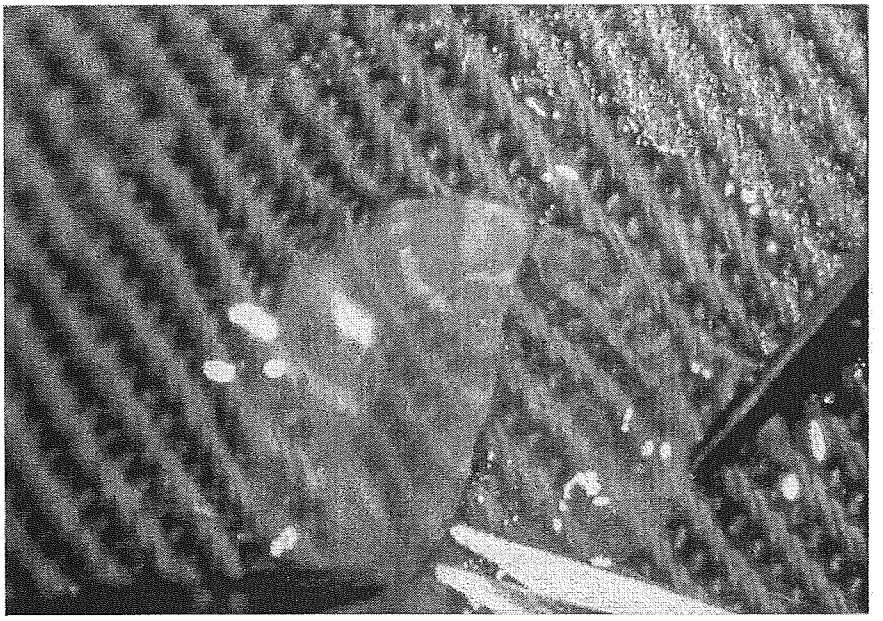

FIGURE 11 The donor endothelium and the Descemet membrane are dissected using toothed forceps and spring-type micro-scissors.

the difference will become greater with time. Intraocular pressure increased more often after PKP than DLKP. This is probably due to the prolonged use of topical corticosteroids after PKP. In DLKP, we used corticosteroid eye drops for approximately six months, after which all medications other than lubricants can be discontinued. The early discontinuation of corticosteroid eye drops may also be beneficial for decreased risk of infection and cataract development. This relatively easy follow-up not only reduces medication-associated side effects, but also increases quality of life. Although none of the eyes studied suffered immunologic rejection, the event is anticipated to occur in approximately 20%–30% of eyes after PKP. In contrast, irreversible visual deterioration caused by rejection is not a major issue after DLKP. It should be noted, however, that DLKP is not free of endothelial damage. Even in uncomplicated cases, approximately 25% loss of endothelial density was noted.

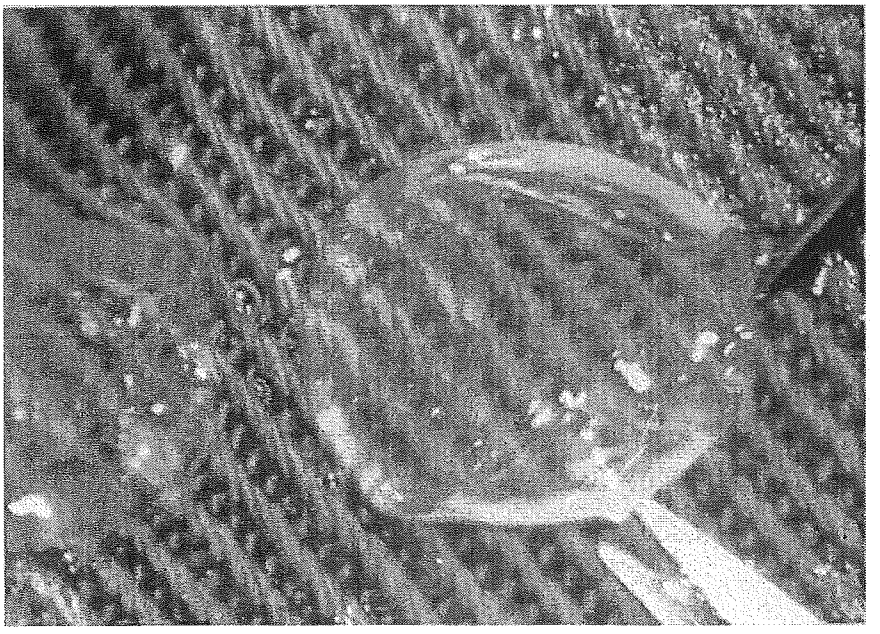

FIGURE 12 Completed dissection procedure.

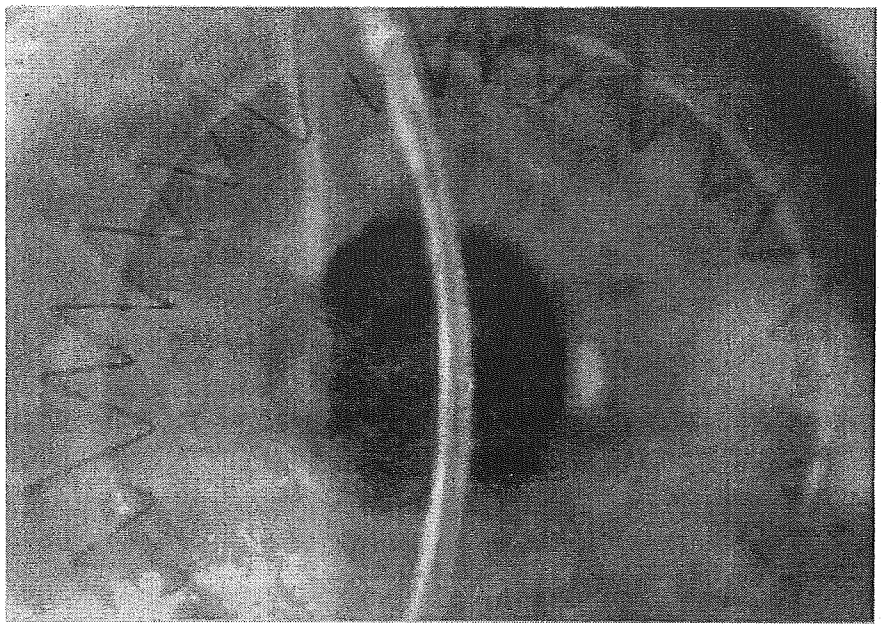

FIGURE 13 The donor buttons are secured by a single, continuous 10-0 nylon suture.

