

Increased conjunctival expression of protease activated receptor 2 (PAR-2) in seasonal allergic conjunctivitis: a role for abnormal conjunctival epithelial permeability in disease pathogenesis?

S Yeoh, M Church, P Lackie, J McGill, M Mota, P Hossain

Division of Infection, Inflammation & Immunity, Faculty of Medicine, University of Southampton, Southampton General Hospital, Southampton, UK

Correspondence to

Parvez Hossain, Division of Infection, Inflammation & Immunity, MP104, Southampton Eye Unit, Faculty of Medicine, University of Southampton, Southampton General Hospital, Southampton SO16 6YD, UK; parvez@soton.ac.uk

Accepted 20 March 2011
Published Online First
10 May 2011

ABSTRACT

Aims Aeroallergen exposure to the conjunctival epithelium in seasonal allergic conjunctivitis (SAC) may induce a cellular stress response that disrupts the barrier properties of the conjunctival epithelium, resulting in allergic disease. Whether such changes occur in SAC is unknown. Epithelial permeability is known to be increased when protease activated receptor 2 (PAR-2) is activated. We evaluated the expression of PAR-2 in patients with SAC-in-season (SACS) and compared it with control non-atopic subjects or those with out-of-season allergic conjunctivitis (OSAC).

Methods Six SACS, eight normal and four OSAC specimens were examined immunohistochemically for PAR-2 and quantified in a masked fashion for the percentage of epithelia stained for each marker using Image-J software. Conjunctival epithelial heights were measured in all groups to confirm the presence of allergic eye disease.

Results Mean percentage staining of PAR-2 was significantly greater in SACS than in normal specimens ($73.4 \pm 15.4\%$ vs $32.8 \pm 30.0\%$, $p=0.038$) or in OSAC ($73.4 \pm 15.4\%$ vs $1.4 \pm 2.2\%$, $p=0.01$). Mean conjunctival epithelial height was significantly raised in SACS ($63.8 \pm 9.0 \mu\text{m}$) versus controls ($44.7 \pm 11.2 \mu\text{m}$) ($p=0.003$, unpaired t test).

Conclusions Conjunctival epithelial PAR-2 is significantly upregulated in SAC. This supports the view that disruption of the barrier properties of the conjunctival epithelium is an important event in SAC pathogenesis.

Allergic eye disease (AED) is a common ocular surface disorder that is increasing in incidence and causes considerable ocular morbidity. Seasonal allergic conjunctivitis (SAC) accounts for 25–50% of cases of AED and is triggered by a type 1 hypersensitivity reaction to aeroallergens that typically occur during the hayfever season.¹

The inflammatory features of AED are associated with the interaction of airborne allergens with conjunctival stromal mast cells. Allergens bind to surface-bound IgE molecules on mast cells, cross-linking them and signalling degranulation, resulting in the release of pre-formed chemical mediators and the subsequent activation of the allergic cascade.

In order for allergens to pass from the external environment and interact with the conjunctival mast cells, they need to cross the conjunctival

epithelium and enter the submucosal space. In other atopic conditions, such as asthma and allergic dermatitis, the epithelial layers are found to be dysregulated, resulting in increased permeability to airborne allergens. These permeability changes have been shown to be related to increased expression of protease-activated receptor-2 (PAR-2), which is an important regulator of epithelial barrier permeability.^{2–4} Whether similar mechanisms are involved in the conjunctival epithelium in AED is not known.

In this paper we investigate the changes in epithelial barrier properties by examining the expression of PAR-2 in patients with AED.

METHODS

Subjects

Conjunctival epithelial biopsy specimens were collected from the following three groups of people: normal (non-atopic) individuals, individuals who had SAC during the pollen season (SACS), and individuals who had SAC but out-of-season (OSAC).

Ethical considerations prevented us from taking repeat biopsies from patients with AED, so to assess whether the effects were from 'in' or 'out' of the pollen season, different patients were used.

The allergen status of each subject was assessed by skin prick testing to a panel of allergen extracts, including house dust mites, rye grass, cat hair and dog hair, to determine their atopic status. Responses to a 0.1% histamine dihydrochloride solution and a saline/albumin diluent were included as positive and negative controls.

Conjunctival biopsy

Conjunctival specimens were taken from the superior bulbar conjunctiva under local anaesthesia (oxybuprocaine hydrochloride) taking a biopsy size of approximately $2 \times 2 \text{ mm}$.

