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Journal of the Chinese Medical Association 79 (2016) 565−569
www.jcma-online.com

Original Article

Evaluation of transforming growth factor-beta1 gene expression in pterygium tissue of atopic patients

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Received August 25, 2014; accepted April 26, 2016

Abstract

Background: The exact pathogenesis of pterygium is still not fully understood. Growth factors are considered to play an important role in the formation of pterygium. Transforming growth factor (TGF)-β1 is considered to be one of the main mediators of fibroblast stimulation and tissue remodeling in allergic conditions. The objective of the present study was to investigate the association between TGF-β1 gene expression and pterygium in atopic and nonatopic participants.
Methods: We used questionnaires to record demographic and clinical information from patients who underwent pterygium excision surgery. Skin prick examination was done to confirm or rule out atopy in 30 patients with atopy (Case Group) and 30 individuals without atopy (Control Group). Additionally, measurement of serum immunoglobulin E, cytokines, including interleukin-4 and interferon-γ, and peripheral blood eosinophil count was performed to confirm atopy in 30 consecutive patients (Case Group). A semiquantitative reverse transcription polymerase chain reaction was performed to determine TGF-β1 gene expression in all individuals.
Results: TGF-β1 mRNA gene expression was significantly higher ($p = 0.0001$) in atopic patients 2.50 ± 1.11 compared to nonatopic individuals 1.40 ± 0.46. Eosinophil count and serum immunoglobulin E were significantly higher ($p = 0.031$ and $p = 0.001$, respectively) in atopic patients compared to the Control Group. Serum interleukin-4 was also significantly higher ($p = 0.01$) in atopic patients compared with nonatopic individuals.
Conclusion: Excess expression of TGF-β1 gene in pterygium tissue of atopic individuals suggests that growth factors play a role in the pathogenesis of pterygium.
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Keywords: atopy; gene expression; pterygium; transforming growth factor-β1

1. Introduction

Pterygium is a benign condition, characterized by invasive fibrovascular growth of the conjunctiva to the cornea, which is generally linked with overexposure to UV radiation.[1] Although some environmental factors (e.g., wind, dust, heat, infection, smoke, chemicals, dry eye, and pollens) are suggested to play a role in the pathogenesis of pterygium,[1−3] the exact etiology of this condition is still not fully understood.

Conflict of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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http://dx.doi.org/10.1016/j.jcma.2016.05.003
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Some investigators have proposed an allergic and immunological basis for the pathogenesis of pterygium.[4,5] Several growth factors, cytokines, and metalloproteinase enzymes are identified in the cornea during the recovery time after photorefractive keratectomy. These biological factors, which are produced by migratory leukocytes, are considered to play an important role in the formation of pterygium.[3] In addition, it has been demonstrated that UV may activate signaling pathways in the epithelial cells of pterygium, resulting in production of cytokines and growth factors.[6]

Both acute and chronic complications have been observed in atopic patients due to the release of inflammatory mediators. Transforming growth factor (TGF)-β1 is considered to be one of the main mediators of tissue remodeling in patients with asthma.[7] It stimulates fibroblasts to produce extracellular matrix proteins and cell-adhesion molecules such as collagen, fibronectin, and integrins; decreases production of collagenase, heparinase, and stromelysin; and results in extracellular matrix deposition. Additionally, it directly induces angiogenesis *in vivo*. Therefore, TGF-β1 is considered an effective regulator of tissue invasion and metastasis.[8] Stroma of pterygium cells and fibroblasts plays a fundamental role in the remodeling process of pterygium tissue. Matrix metalloproteinases have been found in these cells.[9] Furthermore, TGF-β1 enhances matrix metalloproteinase expression and fibroblast activity.[10]

To the best of our knowledge, no previous investigation has considered the association between pterygium and TGF-β1 levels in atopic patients. In the present study, TGF-β1 mRNA gene expression in pterygium tissue of atopic patients was evaluated to investigate its probable association with increased susceptibility for pterygium formation. In addition, the present study aimed to determine if TGF-β1 overexpression in atopic individuals promotes tissue remodeling in pterygium formation. The histopathological differences in pterygium between atopic and nonatopic patients have also been investigated.

