Giuseppe Bisignano[1] & Giacomo Dugo[3]

1 Dipartimento di Scienze del Farmaco e dei Prodotti per la Salute, Università di Messina, Messina, Italy

2 Dipartimento di Scienze Biomediche e delle Immagini Morfologiche e Funzionali, A.O.U. Policlinico "G. Martino", Università di Messina, Messina, Italy

3 Dipartimento di Scienze dell'Ambiente, della Sicurezza, del Territorio, degli Alimenti e della Salute, Università di Messina, Messina, Italy

Corneal inflammatory diseases are often associated to *Staphylococcus aureus* (limbitis, blepharo-conjunctivitis, superficial punctate keratopathy, staphylococcal marginal keratitis, and corneal abscesses). Except for corneal abscesses, *S. aureus* induced corneal inflammation seems to be related to host hypersensitivity rather than to a classical invasive infection. This new approach targeting the immune-modulation of the corneal epithelium seems to be an attractive alternative solution to conventional treatment consisting of corticosteroid drops.

Keywords
immunofluorescence; immunoperoxidase; innate immunity; phytoalexin.

Correspondence
Andreana Marino, Dipartimento di Scienze del Farmaco e dei Prodotti per la Salute, Università di Messina, Polo Annunziata, Messina 98168, Italy.
Tel.: +390906766441;
fax: +390906766405;
e-mail: anmarino@unime.it

Received 3 December 2012; revised 10 April 2013; accepted 2 May 2013. Final version published online 3 June 2013.

doi:10.1111/2049-632X.12046

Editor: Jacques Schrenzel

Abstract

The aim of this study was to evaluate the role of *trans*-resveratrol on *Staphylococcus aureus*-induced keratitis. Rabbit corneas (intact corneas, abraded corneas and abraded corneas exposed to inactivated *S. aureus* strains) were placed in an *ex vivo* culture model. The abraded corneas exposed to *S. aureus* were divided into two 1-h-treatment sub-groups: corneas treated with *trans*-resveratrol and corneas treated with vehicle. The tissues were examined by immunohistochemical analyses and quantitative real-time RT-PCR to determine whether resveratrol could reduce TLR2-mediated recognition of *S. aureus* on epithelial cells and, if so, whether this reduction repressed the expression of inflammatory cytokines. The results demonstrated that resveratrol treatment effectively downregulated cell surface TLR2 on cells stimulated by *S. aureus* and reduced the expression of interleukin-8 gene. In addition, the corneal culture model tested, which is simple and reproducible, could be an alternative to *in vivo* animal testing for the development of novel specific therapies.

Under normal conditions, the cornea is highly resistant to microbial invasion. However, once the epithelial integrity is breached, pathogens may invade the cornea, leading to microbial keratitis (Kumar & Yu, 2006). *Staphylococcus aureus*, a commensal of the wet mucosa and skin, is a leading cause of invasive infection (Heimer *et al.*, 2010). In the field of ophthalmology, ocular infections such as dacryocystitis, conjunctivitis and keratitis, which occur mainly in contact lens wearers and those with corneal injury, are often reported (Heimer *et al.*, 2010; Sotozono *et al.*, 2013). Infectious keratitis and endophthalmitis caused by methicillin-resistant *S. aureus* (MRSA) are increasing problems throughout the world (Major *et al.*, 2010; Sotozono *et al.*, 2013). Because of the incidence of reported antibiotic-resistant strains and failure of antimicrobial peptides treatment to manage keratitis, it has been suggested that a better understanding of the mechanisms by which the pathogen strains induce disease will be critical for the rational design of improved therapeutic strategies (Hazlett, 2004). The ability of corneal epithelial cells to recognize *S. aureus* is largely attributed to the Toll-like receptor 2 (TLR2) which, once activated, triggers pro-inflammatory cytokines expression in a TLR2/MyD88-dependent manner (Sun *et al.*, 2006; Lambiase *et al.*, 2011). Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a phytoalexin whose synthesis in plants can be induced by microbial infections (Alarcón de la Lastra & Villegas, 2005). Several studies within the last few years have shown that resveratrol

exhibits potent anti-inflammatory effects due to downregulation of expression of proinflammatory cytokines by reducing the activities of nuclear factor κB (NF-κB) (Iyori *et al.*, 2008; Speciale *et al.*, 2011). Based on the current knowledge described above, we started experiments to investigate the potential role of *trans*-resveratrol on *S. aureus*-induced keratitis in an *ex vivo* rabbit model.