Conjunctival biopsies were collected from eight patients with SACS and seven normal control subjects. All 15 specimens were assessed for conjunctival epithelial thickness.

A further six patients with SACS, four patients with OSAC and eight control subjects were biopsied. These 18 specimens were assessed by immunohistochemistry, staining primarily for PAR-2, and also for heat shock protein (HSP)-70, HSP-90, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1).

Tissue processing and immunohistochemical analysis

The specimens were transferred and fixed in acetone containing the enzyme inhibitors iodoacetamide (20 mM) and phenyl methyl sulphonyl fluoride (2 mM), and then stored overnight at -20°C before processing into glycolmethacrylate for immunohistochemistry staining. Serial sections were cut at $2\text{ }\mu\text{m}$. The blocks were cut and stained using a standard immunoperoxidase staining protocol.⁵

The details of monoclonal mouse antibodies used in staining the sections are as follows: anti-HSP-70 antibody (dilution 1:300; Santa Cruz Biotechnology, Santa Cruz, California, USA); anti-HSP-90 antibody (dilution 1:160; StressGen Biotechnologies, Victoria, Canada); P2A (a gift from Ahkmed Aslam of Southampton Hospital; dilution 1:400); anti-ICAM-1 (1:40; StressGen Biotechnologies); anti-VCAM-1 (dilution 1:10; StressGen Biotechnologies). The whole staining procedure was performed in 48 h at room temperature. Positive (antibody AA1 staining for mast cells) and negative (omission of primary antibody) controls were used for each biopsy.

Measurements of conjunctival epithelial heights, quantification of immunostaining and statistical analysis

Conjunctival biopsies were collected from eight SACS and seven normals (non-atopic). These were fixed and embedded in glycol methacrylate and conjunctival epithelial thicknesses were measured using an upright light microscope (sections were photographed using a Leica DM-RBE camera $\times 10$ objective) and Image-J software.⁶

An image of a standard 1 mm stage μm scale at $\times 10$ magnification was used to calibrate images used to measure conjunctival epithelial thickness from the basement membrane to the apical surface of the epithelium. Measurements of the conjunctival epithelial thicknesses were taken at regular/consistent intervals and the average was then calculated per field viewed. At least three sections and three separate fields were examined.

Immunohistochemical expression of PAR-2, HSP-70, HSP-90, ICAM and VCAM were quantified as the percentage of epithelia stained using light microscopy (sections were photographed using a Leica DM-RBE camera $\times 40$ objective) and Image-J software. At least two sections and two separate fields of each specimen were viewed. Positive and negative controls were also examined.

Statistical analysis

All analyses were done without the observer knowing the sample type. The Student *t* test was performed on data collected from epithelial heights. Non-parametric testing was performed on data collected from samples stained for PAR-2 and HSP-70 as the data was not normally distributed. A Mann–Whitney *U* test was used to calculate the statistical significance. A *p* value of <0.05 was taken as statistically significant.

RESULTS

Conjunctival epithelial thickness

The mean conjunctival epithelial thicknesses (mean $\pm$ SD) in normal controls and SACS groups were $44.7\pm 11.2\text{ }\mu\text{m}$ and $63.8\pm 9.0\text{ }\mu\text{m}$, respectively. The conjunctival epithelial thicknesses in the SACS group were significantly greater than the normal group ($p=0.003$).

Immunochemical staining

Immunoreactivity for PAR-2, ICAM-1, HSP-70 and HSP-90 was detected in both SACS patient and normal control groups. The OSAC group showed low expression levels for PAR-2 and HSP-70.

VCAM-1 expression was negative in all groups. The positive controls (antibody AA1), stained for mast cells, and the negative controls revealed no staining.

Expression of PAR-2, HSP-70, HSP-90 and ICAM-1 was predominantly seen in the conjunctival epithelium, while in the stroma, weak staining was seen in vascular endothelial cells.

Within the epithelial cells, PAR-2 expressed cytoplasmic staining, HSP-70 showed dense nuclear and cytoplasmic staining, HSP-90 showed cytoplasmic and weak nuclear staining, and ICAM-1 showed cell membrane and cytoplasmic staining.

Staining expression

Percentage staining for the pro-inflammatory molecules tested in the conjunctival epithelium was quantified using Image-J software.