This is not surprising since most of the surgical manipulations are performed immediately above the Descemet membrane, even though higher endothelial density immediately after DLKP has been reported.[57] This indicates that DLKP should not be performed in eyes with decreased endothelial density. We also experienced adverse effects on the endothelium in eyes complicated by the Descemet membrane rupture. Rupture of the Descemet membrane in DLKP is not an uncommon complication, with the incidence reported to be between 0 and 39.2%.[54,59–61] It is reported that surgical maneuvering is possible without significant consequences in most cases after the Descemet rupture.[60] However, our result indicated that severe damage to the endothelium could also occur. We are currently developing a new technique by injecting air vigorously in the deep corneal stroma in the early part of the surgery. We expect the technique may facilitate faster and safer separation between deep stromal tissue and the Descemet membrane. Panda and associates[57] recently reported results of a nonrandomized prospective study comparing DLKP and PKP. The authors reported that the DLKP groups showed better visual acuity, higher rate of clear grafts, less astigmatism, and more endothelial density. According to our experience, DLKP should be strongly recommended for high-risk cases such as eyes with corneal neovascularization. Each surgeon should consider the indication of DLKP with the technical expertise in this surgery. With the development of easier surgical techniques, DLKP may be a first choice of keratoplasty in most high-risk eyes without endothelial abnormalities.

FUTURE EXPECTATIONS

We believe that artificial corneas will dominate ocular surface reconstruction procedures in the future. Various methods have been tried for reconstruction of three-dimensional split and full-thickness corneal equivalents. It has been shown that treatment of corneal stromal keratocytes with ascorbic acid (vitamin C) will cause an increase in the synthesis of collagen, the main noncellular component of the cornea.[62,63] Germain et al.[64] have used these results to develop completely natural cell-based corneal constructs from corneal epithelium and keratocytes without the addition of extracellular matrix proteins.

A prototype replacement cornea that incorporated human corneal cells was previously developed,[65] based on a rabbit corneal equivalent described in the report by Zieske et al.[66] The human prototype was built on a glutaraldehyde-crosslinked, collagen-GAG scaffold, and conserved key physical and physiological features of natural human corneas, including morphology, transparency, biochemical marker expression, ion and fluid transport, and responses to stimuli.

It is our belief that it is possible to fabricate synthetic or composite natural/synthetic polymeric scaffolds that can be used in the development of artificial corneas.

Optimization of the proportion of synthetic to natural polymers, the types of polymers used, and the methods of forming composite scaffolds will lead to achieving an artificial cornea or replacement for different portions of corneas for transplantation in the near future.

REFERENCES

[1] Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986; 103:49–62.

[2] Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 1989; 96:709–722.

[3] Tseng SC. Concept and application of limbal stem cells. *Eye* 1989; 3:141–157.

[4] Holland EJ, Schwartz GS. The evolution of epithelial transplantation for severe ocular surface disease and a proposed classification system. *Cornea* 1996; 15:549–556.

[5] Thoft RA. Keratoepithelioplasty. *Am J Ophthalmol* 1984; 7:1–6.

[6] Turgeon PW, Nauheim RC, Roat MI, Stopak SS, Thoft RA. Indications for keratoepithelioplasty. *Arch Ophthalmol* 1990; 108:233–236.

[7] Tsai RJ, Tseng SC. Human allograft limbal transplantation for corneal surface reconstruction. *Cornea* 1994; 13:389–400.

[8] Tsubota K, Toda I, Saito H, Shinozaki N, Shimazaki J. Reconstruction of the corneal epithelium by limbal allograft transplantation for severe ocular surface disorders. *Ophthalmology* 1995; 102:1486–96.

[9] Croasdale CR, Schwartz GS, Malling JV, Holland EJ. Keratolimbal allograft: recommendations for tissue procurement and preparation by eye banks, and standard surgical technique. *Cornea* 1999; 18:52–8.

[10] Sundmacher R, Reinhard T. Central corneolimbal transplantation under systemic cyclosporine, a cover for severe stem cell deficiencies. *Graefe's Arch Clin Exp Ophthalmol* 1996; 234:122–125.

[11] Reinhard T, Sundmacher R, Spelsberg H, Althaus C. Homologous penetrating central limbo-keratoplasty (HPCLK) in bilateral limbal stem cell insufficiency. *Acta Ophthalmol Scand* 1999; 77:663–7.

[12] Holland EJ. Epithelial transplantation for the management of severe ocular surface disease. *Trans Am Ophthalmol Soc* 1996; 94:677–743.

[13] Schwartz GS, Holland EJ. Iatrogenic limbal stem cell deficiency. *Cornea* 1998; 17:31–7.

[14] Shimazaki J, Shimmura S, Fujishima H, Tsubota K. Association of preoperative tear function with surgical outcome in severe Stevens-Johnson syndrome. *Ophthalmology* 2000; 107:1518–23.

[15] Tsubota K, Goto E, Shimmura S, Shimazaki J. Treatment of persistent corneal epithelial defect by autologous serum application.*Ophthalmology* 1999; 106:1984–9.