Staphylococcus aureus ATCC 6538P and *S. aureus* ATCC 29213 were cultured in tryptic soy broth at 37 °C to reach a concentration of *c.* 5×10^8 CFU mL^{-1}. To inactivate *S. aureus* strains, a UV-germicidal lamp was used for 15 min. The air/liquid organ culture model used in this study was a modification of that published by Foreman *et al.* (1996). The eyes of 24 adult New Zealand rabbits obtained from a local abattoir, enucleated immediately following euthanasia, were maintained in Dulbecco's modified Eagle's medium (DMEM) with antibiotic/antimycotic solution (1 : 200). A demarcated central area (5 mm) of the epithelial layer of the cornea was abraded using a sterile cutter on six eyes within 2 h after enucleation. The entire sclero-corneal ring was excised and mounted on the organ support previously prepared using sterilized contact lens containers filled with DMEM and sterile agar collagen 0.5% (1 : 200). The corneal curvature was maintained during the culture period on the corneal support inverted into sterile dishes containing 2.5 mL of medium. The sclero-corneal rings were then randomly divided into three groups for each strain: intact corneas (three corneal rings); abraded corneas (three corneal rings); activated corneas: abraded corneas exposed to 4 μL of inactivated strains 5×10^8 bacteria mL^{-1} (six corneal rings). The organ cultures were incubated at 37 °C in a humidified atmosphere of 6% CO_2 for 48 h. To moisten the epithelium, 100 μL of medium was added to the surface every 12 h of culture. After incubation, the third group was randomly divided into two 1-h treatment sub-groups, resveratrol-treated samples, corneas treated with a single instillation of 100 μM of *trans*-resveratrol (Sigma–Aldrich), and vehicle-treated samples, corneas treated with a single instillation of solvent control [0.1% dimethylsulfoxide (DMSO) in saline. For the immunohistochemical examinations, the corneas were fixed in 4% paraformaldehyde and embedded in Bioplast (Bio-Optica) and then cut into 5-μm sections.

For immunoperoxidase, the sections were incubated with primary polyclonal antibody anti-TLR2 (active motif 1 : 125), then washed in phosphate-buffered saline (PBS) and incubated in a goat anti-rabbit IgG-peroxidase conjugate (Sigma–Aldrich, 1 : 100). Peroxidase activity was visualized with diaminobenzidine (DAB). The sections were then rinsed and counterstained with Toluidine blue. The observations were performed with Zeiss Axioskop 2 plus microscope. For immunofluorescence study, the sections were incubated in primary antibody (anti-TLR2) and then treated with fluorescent-labelled secondary antibody diluted in PBS and Alexa-Fluor 594 donkey anti-rabbit IgG conjugate (Invitrogen, 1 : 300). After washing, the sections were mounted with ProLong® Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) to prevent photobleaching, and coverslipped. Control experiments were performed in which the primary antibody was excluded. The sections were analyzed and images acquired using a Zeiss LSM 5 DUO confocal laser scanning microscope.

For RNA extraction the superficial layers of the epithelium were removed using a 13-mm pre-autoclaved membrane filter (Redfern *et al.*, 2011). Total RNA from the epithelium was extracted using an RNeasy Mini Kit (Qiagen) following the supplier's instructions. PCR amplification was performed with an Applied Biosystem 7300 Real-Time PCR System, (Monza, Italy) coupled with the SYBR® green JumpStart™ Taq Ready Mix kit using specific primers for TLR2 (Kajikawa *et al.*, 2005), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and interleukin (IL)-8 (Wang *et al.*, 2007) at optimized concentrations and cycling conditions. GAPDH was used as housekeeping gene for normalization. The fold increase was compared with the cells of intact corneas not exposed to *S. aureus* and mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2008). Results were expressed as means ± SD from two experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. Differences in groups and treatments were considered significant for $P < 0.05$.

The external surface of the cornea is covered by a stratified epithelium, composed of an outer layer of squamous cells, an intermediate layer formed by both flattened wing cells, and deeper polygonal cells, as well as a basal layer of columnar cells resting on the Bowman's layer (Fig. 1a). Immunohistochemical analyses were used to label TLR2.

The cells of intact corneas demonstrated an occasional mild immunostaining for TLR2 (Fig. 1a). The cells of abraded corneas presented a stronger staining located on a few cells (Fig. 1b). The cells of corneas activated by both strains revealed a higher staining diffused along the surface of all the epithelium exposed to strains (Fig. 1c and Fig 1d), whereas resveratrol-treated cells showed a pattern similar to those of cells of intact corneas. The images of vehicle-treated samples were not different from those of the activated corneas (data not shown).

These results were confirmed by quantitative RT-PCR. TLR2 mRNA levels were significantly higher in activated cornea cells (2.9 ± 0.3-fold and 3.2 ± 0.2-fold change for *S. aureus* ATCC 6538P and *S. aureus* ATCC 29213, respectively) than in resveratrol-treated cells (0.6 ± 0.1-fold and 1.1 ± 0.2-fold change, for *S. aureus* ATCC 6538P and *S. aureus* ATCC 29213, respectively) compared with abraded cornea cells (1.7 ± 0.2-fold change) or intact cornea cells (1.0 ± 0.2-fold change).