The percentage staining expression of PAR-2 (mean $\pm$ SD) was significantly greater in SACS compared with OSAC ($73.4\pm 15.4\%$ vs $1.36\pm 2.2\%$, $p=0.01$) and the normal control group ($73.4\pm 15.4\%$ vs $32.8\pm 30.0\%$, $p=0.038$) (figures 1A and 2). Similarly, there was increased expression of HSP-70 in SACS compared with OSAC group ($73.3\pm 22.21\%$ vs $22.0\pm 7.76\%$, $p=0.013$) (figures 1B and 2). No significant difference was found between any of the groups for HSP-90 or for ICAM-1 (figure 1C,D).

Distribution of staining within the normal controls

There was a wide distribution of PAR-2, HSP-70 and HSP-90 expression in the control group.

DISCUSSION

We have shown for the first time that there is a significant increase in the expression of PAR-2 and HSP-70 in the conjunctival epithelium of SAC patients when exposed to pollen.

In the absence of allergenic stimuli, patients with OSAC have low levels of HSP-70 and PAR-2 expressions, suggesting that allergen challenge increases the protein levels in the conjunctival epithelium. Other atopic conditions such as asthma, allergic rhinitis and contact dermatitis show similarities where high levels of PAR-2 and HSP-70 are found in the epithelial layers.^{7–11} In addition, increased expression of HSP-70 and PAR-2 is associated with an increase in conjunctival epithelial thickness. Previously published data suggest that the changes in conjunctival epithelium thickness are due to a primary increase in epithelial cell volume, rather than conjunctival epithelium hyperplasia.¹²

The conjunctival epithelium of healthy subjects, with no atopic background, has shown variability in constitutive expression of PAR-2 and HSP-70 for reasons yet unknown. A study by Knight *et al*⁷ who examined levels of PAR-1, PAR-2 and PAR-3 in the bronchial epithelium of non-atopic subjects, also demonstrated the variability of these markers. Trypsin, an enzyme known to activate PAR-2, is found in proteases produced by a variety of organisms such as house dust mites, fungi and bacteria (eg, *Staphylococcus aureus*). It is well known that 20% of the population are chronic carriers of *Staphylococcus aureus*, which are commensals of skin and nasal passages. Therefore, we suspect a non-specific action from these common organisms could explain why PAR-2 staining variability is demonstrated in the specimens from non-atopic individuals.

Although our sample sizes are small, statistical power tests indicate that the differences in PAR-2 expression that we found were above a confidence level of 95% (in the comparison between SACS and non-atopic controls) and close to 100%