[16] Tsubota K, Goto E, Fujita H, Ono M, Inoue H, Saito I, Shimmura S. Treatment of dry eye by autologous serum application in Sjogren's syndrome. *Br J Ophthalmol* 1999; 83:390–5.

[17] Tsubota K. Tear dynamics and dry eye. *Prog Retin Eye Res* 1998 Oct; 17:565–96.

[18] Tsubota K, Higuchi A. Serum application for the treatment of ocular surface disorders. *Int Ophthalmol Clin* 2000; 40:113–22.

[19] Tsubota K, Shimazaki J. Surgical treatment of children blinded by Stevens-Johnson syndrome. *Am J Ophthalmol* 1999; 128:573–81.

[20] Wei ZG, Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells are preferentially located in fornical epithelium: implications on conjunctival epithelial homeostasis. *Invest Ophthalmol Vis Sci* 1995; 36:236–46.

[21] Wei ZG, Sun TT, Lavker RM. Rabbit conjunctival and corneal epithelial cells belong to two separate lineages. *Invest Ophthalmol Vis Sci* 1996; 37:523–33.

[22] Kenyon KR. Limbal autograft transplantation for chemical and thermal burns. *Dev Ophthalmol* 1989; 18:53–8.

[23] Tsubota K, Fukagawa K, Fujihara T, Shimmura S, Saito I, Saito K, Takeuchi T. Regulation of human leukocyte antigen expression in human conjunctival epithelium. *Invest Ophthalmol Vis Sci* 1999; 40:28–34.

[24] Tseng SC, Prabhasawat P, Barton K, Gray T, Meller D. Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. *Arch Ophthalmol* 1998; 116(4):431–41.

[25] Kinoshita S, Kiritoshi A, Ohji M, et al. Disappearance of palisades of Vogt in ocular surface disease. *Jpn J Clin Ophthalmol* 1986; 40:363–366.

[26] Tsubota K, Satake Y, Kaido M, Shinozaki N, Shimmura S, Bissen-Miyajima H, Shimazaki J. Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation. *N Engl J Med* 1999; 340:1697–703.

[27] Kim JC, Tseng SC. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. *Cornea* 1995; 14:473–84.

[28] Pires RT, Chokshi A, Tseng SC. Amniotic membrane transplantation or conjunctival limbal autograft for limbal stem cell deficiency induced by 5-fluorouracil in glaucoma surgeries. *Cornea* 2000; 19:284–7.

[29] Tsubota K, Satake Y, Ohyama M, Toda I, Takano Y, Ono M, Shinozaki N, Shimazaki J. Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. *Am J Ophthalmol* 1996; 122:38–52.

[30] De Rotth A. Plastic repair of conjunctival defects with fetal membrane. *Arch Ophthalmol* 1940; 23:522–525.35.

[31] Shimmura S, Shimazaki J, Ohashi Y, Tsubota K. Antiinflammatory effects of amniotic membrane transplantation in ocular surface disorders. *Cornea* 2001; 20:408–13.

[32] Koizumi NJ, Inatomi TJ, Sotozono CJ, Fullwood NJ, Quantock AJ, Kinoshita S. Growth factor mRNA and protein in preserved human amniotic membrane. *Curr Eye Res* 2000; 20:173–7.

[33] Gabric N, Mravicic I, Dekaris I, Karaman Z, Mitrovic S. Human amniotic membrane in the reconstruction of the ocular surface. *Doc Ophthalmol* 1999; 98:273–83.

[34] Honavar SG, Bansal AK, Sangwan VS, Rao GN. Amniotic membrane transplantation for ocular surface reconstruction in Stevens-Johnson syndrome. *Ophthalmology* 2000; 107:975–9.

[35] Tseng SC, Li DQ, Ma X. suppression of transforming growth factor-beta isoforms, TGF-beta receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. *J Cell Physiol* 1999; 179:325–35.

[36] Solomon A, Meller D, Prabhasawat P, John T, Espana EM, Steuhl KP, Tseng SC. Amniotic membrane grafts for nontraumatic corneal perforations, descemetoceles, and deep ulcers. *Ophthalmology* 2002; 109:694–703.

[37] Heiligenhaus A, Bauer D, Meller D, Steuhl KP, Tseng SC. Improvement of HSV-1 necrotizing keratitis with amniotic membrane transplantation. *Invest Ophthalmol Vis Sci* 2001; 42:1969–74.

[38] Tseng SC. Amniotic membrane transplantation for persistent corneal epithelial defect. *Br J Ophthalmol* 2001; 85:1400–1.

[39] Meallet MA, Espana EM, Grueterich M, Ti SE, Goto E, Tseng SC. Amniotic membrane transplantation with conjunctival limbal autograft for total limbal stem cell deficiency. *Ophthalmology* 2003; 110:1585–92.

[40] Tseng SC. Amniotic membrane transplantation for ocular surface reconstruction. *Biosci Rep* 2001; 21:481–9.

[41] Mejia LF, Acosta C, Santamaria JP. Use of nonpreserved human amniotic membrane for the reconstruction of the ocular surface. *Cornea* 2000; 19:288–91.

[42] Chen HJ, Pires RT, Tseng SC. Amniotic membrane transplantation for severe neurotrophic corneal ulcers. *Br J Ophthalmol* 2000; 84:826–33.

[43] Kruse FE, Rohrschneider K, Volcker HE. Multilayer amniotic membrane transplantation for reconstruction of deep corneal ulcers. *Ophthalmology* 1999; 106:1504–10.