To study the TLR signalling efficiency and downstream effector responsiveness to *S. aureus*, the expression of IL-8 mRNA was measured. The results showed a significant upregulation of IL-8 mRNA levels in activated cornea cells (7.8 ± 1.9-fold and 3.6 ± 0.1-fold change for *S. aureus* ATCC 6538P and *S. aureus* ATCC 29213, respectively) against resveratrol-treated cells (0.5 ± 0.1-fold and 0.9 ± 0.2-fold change for *S. aureus* ATCC 6538P and *S. aureus* ATCC 29213, respectively) when compared with abraded cornea cells (1.9 ± 0.3-fold change) or intact cornea cells (1.0 ± 0.1-fold change). DMSO did not

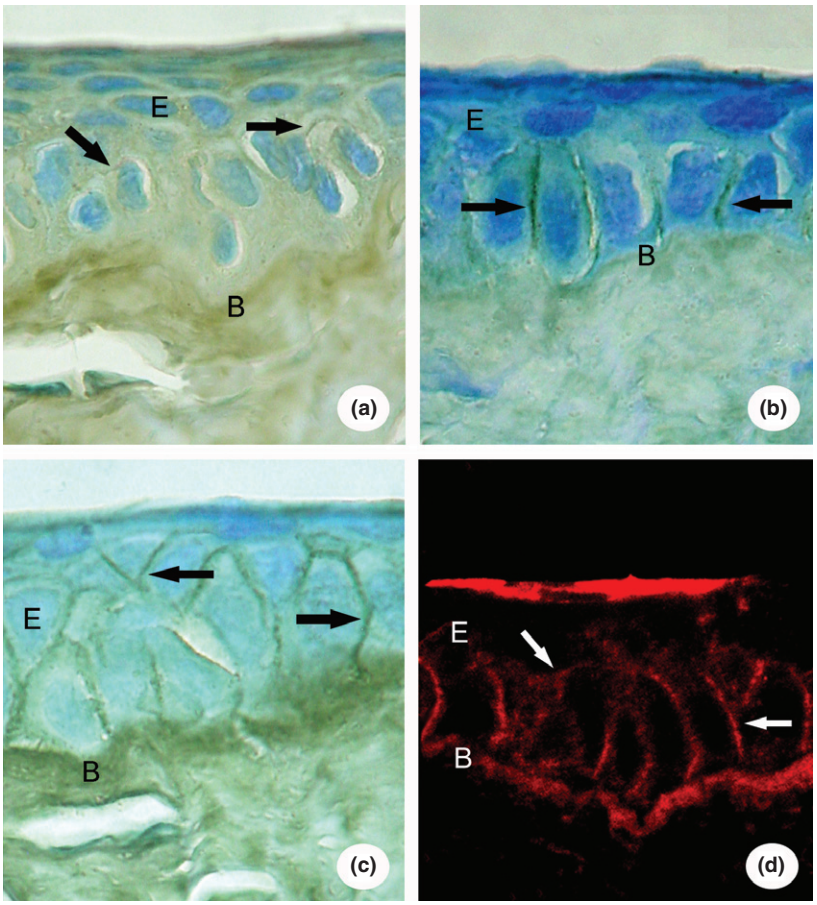

Fig. 1 Expression of TLR2 on cell surface in sections of rabbit corneal epithelium. Peroxidase immunostaining with anti-TLR2 antibodies: intact cornea (a); abraded cornea (b); abraded cornea exposed to inactivated *Staphylococcus aureus* (c). Immunofluorescence staining with anti-TLR2 antibodies on abraded cornea exposed to inactivated *S. aureus* (d). E, corneal epithelium; (B) Bowman's layer; Arrow, TLR2 immunostaining. Original magnification: ×100.

influence the TLR2 or IL-8 expression in vehicle-treated samples (data not shown).

Human corneal epithelial cells express the TLR2, which recognize and respond to *S. aureus* infection by expression and secretion of pro-inflammatory cytokines (IL-8) in a TLR2/MyD88-dependent manner (Lambiase *et al.*, 2011). The anti-inflammatory properties of resveratrol against bacterial infection are due to inhibition of both transcriptional activity and translocation into nuclei of NF-κB functioning downstream of TLR2 (Iyori *et al.*, 2008).