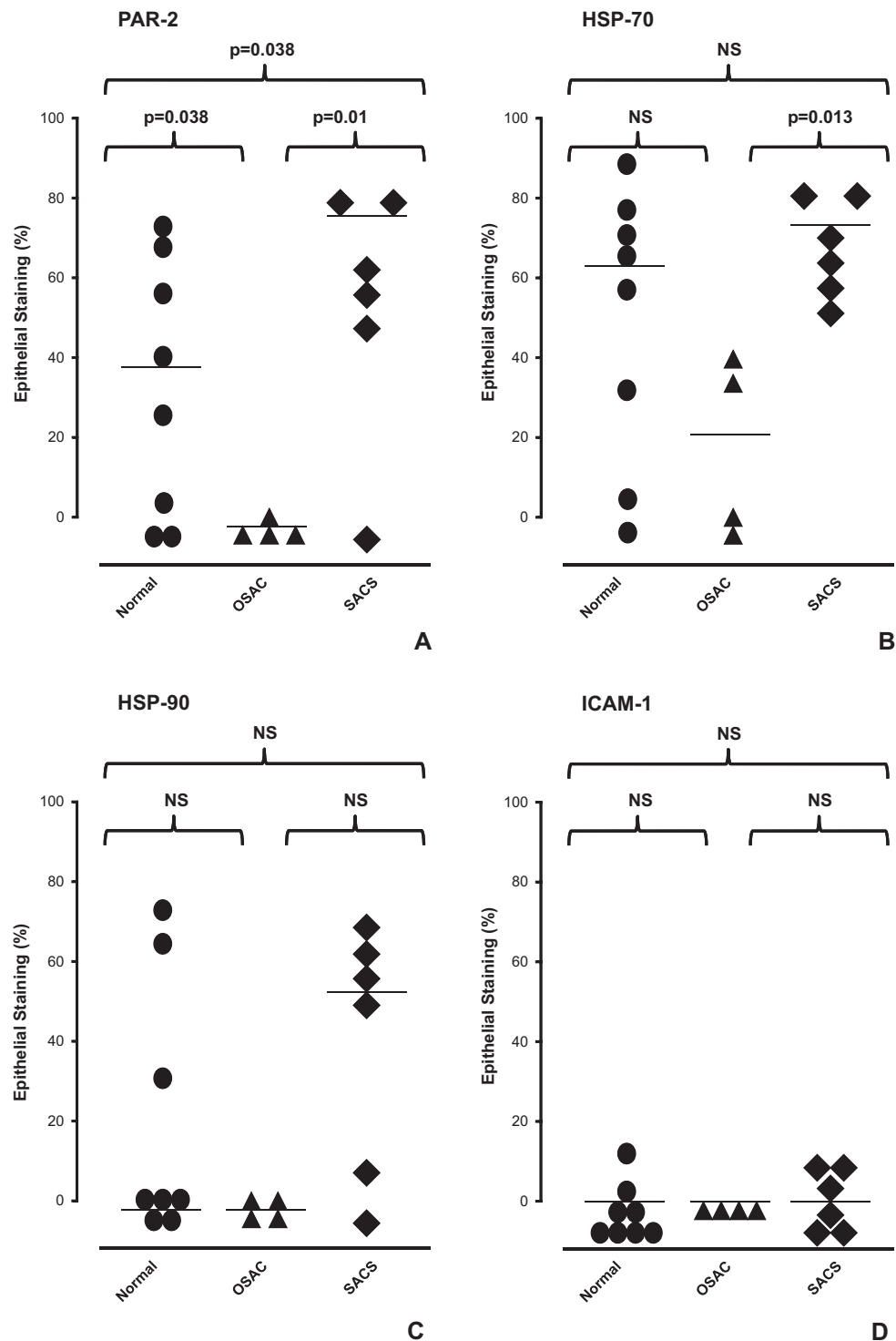


Figure 1 The expression of protease activated receptor 2 (PAR-2) (A), heat shock protein (HSP)-70 (B), HSP-90 (C) and intracellular adhesion molecule (ICAM)-1 (D) in the conjunctival epithelia of seasonal allergic conjunctivitis (SACS) patients 'in-season', out-of-season allergic conjunctivitis (OSAC) patients 'out-of-season', and normal controls. Bars indicate mean values. NS denotes no significance.

(when comparing SACS and OSACS). Similarly, for the difference in HSP-70 expression found comparing SACS and OSAC, the significance probability was also close to 100%.

Unfortunately, due to ethical reasons repeat biopsies from the same patient was not allowed, so we were not able to take biopsies in the same atopic patient 'in-season' and 'out-of-season'.

ICAM-1 and VCAM-1 are typically expressed on the vascular endothelium at sites of active inflammation. Increased expression in epithelial cells has also been reported in other studies in

bronchial and renal tissues.^{13 14} The poor expression of ICAM-1 and VCAM-1 in both normal and 'out-of-season' AED epithelia in our study confirms that there is little inflammatory activity present in our study population. Hence, the changes in PAR-2 and HSP-70 are likely to reflect that the epithelial layer has abnormal barrier properties, thus creating a predisposition to inflammatory disease.

HSP-90 expression in the conjunctival epithelium, although not statistically significant, shows seasonal variability in levels