[44] Hanada K, Shimazaki J, Shimmura S, Tsubota K. Multilayered amniotic membrane transplantation for severe ulceration of the cornea and sclera. *Am J Ophthalmol* 2001; 131:324–31.
[45] Su CY, Lin CP. Combined use of an amniotic membrane and tissue adhesive in treating corneal perforation: a case report. *Ophthalmic Surg Lasers* 2000; 31:151–4.
[46] Dogru M, Yildiz M, Baykara M, Ozcetin H, Ertuk H. Corneal sensitivity and ocular surface changes following preserved amniotic membrane transplantation for nonhealing corneal ulcers. *Eye* 2003; 17:139–148.
[47] Meller D, Pires RT, Mack RJ, Figueiredo F, Heiligenhaus A, Park WC, Prabhasawat P, John T, McLeod SD, Steuhl KP, Tseng SC. Amniotic membrane transplantation for acute chemical or thermal burns. *Ophthalmology* 2000; 107:980–9.
[48] Sridhar MS, Bansal AK, Sangwan VS, Rao GN. Amniotic membrane transplantation in acute chemical and thermal injury. *Am J Ophthalmol* 2000; 130:134–7.
[49] Tsai RJ, Tseng SC. Effect of stromal inflammation on the outcome of limbal transplantation for corneal surface reconstruction. *Cornea* 1995; 14:439–49.
[50] Gabler B, Lohmann CP. Hypopyon after repeated transplantation of human amniotic membrane onto the corneal surface. *Ophthalmology* 2000; 107:1344–6.
[51] Arentsen JJ. Corneal transplant allograft reaction: possible predisposing factors. *Trans Am Ophthalmol Soc* 1983; 81:361–402.
[52] Archila EA. Deep lamellar keratoplasty dissection of host tissue with intrastromal air injection. *Cornea* 1984–85; 3:217–8.
[53] Panda A, Singh R. Intralamellar dissection techniques in lamellar keratoplasty. *Cornea* 2000; 19:22–5.
[54] Melles GR, Lander F, Rietveld FJ, Remeijer L, Beekhuis WH, Binder PS. A new surgical technique for deep stromal, anterior lamellar keratoplasty. *Br J Ophthalmol* 1999; 83:327–33.
[55] Amayem AF, Anwar M. Fluid lamellar keratoplasty in keratoconus. *Ophthalmology* 2000; 107:76–9; discussion 80.
[56] Manche EE, Holland GN, Maloney RK. Deep lamellar keratoplasty using viscoelastic dissection. *Arch Ophthalmol* 1999; 117:1561–5.
[57] Panda A, Bageshwar LM, Ray M, Singh JP, Kumar A. Deep lamellar keratoplasty versus penetrating keratoplasty for corneal lesions. *Cornea* 1999; 18:172–5.
[58] Shimmura S, Tsubota K. Ocular surface reconstruction update. *Curr Opin Ophthalmol* 2002; 13:213–9.
[59] Tsubota K, Kaido M, Monden Y, Satake Y, Bissen-Miyajima H, Shimazaki J. A new surgical technique for deep lamellar keratoplasty with single running suture adjustment. *Am J Ophthalmol* 1998; 126:1–8.
[60] Sugita J, Kondo J. Deep lamellar keratoplasty with complete removal of pathological stroma for vision improvement. *Br J Ophthalmol* 1997; 81:184–8.
[61] Morris E, Kirwan JF, Sujatha S, Rostron CK. Corneal endothelial specular microscopy following deep lamellar keratoplasty with lyophilised tissue. *Eye* 1998; 12:619–22.
[62] Grinnell F, Fukamizu H, Pawelek P, Nakagawa S. Collagen processing, crosslinking, and fibril bundle assembly in matrix produced by fibroblasts in long-term cultures supplemented with ascorbic acid. *Exp Cell Res* 1989; 181:483–91.
[63] Geesin JC, Hendricks LJ, Gordon JS, Berg RA. Modulation of collagen synthesis by growth factors: the role of ascorbate-stimulated lipid peroxidation. *Arch Biochem Biophys* 1991; 289:6–11.
[64] Germain L, Carriere P, Giasson M, et al. A new method for in vitro production of human reconstructed cornea for tissue engineering. *Invest Opthalmol Vis Sci* 1999; S329 (abstract no. 1745).
[65] Griffith M, Osborne R, Munger R, Xiong X, Doillon CJ, Laycock NL, Hakim M, Song Y, Watsky MA. Functional human corneal equivalents constructed from cell lines. *Science* 1999; 286:2169–72.
[66] Zieske JD, Mason VS, Wasson ME, Meunier SF, Nolte CJ, Fukai N, Olsen BR, Parenteau NL. Basement membrane assembly and differentiation of cultured corneal cells: importance of culture environment and endothelial cell interaction. *Exp Cell Res* 1994; 214:621–33.

Survival Analysis of Conjunctival Limbal Grafts and Amniotic Membrane Transplantation in Eyes With Total Limbal Stem Cell Deficiency

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OCULAR SURFACE RECONSTRUCTION HAS BECOME common terminology among corneal surgeons in the treatment of severe ocular surface disease, which is mostly refractory to conventional medical therapeutic modalities. The challenge in the field was initiated especially by the necessity to find a cure for patients with Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid, and chemical or thermal burns.