2. Methods

2.1. Study population

This study was conducted in accordance with the Helsinki Declaration of 1975 (as revised in 1983) and approved by the Research Ethics Committee of Islamic Azad University of Mashhad, Mashhad, Iran. Informed consent was obtained from all participants after the nature of the study was explained. Predesigned questionnaires were used to record demographic information and past medical history of individuals who were diagnosed with pterygium and referred to the Eye Hospital of Mashhad University of Medical Sciences from June 2010 to May 2011. Thereafter, 30 pterygium patients without any history of allergic reactions were enrolled in the Control Group. Those with a history of at least one allergic condition (e.g., asthma, allergic rhinitis, atopic dermatitis, hives or angioedema, or food allergies) underwent skin prick testing and measurement of total serum immunoglobulin (Ig)E level. Correspondingly, 30 pterygium patients with positive skin prick test and IgE level > 100 IU/mL were included in the Case Group. According to ophthalmological examination, surgical excision of pterygium was performed on all 60 patients who were enrolled in the study. The exclusion criteria were previous treatment with corticosteroids during the past 2 months, immunodeficiency, and absence of indication for excisional surgery. Participants aged ≥ 60 years were also excluded from the study population due to decreased wheal and flare in skin prick tests.[11] In addition, patients were advised to withdraw from the use of drugs and medications at least 48 hours before the skin test because some topical corticosteroids or antihistamines may affect the validity of skin prick tests.[12] Additionally, we certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research.

2.2. Immunological assessment

All patients were evaluated by skin prick test on the inner forearm, using 19 common standard allergen extracts (Stallergenes, Antony, France; Hollister−Stier Laboratories, Spokane, WA, USA). Blood samples were obtained by

Table 1
Sequence of primers used in this study.

mRNA	Primer
TGF-β1 mRNA (137 bp, RT-PCR)	Sense primer: 5′-AAGGACCTCGGCTGGAAGTG-3′ Anti-sense primer: 5′-CCCGGGTTATGCTGGTTGTA-3′
GAPDH mRNA (266 bp, RT-PCR)	Sense primer: 5′-GGAAGGTGAAGGTCGGAGTCA-3′ Anti-sense primer: 5′-GTCATTGATGGCAACAA TATCCACT-3′

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; RT-PCR = reverse transcription polymerase chain reaction; TGF-β1 = transforming growth factor-β1.

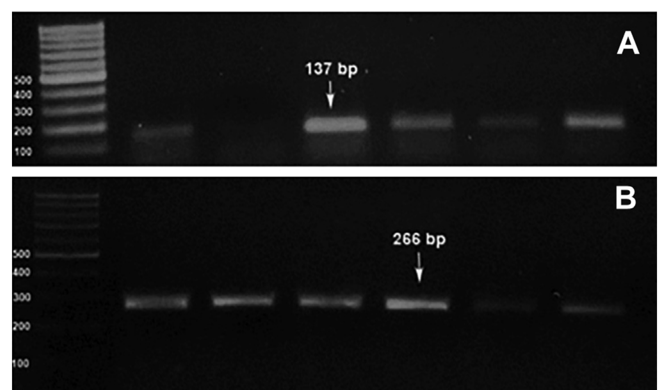

Fig. 1. Transforming growth factor (TGF)-β1 gene expression compared with the ladder was measured using reverse transcription polymerase chain reaction (RT-PCR). RT-PCR products from TGF-β1 mRNA with 137-bp fragment (A) and glyceraldehyde-3-phosphate dehydrogenase mRNA with 266-bp fragment (B) were separated on agarose gels and stained with green viewer. According to the Mann−Whitney *U* test, mean relative expression level of TGF-β1 mRNA was significantly higher ($p = 0.0001$) in atopic patients (2.50 ± 1.11) compared with that in nonatopic individuals (1.40 ± 0.46).