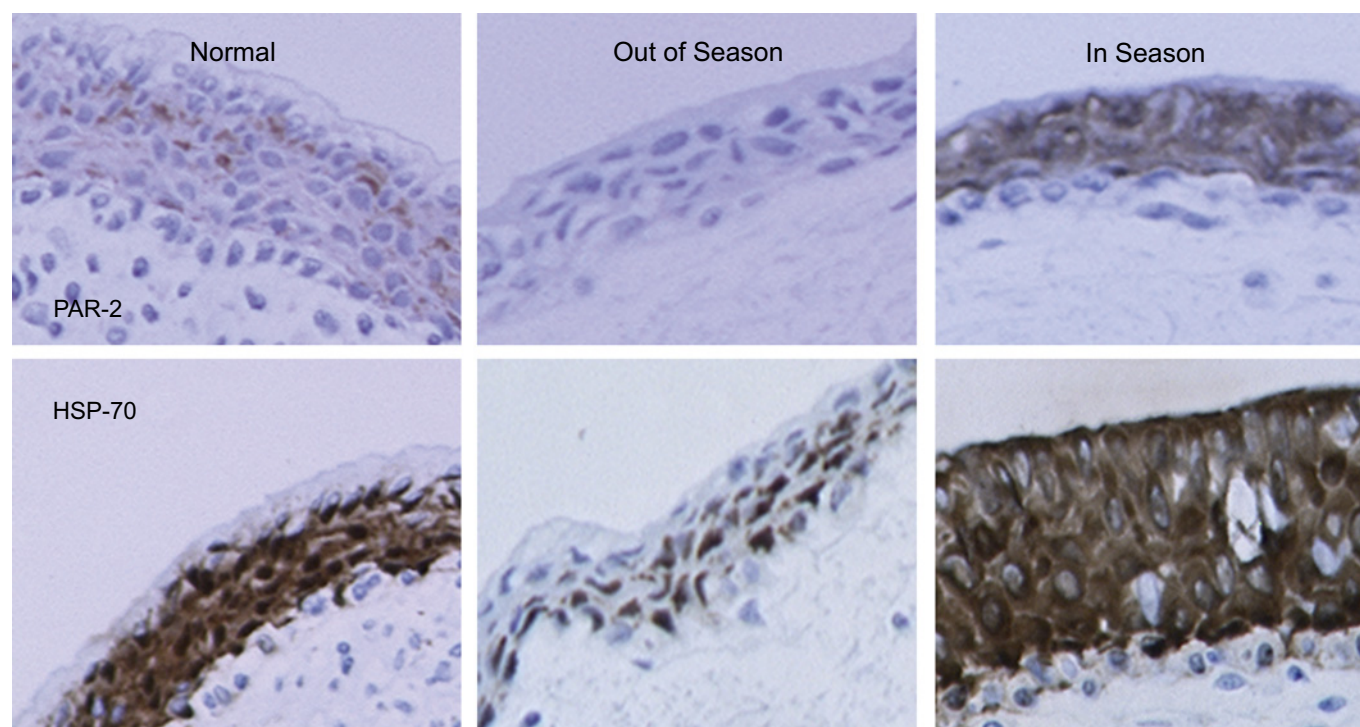


Figure 2 Photomicrographs of sections of conjunctival epithelia, demonstrating that the epithelia have different levels of expression for protease activated receptor 2 (PAR-2) and heat shock protein (HSP)-70 in normal controls and seasonal allergic conjunctivitis (SACS) 'in-season' and 'out-of-season'.

of expression in the atopic groups similar to that seen for PAR-2 and HSP-70. This suggests that HSP-90—like HSP-70—is an inflammatory marker in the conjunctival epithelium, but is not a significant factor in AED.

In order to initiate the allergic response, allergens need to cross the epithelial surfaces to gain access to the sub-epithelial layer. Several studies of atopic conditions have shown that PAR-2 alters the epithelial barrier function to facilitate the passage of an allergen.^{3 4 15} The epithelia in atopic subjects with conditions such as asthma, allergic rhinitis and atopic dermatitis have been shown to express high levels of PAR-2.^{3 8 16}

PAR-2 is a seven-transmembrane G protein coupled receptor, abundantly expressed on surfaces such as those lining the airways, gut and the skin. These epithelia surfaces are frequently exposed to environmental allergens containing enzymes responsible for activating these receptors. The N-amino terminus component of PAR-2 is activated by proteolytic cleavage by serine proteases found in house dust mites, pollen and mast cells.¹⁷ Atopic patients who have upregulated PAR-2 manifest their allergic symptoms when exposed to serine proteases found in these aeroallergens.^{18 19} In a murine model, infusion of PAR-2-activating peptide (PAR-2-AP) into the airway and intestine initiated an inflammatory response that increased both lung endothelial/epithelial and intestinal permeability to macromolecules.²⁰ Disorders with high levels of epithelial PAR-2 expression have altered epithelial permeability, which may also be reflected in the conjunctival epithelium in active AED.