The common point that is shared by these patients is the depletion of the corneal epithelial stem cells that are located in the limbal area surrounding the cornea. Patients with total limbal stem cell deficiency invariably complain of pain and photophobia and have severe loss of vision that is caused by the invading vascularized conjunctiva.[1]

There is no doubt that standard penetrating keratoplasty is a contraindication in surgical treatment of such patients because the life span of corneal epithelial progenitor cells from a corneal graft is limited. The transplanted corneal button is replaced inevitably by invading vascularized tissue, which is complicated further by immunologic rejection and secondary glaucoma. Penetrating keratoplasty and lamellar keratoplasty in these patients recently have regained their status as a surgical tool, only in conjunction with limbal transplantation.[1]

Limbal transplantation, which was first described as an autologous procedure from the healthy fellow eye,[2] is a means to restore corneal epithelial stem cells in the diseased eye. When the stem cell deficiency is bilateral, corneal surface reconstruction relies on transplantation of an allogeneic source of limbal epithelial stem cells by limbal conjunctival allograft. In this procedure, conjunctiva acts as a vessel that conveys the fragile epithelial stem cells from the healthy eye of a living relative. The use of living related donors should afford some degree of tissue matching. In addition, the tissue is fresh, which means that the stem cells may be healthier than those that can be obtained from stored tissue as in keratolimbal allografts (KLAL) that are obtained from cadaver donors.[2] The disadvantages of allogeneic limbal transplantation are the availability of a relative who is willing to donate and the limited availability of transplanted stem cells in a cadaver donor procedure, because caution must be used to prevent the subsequent development of iatrogenic deficiency of epithelial stem cells in the donor.[2]

In this issue of the JOURNAL, Santos and associates[3] present the results of their study of epithelial stem cell transplantation for severe ocular surface disease in patients with chemical burns and SJS. They evaluated 33 eyes of 31 patients with total limbal stem cell deficiency that underwent conjunctival limbal allografts with amniotic membrane transplantation (AMT). Keratoplasty was done in 48.5% of the eyes, and systemic immunosuppression with cyclosporine A was introduced in 51.5% of the cases. Santos and associates should be commended for (1) recruiting subjects of the same disease severity; (2) performing HLA typing and matching of class I and II antigens; (3) relating graft survival probability to cause, tear function, ocular surface keratinization, and eyelid abnormalities; and (4) following their patients with impression cytology persistently for a mean period of 33 months.

The survival of the ocular surface transplantation in this study was 46% at 1 year, with a cumulative survival of 39% after 3 years. We previously published that the overall success rate, which was measured as the rate of corneal epithelization, of KLAL with AMT in patients with SJS and chemical burns was 51%. After an average follow-up period of 3 years, we noted that 28% of the patients with SJS and 50% of the patients with chemical injury had clear corneas and that 60% of the patients had visual acuity improvement by two or more lines.[4,5] Because the life span of transient amplifying cells is believed to be <1 year, maintenance of a normal corneal epithelium for >1 year suggested the sustained viability of stem cell grafts.[6] Similar to our previously reported observations, the univariate analysis in this study revealed a significant impact on graft survival for patients with SJS, dry eye, keratinization, the presence of lid abnormalities, and allogeneic conjunctival limbal transplantation.[7,8]

The implication of preoperative dry eye as the most important prognostic factor on surgical outcome in this study underlines the importance of perioperative epithelial maintenance procedures once again and backs up our

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previous reports that tear function affected the surgical outcome and success of KLAL and AMT in patients with SJS.[9] It is our experience that combined efforts that involve the use of autologous serum eye drops, nonpreserved 0.1% sodium hyaluronate eye drops, and artificial tears and punctum plug insertion increases the probability of limbal and corneal graft survival in ocular surface reconstruction.[10] The survival rate of the corneal graft in the current study was 25%, but 70% of the procedures were performed in the same session. It is our impression that the success rate of such transplantations is higher when the epithelium on the transplanted ocular surface is allowed to stabilize for 6 months before an attempt is made to perform corneal transplantation.[9] In our experience, the poor prognosis of corneal graft survival is due to increased exposure of the host immune system to donor corneal antigens through the recognition limbal allograft antigens and the addition of penetrating keratoplasty at the same setting that increases the wound healing response and inflammation, which we believe are detrimental to the limbal stem cells. Although HLA typing and immunosuppression were carried out in the current study, both factors were not found to have a significant impact on surgical outcome. Better allogeneic graft survival may be achieved by matching all major HLA major loci and split and minor antigens.[11] Although possibly beneficial, such practices are impractical and might decrease the pool of potential donor eyes and increase the time a patient would have to wait for the surgical procedure.[11]

Other important issues to be addressed in relation to limbal allograft transplantation are the length of immunosuppression, optimal protocols to facilitate graft survival, and further research and provision of comparative data on various regimens. We use an immunosuppression regimen of systemic cyclosporine A indefinitely and a short-term regimen of systemic corticosteroids in our patients, but the recent trend is toward combining cyclosporine A with newer agents, such as mycophenolate mofetil and tacrolimus.[12]

The use of AMT in conjunction with limbal transplants is definitely essential in our view because of antiapoptotic effects on epithelial cells and fibroblasts, rapid epithelization, reduced inflammation, vascularization, and scarring.

Amniotic membrane actions (such as restoration of normal conjunctival epithelial phenotype with an increase in goblet cell density) are all desired effects that facilitate the clinical outcome in limbal graft surgery.[12,13]

In the past, little could be done to restore the vision of a patient with severe ocular surface disease. Through recent advances, many such patients can regain useful ambulatory vision. For instance, the transplantation of oral mucosal epithelial sheets, which provides the ability to expand limbal epithelial stem cells in an ex-vivo environment on an amniotic membrane carrier, seems to be a promising alternative to current limbal allograft transplantation procedures.[14] It is our belief that tissue engineering that involves artificial corneal stroma with or without epithelial cells may prove to be another future avenue for ocular surface reconstruction. Keratoprosthesis is another alternative for patients with dry eye in whom the ocular surface is severely keratinized and chances of graft failure are high.[15] The encouraging results by Santos and associates[3] provide a basis for further progress and research in the field.