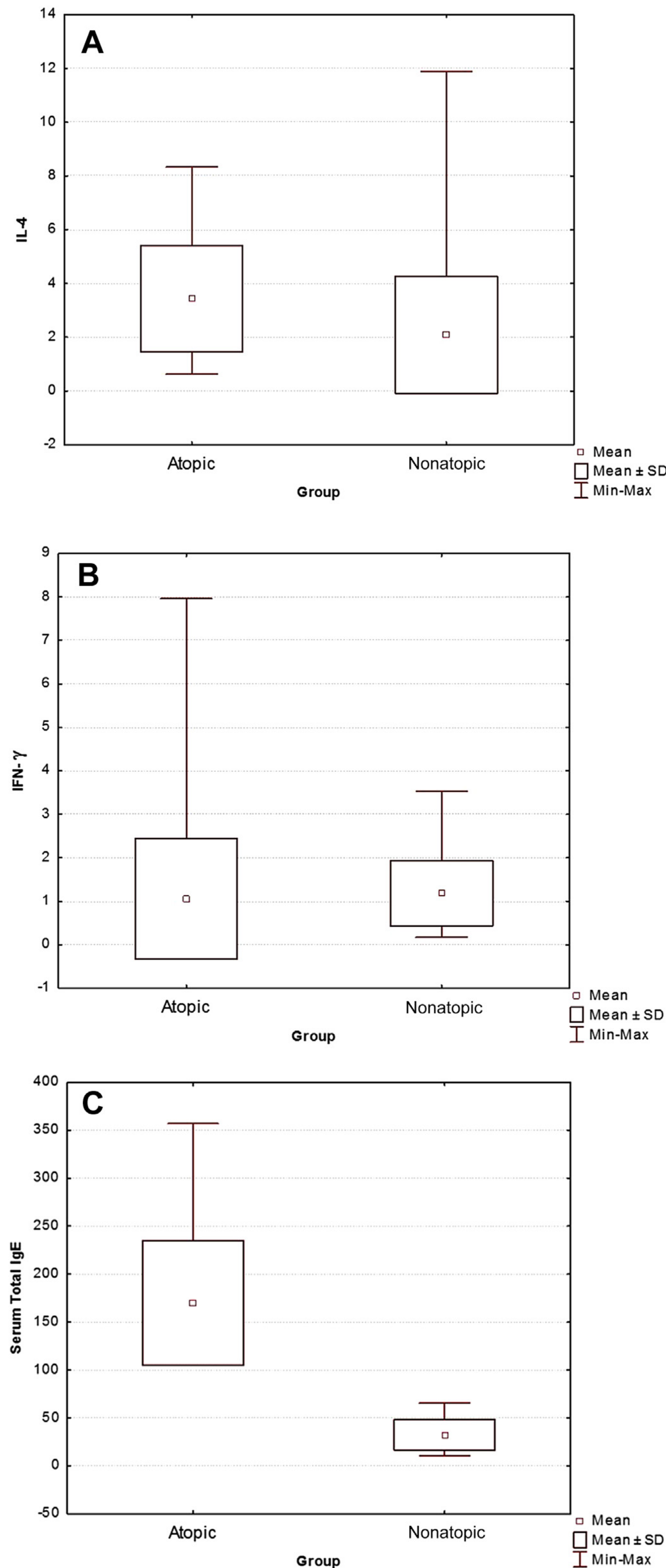

Fig. 2. Comparison of serum cytokines interleukin (IL)-4 (A), interferon (IFN)-γ (B), and total immunoglobulin (Ig)E (C) levels between pterygium patients with ($n = 30$) and without ($n = 30$) atopy. Cytokines and total IgE levels were measured by enzyme-linked immunoassay. Data are mean ± standard error. (A) Mean serum level of IL-4 in atopic patients (3.43 ± 1.98 pg/mL) was significantly higher ($p = 0.01$) than in nonatopic individuals (2.09 ± 2.18 pg/mL). (B) Mean serum level of IFN-γ in atopic patients (1.06 ± 1.39 pg/mL) did not differ significantly ($p = 0.06$) from that in nonatopic individuals (1.18 ± 0.74 pg/mL). (C) Mean serum level of IgE in atopic patients (170.04 ± 65.0 IU/mL) was significantly higher ($p = 0.001$) than that in nonatopic individuals (32.21 ± 15.96 IU/mL).

venipuncture for eosinophil count and cytokine assay. The eosinophil count was measured using a Sysmex KX-21N cell counter (Sysmex Corporation, Kobe, Japan). Aliquots of serum were stored at −70°C until analyzed for cytokines and IgE level. Enzyme-linked immunosorbent assay was performed to determine serum IgE levels (Monobind, Lake Forest, CA, USA) and interleukin (IL)-4 and interferon (IFN)-γ (Amersham BioSciences, Little Chalfont, Buckinghamshire, UK). Thirty individuals with positive skin prick tests and serum IgE > 100 IU/mL were classified as atopic and included in the Case Group.