PAR-2 is known to alter epithelial permeability by the disruption of epithelial E-cadherin adhesion molecules, which are important components of tight junctions. Hughes *et al* have shown that baseline E-cadherin expression is lower in subjects with OSAC than in normal non-atopic subjects.¹²

We have shown that PAR-2 is elevated in atopic individuals in the presence of pollen. If we combine the data from Hughes *et al*

and the present study, we can hypothesise that there is an inverse relationship between PAR-2 and E-cadherin expression, and that levels are dependent on seasonal state. For example, in the presence of pollen, PAR-2 levels in atopic subjects are elevated; this effect mediates further PAR-2-induced E-cadherin down-regulation, to levels below those of the quiescent state. This would explain the increased permeability of a structurally weaker epithelium to pollen penetration. Once in the sub-epithelial layer, pollen antigens cross-link with mast cells, resulting in release of tryptase to promote further upregulation of PAR-2.

The overexpression of HSP-70 in AED subjects in this study, following pollen exposure, suggests that these proteins also play an important role in the regulation of the allergic response in AED. HSP-70 seems to have an important role in other atopic conditions, as overexpression of HSP-70 is found in peripheral lymphocytes of patients with allergic rhinitis and bronchi of asthmatic patients.^{21 22} HSP-70 has an important role in antigen processing and/or presentation to antigen-presenting cells, which is essential for the initiation and modulation of the allergic response.²³ Studies have shown the ability of HSP-70 to transduce its pro-inflammatory signals by utilising Toll-like receptors (TLRs).²⁴ The signalling pathways of TLRs are involved in the front-line defence against invading microorganisms. Recent studies have shown involvement of TLRs with atopic diseases. Interestingly, TLR-4 has been shown to be upregulated in human allergic conjunctiva in the stroma of patients with vernal keratoconjunctivitis.²⁵ Further work is required to assess whether TLRs have a role in the conjunctival epithelium in AED.

This study demonstrates that PAR-2 and HSP-70 are good markers of allergic properties in the conjunctival epithelium in AED. We have shown that there are alterations in the barrier properties of the conjunctival epithelium after aeroallergen exposure. The increased expression of PAR-2 suggests that the

conjunctival epithelium is structurally weaker and may exacerbate the allergic response. Further investigations are required to determine whether predisposition to AED is related to primary structural changes in the conjunctival epithelium.

Funding Funded by University of Southampton.

Competing interests None.