REFERENCES

1. Tsubota K, Toda I, Saito H, Shinozaki N, Shimazaki J. Reconstruction of the corneal epithelium by limbal allograft transplantation for severe ocular surface disorders. Ophthalmology 1995;102:1486–1496.
2. Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. Ophthalmology 1989;96:709–722.
3. Santos MS, Gomes JAP, Hofling-Lima AL, Rizzo LV, Romano AC, Belfort R. Survival analysis of conjunctival limbal grafts and amniotic membrane transplantation in eyes with total limbal stem cell deficiency. Am J Ophthalmol 2005;140:223–230.
4. Tsubota K, Satake Y, Ohyama M, et al. Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. Am J Ophthalmol 1996;122:38–52.
5. Tsubota K, Satake Y, Kaido M, et al. Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation. N Engl J Med 19993;340:1697–1703.
6. Tsubota K. Corneal epithelial stem cell transplantation. Lancet 1997;349:1556.
7. Tseng SC, Prabhasawat P, Barton K, Gray T, Meller D. Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. Arch Ophthalmol 1998;116:431–441.
8. Kenyon KR. Limbal autograft transplantation for chemical and thermal burns. Dev Ophthalmol 1989;18:53–58.
9. Shimazaki J, Shimmura S, Fujishima H, Tsubota K. Association of preoperative tear function with surgical outcome in severe Stevens-Johnson syndrome. Ophthalmology 2000;107:1518–1523.
10. Tsubota K, Goto E, Shimmura S, Shimazaki J. Treatment of persistent corneal epithelial defect by autologous serum application. Ophthalmology 1999;106:1984–1989.
11. Reinhard T, Sundmacher R, Spelsberg H, Althaus C. Homologous penetrating central limbo-keratoplasty (HPCLK) in bilateral limbal stem cell insufficiency. Acta Ophthalmol Scand 1999;77:663–667.
12. Solomon A, Ellies P, Anderson DF, et al. Long term outcome of keratoloimbal allograft with or without penetrating keratoplasty for total limbal stem cell deficiency. Ophthalmology 2002;109:1159–1166.
13. Kim JS, Kim JC, Na BK, Jeong JM, Song CY. Amniotic membrane patching promotes healing and inhibits proteinase activity on wound healing following acute corneal alkali burn. Exp Eye Res 2000;70:329–337.
14. Kinoshita S, Koizumi N, Nakamura T. Transplantable cultivated mucosal epithelial sheet for ocular surface reconstruction. Exp Eye Res 2004;78:483–491.
15. Dohlman CH, Terada H. Keratoprosthesis in pemphigoid and Stevens-Johnson syndrome. Adv Exp Med Biol 1998; 438:1021–1025.

Prostaglandin D_2 Induces Chemotaxis in Eosinophils Via Its Receptor CRTH2 and Eosinophils May Cause Severe Ocular Inflammation in Patients With Allergic Conjunctivitis

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Purpose: Eosinophils are known to have important roles in the pathogenesis of allergic conjunctivitis. Prostaglandin (PG) D_2, which has been implicated as a factor in allergic diseases, is known to have chemotactic activity for eosinophils. Its receptor, chemoattractant receptor homologous molecule expressed on T_H2 (CRTH2), serves as a receptor for PGD_2 and has been reported to mediate PGD_2-dependent migration of eosinophils. In the present study, both eosinophil toxic activity for corneal epithelial cells and chemotaxis induced by PGD_2 in normal volunteers were investigated. Expression of CRTH2 in normal subjects was also measured.

Methods: Primary cultured corneal epithelial cells and eosinophils in serum from normal volunteers were used and a human corneal epithelial cell line was established. Studies were performed with/without amniotic membrane. CRTH2 expression on eosinophils was assessed by flow cytometry. Chemotaxis experiments were performed using a modified Boyden chamber technique.

Results: Corneal epithelial cells cultured with eosinophils showed higher floating epithelial cells and epithelial defect than those cultured in the absence of eosinophils. Flow cytometry analysis revealed that eosinophils expressed CRTH2. PGD_2 induced chemotaxis of eosinophils.

Conclusions: Corneal epithelial damage might be caused by eosinophils, which are recruited by PGD_2 secretion via CRTH2 expressed on eosinophils.

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Eosinophils are believed to be major effector cells in the late phase of allergic inflammation and have been implicated in the pathogenesis of corneal lesions in severe ocular allergies.[1] We previously reported that eosinophils are found in the tears of patients with severe atopic keratoconjunctivitis (AKC), human corneal keratocytes are capable of producing eotaxin by stimulation with IL-4, and differential regulation of chemokine production from corneal cells induced by IL-4 might relate to selective recruitment of eosinophils to the ocular surface.[2,3] Hence, eosinophils might perform an important role; however, no studies have conclusively demonstrated this.

Prostaglandin (PG) D_2 is a major cyclooxygenase metabolite of arachidonic acid, which is produced by mast cells in response to antigen challenge.[4] PGD_2 acts as a mediator in allergic asthma and plays important roles in other allergic disorders such as allergic rhinitis and atopic dermatitis.[5] It is also known that PGD_2 is released into tears during episodes of allergic conjunctivitis.[6] Accumulation of eosinophils to the ocular surface involves the production of eosinophil chemoattractants at the site of allergic inflammation; however, the precise mechanisms have not been clarified.

Recently, Nagata et al[7] cloned a novel putative chemoattractant receptor named CRTH2, a 7-transmembrane G protein–coupled receptor structurally related to members of the *N*-formyl peptide receptor subfamily. In this study, we investigated the hypothesis that eosinophils cause direct corneal damage and that eosinophil recruitment is mediated by PGD_2-dependent chemotaxis via CRTH2.