2.3. RNA extraction and reverse transcription polymerase chain reaction

After pterygium excision surgery, the tissues were collected and immersed in RNA later solution at −20°C until extraction. An expert clinical pathologist histologically confirmed all specimens as pterygium. RNeasy Kit (Fibrous Tissue, Qiagen, Hilden, Germany) was used for purification of total RNA from pterygium biopsy samples. Also, the quality and quantity of the RNA was photometrically confirmed. Subsequently, cDNA was generated with Oligo-dt16 (Pars Tous, Iran). Reverse transcription polymerase chain reaction (PCR) was performed in a total volume of 20 μL containing 2 μL 10 × PCR buffer, 2 mmol/L magnesium chloride, 0.2 mmol/L dNTP mixture, 1.5 U Hot-Start Taq DNA polymerase, 0.5 mmol/L each primer, and 2 μL cDNA. A fragment of TGF-β1 gene (137 bp) was amplified in the following PCR conditions: 30 seconds at 94°C (denaturation), 30 seconds at 60°C (annealing), and 30 seconds at 72°C (extension) for 35 cycles with the primers listed in Table 1.

To normalize expression of TGF-β1 mRNA, a 266-bp fragment length of glyceraldehyde-3-phosphate dehydrogenase was amplified as follows: 30 seconds at 94°C (denaturation), 30 seconds at 56°C (annealing), and 30 seconds at 72°C (extension) for 35 cycles with the primers listed in Table 1. A 2.5% agarose gel was used to separate PCR products and to visualize under UV illumination, stained with green viewer (Pars Tous). The mRNA expression of each gene was determined using Kodak 1D Image Analysis Software (Kodak, Stuttgart, Germany). The band intensity was demonstrated as an absolute integrated optical density. The integrated optical density of each PCR product was normalized to that of glyceraldehyde-3-phosphate dehydrogenase for the same biopsy sample (Fig. 1A and 1B).

2.4. Statistical analysis

Data were analyzed using SPSS version 18 (IBM Corporation, Armonk, NY, USA) and displayed as mean ± standard deviation. To check the normality of the data, the Kolmogorov−Smirnov and Lilliefors test (for the correction of p values

related to Kolmogorov−Smirnov) were applied. The Student *t* test was performed for data with normal distribution, and the Mann−Whitney *U* test was used to compare non-normal variables. Statistical analyses for the distributions of optical densities in pterygium patients with and without atopy were carried out using the χ^2 test or Fisher's exact test. A *p* value < 0.05 was considered statistically significant.

3. Results

Mean ± standard deviation of age in patients with and without atopy were 52.2 ± 12.0 years and 53.4 ± 16.6 years, respectively. In both groups, 21 individuals were male (70.0%) and nine were female (30.0%). Considering age and gender, no statistically significant difference was noted between the groups. Eosinophil count was significantly higher ($p = 0.031$) in atopic patients ($1.87 \pm 0.73 \times 10^9$/L) compared to non-atopic individuals ($1.47 \pm 0.51 \times 10^9$/L). Similarly, serum IgE level was significantly higher ($p = 0.001$) in atopic patients, as it was 170.04 ± 65.00 IU/mL in allergic patients compared with 32.21 ± 15.96 units/mL in the Control Group (Fig. 2C).

As illustrated in Fig. 2, the Student *t* test confirmed that serum IL-4 was significantly higher ($p = 0.01$) in atopic patients (3.43 ± 1.98 pg/mL) compared with nonatopic individuals (2.09 ± 2.18 pg/mL). However, no significant difference was found ($p = 0.06$) in the serum IFN-γ level between the two groups (1.06 ± 1.39 pg/mL in the Case Group and 1.18 ± 0.74 pg/mL in the Control Group; Fig. 2A and 2B).