Our results demonstrated that, in response to *S. aureus*, epithelial cells increased expression of TLR2s and, at the same time, significantly upregulated expression of the IL-8 gene. Since the IL-8 gene has the consensus sequence in its promoter κB, it is possible to hypothesize the involvement of the transcription factor NF-κB by the TLR2/MyD88 pathway in the activation of the inflammatory response triggered by *S. aureus* (Johnson *et al.*, 2005). The resveratrol treatment significantly decreased cell surface TLR2 and downregulated expression of IL-8 gene in epithelial cells stimulated by *S. aureus* with respect to untreated cells.

Our study is the first to demonstrate the anti-inflammatory effects of resveratrol against *S. aureus*-induced corneal inflammation. The present results are very interesting and, although obtained on two strains of *S. aureus*, suggest that resveratrol should be evaluated not only for its anti-inflammatory effects but also for its potential innate immunesuppressive properties. Further detailed studies will be needed to confirm the anti-inflammatory activity of resveratrol towards a greater number of strains. The experimental approach described here and based on *ex vivo* corneal culture can be an alternative to *in vivo* animal testing for achieving an understanding of the molecular events of bacterial–epithelial interactions and their inflammatory consequences. The corneal culture model tested can also be used to develop novel specific therapies.

References

Alarcón de la Lastra C & Villegas I (2005) Resveratrol as an anti-inflammatory and anti-aging agent: mechanisms and clinical implications. *Mol Nutr Food Res* 49: 405–430.

Foreman DM, Pancholi S, Jarvis-Evans J, McLeod D & Boulton ME (1996) A simple organ culture model for assessing the effects of growth factors on corneal re-epithelialization. *Exp Eye Res* 62: 555–564.

Hazlett LD (2004) Corneal response to *Pseudomonas aeruginosa* infection. *Prog Retin Eye Res* 23: 1–30.

Heimer SR, Yamada A, Russell H & Gilmore MS (2010) Response of corneal epithelial cells to *Staphylococcus aureus*. *Virulence* 1: 223–235.

Iyori M, Kataoka H, Shamsul HM, Kiura K, Yasuda M, Nakata T, Hasebe A & Shibata K (2008) Resveratrol modulates phagocytosis of bacteria through an NF-κB-dependent gene program. *Antimicrob Agents Chemother* 52: 121–127.

Johnson AC, Heinzel FP, Diaconu E, Sun Y, Hise AG, Golenbock D, Lass JH & Pearlman E (2005) Activation of Toll-like receptor (TLR) 2, TLR4, and TLR9 in the mammalian cornea induces MyD88-dependent corneal inflammation. *Invest Ophthalmol Vis Sci* 46: 589–595.

Kajikawa O, Frevert CW, Lin SM, Goodman RB, Mongovin SM, Wong V, Ballman K, Daubeuf B, Elson G & Martin TR (2005) Gene expression of Toll-like receptor 2, Toll-like receptor 4, and MD2 is differentially regulated in rabbits with *Escherichia coli* pneumonia. *Gene* 344: 193–202.

Kumar A & Yu FS (2006) Toll like receptors and corneal innate immunity. *Curr Mol Med* 6: 327–337.

Lambiase A, Micera A, Sacchetti M, Mantelli F & Bonini S (2011) Toll-like receptors in ocular surface diseases: overview and new findings. *Clin Sci (Lond)* 120: 441–450.

Livak KJ & Schmittgen TD (2008) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25: 402–408.

Major JC Jr, Engelbert M, Flynn HW Jr, Miller D, Smiddy WE & Davis JL (2010) *Staphylococcus aureus* endophthalmitis: antibiotic susceptibilities, methicillin resistance, and clinical outcomes. *Am J Ophthalmol* 149: 278–283.

Redfern RL, Reins RY & McDermott AM (2011) Toll-like receptor activation modulates antimicrobial peptide expression by ocular surface cells. *Exp Eye Res* 92: 209–220.

Sotozono C, Fukuda M, Ohishi M, Yano K, Origasa H, Saiki Y, Shimomura Y & Kinoshita S (2013) Vancomycin ophthalmic ointment 1% for methicillin-resistant *Staphylococcus aureus* or methicillin-resistant *Staphylococcus epidermidis* infections: a case series. *BMJ Open* 3: e001206.

Speciale A, Chirafisi J, Saija A & Cimino F (2011) Nutritional antioxidants and adaptive cell responses: an update. *Curr Mol Med* 11: 770–789.

Sun Y, Hise AG, Kalsow CM & Pearlman E (2006) *Staphylococcus aureus*-induced corneal inflammation is dependent on Toll-like receptor 2 and myeloid differentiation factor 88. *Infect Immun* 74: 5325–5332.

Wang Y, Zhong M, Tan XX, Yang YJ, Chen WJ, Liu W & Zheng K (2007) Expression change of interleukin-8 gene in rabbit basilar artery after subarachnoid hemorrhage. *Neurosci Bull* 23: 151–155.