METHODS

Primary Culture

Human corneas were obtained from the American Eye Bank Association. Human corneal epithelial cells and keratocytes were established in culture as previously described by

Cubitt et al.[8] In brief, corneas with the adjacent limbus were dissected into small pieces and cultured in collagen-coated 35-mm culture dishes (Iwaki Co., Tokyo, Japan) in supplemental hormonal epithelial medium consisting of an equal volume of HEPES-buffered DMEM and Ham's F12 containing bicarbonate, 0.5% dimethyl sulfoxide, human epidermal growth factor 10 ng/mL, insulin 5 μg/mL, cholera toxin A subunit 0.1 μg/mL, 10% FCS, and penicillin G–streptomycin. Cultures were performed in standard conditions (37°C; 95% humidified air and 5% CO_2) to the confluent. Cells were used at second to fifth passages. Purity of each cell type was assessed based on cell morphology and differential response to anti-cytokeratin antibodies. Corneal epithelial cells exhibited staining for anti-keratin AE1/AE3 (Progen Biotechnik GmBH, Heidelberg, Germany) and to anti-vimentin antibodies (Roche Molecular Biochemicals, Indianapolis, IN). Keratocytes exhibited staining for anti-vimentin but not for anti-keratin AE1/AE3 antibodies. Corneal epithelial cells were resuspended at a concentration of 3×10^5 cells/mL with FBS-free RCGM medium. Keratocytes were resuspended in DMEM containing 15% FBS (GIBCO BRL, Grand Island, NY).[9,10]

Eosinophils

All experiments followed the tenets of the Declaration of Helsinki. Human granulocytes were isolated from heparin-anticoagulated venous blood samples obtained from normal subjects. The granulocytes underwent Percoll density gradient centrifugation at room temperature, and CD16-positive cells (neutrophils) were removed by immunomagnetic beads as previously described.[11] Subjects had no history of allergic diseases and no other systemic diseases, and both their total serum IgE and antigen-specific IgE were normal. Written informed consent was obtained from all subjects before participation. Eosinophil purity of cytocentrifuge preparations was determined by Diff-Quick staining (American Scientific Products, McGraw Park, IL) and was confirmed >97%.

Eosinophil Damage to Corneal Epithelial Cells

Corneal epithelial cells (1×10^6 cells/dish) were preincubated with 30 ng/mL of IL-4 plus TNF-α, then 1×10^6 cells/mL/dish of eosinophils were added to the culture medium and observed for 24 hours. Fluorescein staining was performed to observe corneal epithelial damage.

Reagents

PGD_2 was obtained from Cayman Chemical Co. (Ann Arbor, MI). CRTH2-specific rat mAb BM16 was obtained by the previously described procedure.[7] Immunizing Wistar rats with CRTH2-transfected rat T-cell line TART-1 generated mAb, but their epitopes were unclear.

Flow Cytometer Analysis

Flow cytometry (FACScan, Becton Dickinson, San Jose, CA) was conducted at fixed laser power (600 mW at 488 nm) using a previously reported method.[7] Briefly, eosinophils (1×10^5 cells) were first washed with FACS buffer and preincubated with human IgG (ICN Biomedicals Inc., Costa Mesa, CA). The samples were then incubated with biotinylated BM16 or with conjugated biotinylated rat IgG as control (Bio Source International, Camarillo, CA) at room temperature for 20 minutes. The samples were washed twice with FACS buffer and incubated with PE-labeled streptavidin at room temperature for 20 minutes. After washing with FACS buffer, the samples were incubated with goat anti-mouse Ig FITC Ab (Becton Dickinson) and run on the flow cytometer. Mean fluorescence intensity of the positive cell population, which was due to binding of the FITC-labeled antibodies to specific antigen, was used to quantify the presence of CRTH2 on the eosinophils.

Chemotactic Experiments

Chemotactic experiments were performed using a modified Boyden chamber technique. A 96-well microchemotaxis chamber equipped with a 5-μm millipore filter (Nucleopore, Pleasanton, CA) was used according to the manufacturer's instructions. Briefly, eosinophils (5×10^4/50 μL) resuspended in RPMI1640 were placed in each well of the upper chamber on the upper surface of the membrane. PGD_2 in RPMI1640

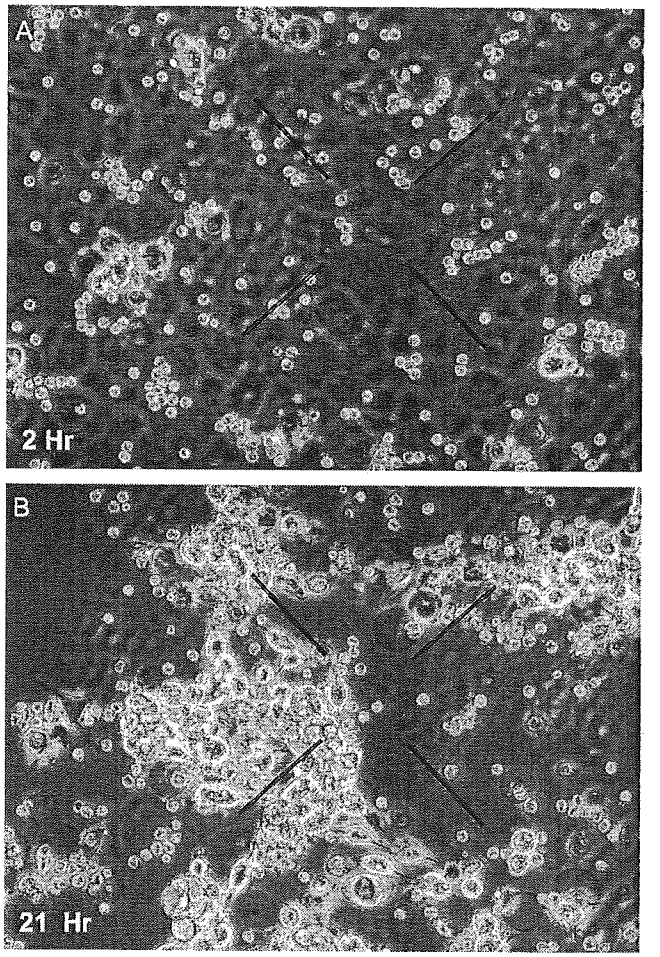

FIGURE 1. Corneal epithelial cell damage by eosinophils. When eosinophils were added into corneal epithelial culture medium (t = 2 hours; top), normal eosinophils were observed and no floating epithelial cells were seen. After 21 hours, a number of floating spindle-shaped or filamentous corneal epithelial cells were seen along with eosinophils (t = 21 hours; bottom).