Results from the Mann−Whitney *U* test demonstrated that the mean relative expression level of TGF-β1 mRNA was significantly higher ($p = 0.0001$) in atopic patients (2.50 ± 1.11) compared with nonatopic individuals (1.40 ± 0.46; (Fig. 1A).

Finally, the eosinophils and mast cells in pterygium tissues of atopic patients were higher; however, these changes did not reach statistical significance in any cytological contents in pterygium tissues of patients with and without atopy (data not shown).

4. Discussion

In this study, expression of TGF-β1 mRNA in pterygium tissues of atopic patients was significantly higher than in nonatopic individuals. Although the pathogenesis of pterygium is still not completely understood, some risk factors such as UV light exposure, immunoinflammatory processes, viral infections, and DNA damage are suggested to play a role.[13] In addition, pterygium can be considered as a benign neoplastic condition due to the local invasion, epithelial cell metaplasia, and abnormal expression of p53 tumor suppressor gene, which are found in pterygium tissue.[14−17]

High levels of TGF-β1 suppress the immune response and increase tumor invasiveness (metastasis), cell motility, angiogenesis, and interaction of tumor cells with the extracellular matrix.[8] Therefore, it is proposed that the increased level of TGF-β1 is an indicator of invasiveness of various late-stage cancers.[18,19] In the present study, the relative increase in the level of TGF-β1 mRNA among pterygium patients with atopy suggests one possible explanation for the promotion of pterygium growth. Meanwhile, it is well established that UV, smoke, and pollens are major risk factors in the etiology of pterygium. The pathogenesis of pterygium is that TGF-β1 worsens the pathology that has already been shown by other risk factors such as UV light, smoke, pollens, and viruses. TGF-β1 suppresses tumorigenesis; however, after tumor cells become resistant to growth inhibition, overexpression of TGF-β1 results in angiogenesis, invasion, and excess production of extracellular matrix.

Expression of TGF-β1 gene is correlated with tissue fibrosis and airway remodeling.[20] In addition, TGF-β1, tumor necrosis factor-α, IL-4, and histamine release in vernal keratoconjunctivitis are responsible for the previously described pathological changes such as fibrillar collagen production, giant papillae formation, and conjunctival tissue remodeling.[21] Based on the current results, it may be concluded that TGF-β1 as a potent mediator of tissue remodeling can induce pathological changes, such as fibrovascular advancement and elastotic degeneration, which are found in pterygium.

TGF-β1 also plays an important role in wound healing. It stimulates fibroblasts to proliferate, migrate, and gradually make a collagenous matrix in the wound tissue. However, TGF-β1, along with insulin-like growth factor 1 and IL-1, may result in fibroproliferative disorders such as keloids and hypertrophic scars. Inflammation, remodeling of the wound matrix, increased synthesis of extracellular-matrix proteins and fibrogenic cytokines, abnormalities in cell migration and proliferation, and increased response to cytokines are considered responsible for these conditions.[22] It has been shown that photoreactive keratectomy can induce rapid growth of pterygium, possibly because of the existence of several fibrogenic cytokines during the recovery period.[3]

Finally, we suggest that one possible mechanism of pterygium formation is through fibrogenic cytokines such as TGF-β that it promotes with atopic conditions. Therefore, physicians should consider incorporating into their therapies inhibition of the effect of growth factors after pterygium tissue excision, before or after surgery, to reduce the recurrence rate of this condition.

Acknowledgments

The authors would like to acknowledge the financial support of the Vice-Chancellor for Research of Islamic Azad University of Mashhad. Dr. Shayegan was supported by a grant from Islamic Azad University − Mashhad Branch (m/1157).

References

1. Threlfall TJ, English DR. Sun exposure and pterygium of the eye: a dose-response curve. *Am J Ophthalmol* 1999;**128**:280−7.
2. Abelson MB, Turner D. A randomized, double-blind, parallel-group comparison of olopatadine 0.1% ophthalmic solution versus placebo for controlling the signs and symptoms of seasonal allergic conjunctivitis and rhinoconjunctivitis. *Clin Ther* 2003;**25**:931−47.