Patient consent Obtained

Ethics approval This study was conducted with the approval of the Southampton and South West Joint Research Ethics Committee (REC 036/02) and was conducted in accordance with the tenets of the Declaration of Helsinki.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. McGill JI, Holgate ST, Church MK, *et al.* Allergic eye disease mechanisms. *Br J Ophthalmol* 1998;**82**:1203–14.
2. Winter MC, Shasby SS, Ries DR, *et al.* PAR2 activation interrupts E-cadherin adhesion and compromises the airway epithelial barrier: protective effect of beta-agonists. *Am J Physiol Lung Cell Mol Physiol* 2006;**291**:L628–35.
3. Jeong SK, Kim HJ, Youm JK, *et al.* Mite and cockroach allergens activate protease-activated receptor 2 and delay epidermal permeability barrier recovery. *J Invest Dermatol* 2008;**128**:1930–9.
4. Roelandt T, Heughebaert C, Hachem JP. Proteolytically active allergens cause barrier breakdown. *J Invest Dermatol* 2008;**128**:1878–80.
5. Britten KM, Howarth PH, Roche WR. Immunohistochemistry on resin sections: a comparison resin embedding technique for small mucosal biopsies. *Biotech Histochem* 1993;**68**:271–80.
6. Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with Image J. *Biophotonics* 2004;**11**:36–42.
7. Knight DA, Lim S, Scaffidi AK, *et al.* Protease-activated receptors in human airways: upregulation of PAR-2 in respiratory epithelium from patients with asthma. *J Allergy Clin Immunol* 2001;**108**:797–803.
8. Lee HM, Kim HY, Kang HJ, *et al.* Up-regulation of protease-activated receptor 2 in allergic rhinitis. *Ann Otol Rhinol Laryngol* 2007;**116**:554–8.
9. Vignola AM, Chanez P, Polla BS, *et al.* Increased expression of heat shock protein 70 on airway cells in asthma and chronic bronchitis. *Am J Respir Cell Mol Biol* 1995;**13**:683–91.
10. Chalastris T, Nicolopoulou-Stamati P, Patsouris E, *et al.* Expression of substance P, vasoactive intestinal peptide and heat shock protein 70 in nasal mucosal smears of patients with allergic rhinitis: investigation using a liquid-based method. *J Laryngol Otol* 2008;**122**:700–6.
11. Yusuf N, Nasti TH, Huang CM, *et al.* Heat shock proteins HSP27 and HSP70 are present in the skin and are important mediators of allergic contact hypersensitivity. *J Immunol* 2009;**182**:675–83.
12. Hughes JL, Lackie PM, Wilson SJ, *et al.* Reduced structural proteins in the conjunctival epithelium in allergic eye disease. *Allergy* 2006;**61**:1268–74.
13. Atsuta J, Sterbinsky SA, Plitt J, *et al.* Phenotyping and cytokine regulation of the BEAS-2B human bronchial epithelial cell: demonstration of inducible expression of the adhesion molecules VCAM-1 and ICAM-1. *Am J Respir Cell Mol Biol* 1997;**17**:571–82.
14. Seron D, Cameron JS, Haskard DO. Expression of VCAM-1 in the normal and diseased kidney. *Nephrol Dial Transplant* 1991;**6**:917–22.
15. Vroling AB, Jonker MJ, Luiten S, *et al.* Primary nasal epithelium exposed to house dust mite extract shows activated expression in allergic individuals. *Am J Respir Cell Mol Biol* 2008;**38**:293–9.
16. Reed CE, Kita H. The role of protease activation of inflammation in allergic respiratory diseases. *J Allergy Clin Immunol* 2004;**114**:997–1008;quiz 1009.
17. Coelho AM, Ossovskaya V, Bunnett NW. Proteinase-activated receptor-2: physiological and pathophysiological roles. *Curr Med Chem Cardiovasc Hematol Agents* 2003;**1**:61–72.
18. Sun G, Stacey MA, Schmidt M, *et al.* Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol* 2001;**167**:1014–21.
19. Kato T, Takai T, Fujimura T, *et al.* Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes. *Allergy* 2009;**64**:1366–74.
20. Su X, Camerer E, Hamilton JR, *et al.* Protease-activated receptor-2 activation induces acute lung inflammation by neuropeptide-dependent mechanisms. *J Immunol* 2005;**175**:2598–605.
21. Liu L, Xiao C, Zhang M, *et al.* Expression of HSP70 in peripheral lymphocytes of the patients with allergic rhinitis. *J Huazhong Univ Sci Technol Med Sci* 2003;**23**:310–12.
22. Tong W, Luo W. Heat shock proteins mRNA expressions by peripheral blood mononuclear cells in asthma and chronic bronchitis. *Chin Med J (Engl)* 2000;**113**:175–7.
23. Bertorelli G, Bocchino V, Zhuo X, *et al.* Heat shock protein 70 upregulation is related to HLA-DR expression in bronchial asthma. Effects of inhaled glucocorticoids. *Clin Exp Allergy* 1998;**28**:551–60.
24. Asea A, Rehli M, Kabingu E, *et al.* Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 2002;**277**:15028–34.
25. Bonini S, Micera A, Iovieno A, *et al.* Expression of toll-like receptors in healthy and allergic conjunctiva. *Ophthalmology* 2005;**112**:1528;discussion 1548–9.



Increased conjunctival expression of protease activated receptor 2 (PAR-2) in seasonal allergic conjunctivitis: a role for abnormal conjunctival epithelial permeability in disease pathogenesis?

S Yeoh, M Church, P Lackie, et al.

Br J Ophthalmol 2011 95: 1304-1308 originally published online May 10, 2011

doi: 10.1136/bjo.2010.191221

Updated information and services can be found at:

<http://bjo.bmj.com/content/95/9/1304.full.html>

These include:

References

This article cites 25 articles, 10 of which can be accessed free at:

<http://bjo.bmj.com/content/95/9/1304.full.html#ref-list-1>

Email alerting service

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections

Articles on similar topics can be found in the following collections

[Conjunctiva](#) (166 articles)

[Ocular surface](#) (436 articles)

Notes

To request permissions go to:

<http://group.bmj.com/group/rights-licensing/permissions>

To order reprints go to:

<http://journals.bmj.com/cgi/reprintform>

To subscribe to BMJ go to:

<http://group.bmj.com/subscribe/>