3. Pang Y, Rose T. Rapid growth of pterygium after photorefractive keratectomy. *Optometry* 2006;**77**:499−502.
4. Pinkerton OD, Hokama Y, Shigemura LA. Immunologic basis for the pathogenesis of pterygium. *Am J Ophthalmol* 1984;**98**:225−8.
5. Isaji M, Kikuchi S, Miyata H, Ajisawa Y, Araki-Inazawa K, Tsukamoto Y, et al. Inhibitory effects of tranilast on the proliferation and functions of human pterygium-derived fibroblasts. *Cornea* 2000;**19**:364−8.
6. Di Girolamo N, Wakefield D, Coroneo MT. UVB-mediated induction of cytokines and growth factors in pterygium epithelial cells involves cell surface receptors and intracellular signaling. *Invest Ophthalmol Vis Sci* 2006;**47**:2430−7.
7. Halwani R, Al-Muhsen S, Al-Jahdali H, Hamid Q. Role of transforming growth factor-beta in airway remodeling in asthma. *Am J Respir Cell Mol Biol* 2011;**44**:127−33.
8. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;**342**:1350−8.
9. Dushku N, John MK, Schultz GS, Reid TW. Pterygia pathogenesis: corneal invasion by matrix metalloproteinase expressing altered limbal epithelial basal cells. *Arch Ophthalmol* 2001;**119**:695−706.
10. Lee CH, Hong CH, Yu HS, Chen GS, Yang KC. Transforming growth factor-beta enhances matrix metalloproteinase-2 expression and activity through AKT in fibroblasts derived from angiofibromas in patients with tuberous sclerosis complex. *Br J Dermatol* 2010;**163**:1238−44.
11. Skassa-Brociek W, Manderscheid JC, Michel FB, Bousquet J. Skin test reactivity to histamine from infancy to old age. *J Allergy Clin Immunol* 1987;**80**:711−6.
12. Kränke B, Aberer W. Skin testing for IgE-mediated drug allergy. *Immunol Allergy Clin N Am* 2009;**29**:503−16.
13. Detorakis ET, Drakonaki EE, Spandidos DA. Molecular genetic alterations and viral presence in ophthalmic pterygium. *Int J Mol Med* 2000;**6**:35−41.
14. Chan CM, Liu YP, Tan DT. Ocular surface changes in pterygium. *Cornea* 2002;**21**:38−42.
15. Yeung SN, Kim P, Lichtinger A, Amiran MD, Cote E, Teitel S, et al. Incidence of ocular surface squamous neoplasia in pterygium specimens: an 8-year survey. *Br J Ophthalmol* 2011;**95**:592.
16. Weinstein O, Rosenthal G, Zirkin H, Monos T, Lifshitz T, Argov S. Overexpression of p53 tumor suppressor gene in pterygia. *Eye* 2002;**16**:619−21.
17. Siak JJ, Ng SL, Seet LF, Beuerman RW, Tong L. The nuclear-factor kappaB pathway is activated in pterygium. *Invest Ophthalmol Vis Sci* 2011;**52**:230−6.
18. Maehara Y, Kakeji Y, Kabashima A, Emi Y, Watanabe A, Akazawa K, et al. Role of transforming growth factor-beta 1 in invasion and metastasis in gastric carcinoma. *J Clin Oncol* 1999;**17**:607−14.
19. Picon A, Gold LI, Wang J, Cohen A, Friedman E. A subset of metastatic human colon cancers expresses elevated levels of transforming growth factor beta1. *Cancer Epidemiol Biomarkers Prev* 1998;**7**:497−504.
20. Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, et al. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Resp Cell Mol Biol* 1997;**17**:326−33.
21. Leonardi A, Di Stefano A, Motterle L, Zavan B, Abatangelo G, Brun P. Transforming growth factor-beta/Smad − signalling pathway and conjunctival remodelling in vernal keratoconjunctivitis. *Clin Exp Allergy* 2011;**41**:52−60.
22. Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med* 1999;**341**:738−46.