was placed in the lower wells. The chamber was incubated at 37°C for 30 minutes in a mixture of 5% CO_2 and air and then disassembled. The membrane was removed and washed in PBS to remove nonmigrating eosinophils from the upper surface, scraped, and stained with Diff-Quick staining. Eosinophils were counted in 5 random high-power fields (hpf) using light microscopy, and chemotactic activity was expressed as mean number of eosinophils/hpf.

RESULTS

Corneal Epithelial Cell Damage by Eosinophils

When eosinophils were added to corneal epithelial culture medium (t = 2 hours; Fig. 1A), round eosinophils were observed and no floating epithelial cells were seen. After 21 hours, a number of floating corneal epithelial cells appeared as spindle-shaped or filamentous cells (Fig. 1B). These cells were detached from the cultured epithelial cells, which had located to the bottom of the incubator.

Eosinophil Coculture in Chamber

When eosinophils were added to cultured epithelial cells incubated on amniotic membrane for 24 hours, an epithelial defect was observed by fluorescein staining (Fig. 2).

CRTH2 Expression on Eosinophils

FACS analysis showed expression of CRTH2 on eosinophils obtained from normal subjects (Fig. 3).

Eosinophil Count and Chemotactic Response to PGD_2

Eosinophils migrating through a nitrocellulose filter in Boyden chamber assay were stained and counted (Fig. 4), revealing that PGD_2 was a potent eosinophil chemoattractant as previously reported.[12]

DISCUSSION

Allergic diseases affecting the eye are common. Some severely allergic patients have cobblestone-like papillae and corneal ulcers and present with symptoms of ocular pain and loss of visual acuity, resulting in disruption of normal lifestyle. These patients often are very difficult to treat.[13]

We hypothesized that corneal allergic disease might involve direct immunologic responses such as eosinophil infiltration and investigated eosinophil toxicity on corneal epithelial cells. Eosinophils were shown to cause corneal epithelial cell damage in vitro. Damaged corneal epithelial cells floated in the medium and fluorescein staining showed that eosinophils caused epithelial defect after 24 hours. Following eosinophil-mediated damage to corneal epithelial cells, more inflammatory proteins may be produced from fibroblasts located beneath the epithelium, which may in turn cause further corneal problems such as ulcer.

In late-phase allergic reaction, PGD_2 released from mast cells in the early phase may have an important role as an eosinophil chemotactic factor. Thus, we investigated expression of CRTH2 on eosinophils and eosinophil chemotaxis mediated by PGD_2. FACS analysis showed CRTH2 expression on eosinophils from normal subjects and PGD_2 caused eosinophil chemotaxis in a dose-dependent manner. Our results suggest that CRTH2 might contribute to the pathogenesis of allergic disease through eosinophil chemotaxis induced by PGD_2.

Eosinophil infiltration may play a role in hyperreactivity at the late stage[14,15] as well as corneal damage.[16] Nagata et al[7] reported that CRTH2 is selectively expressed in vivo in

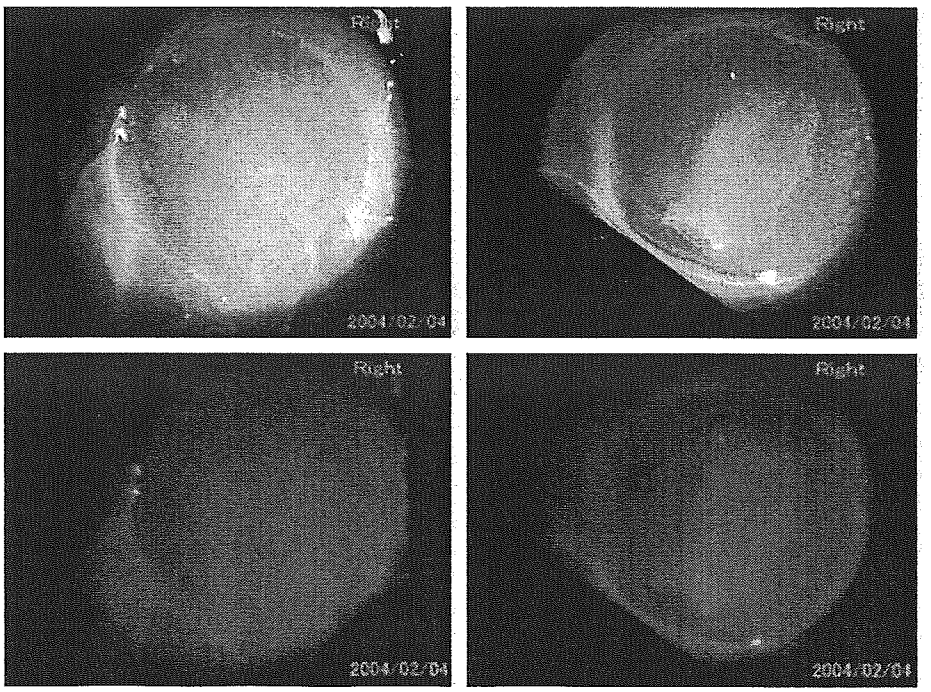

FIGURE 2. Eosinophil coculture in chamber. When eosinophils were added to the tube in which cultured epithelial cells were incubated on amniotic membrane, corneal epithelial defect was observed by fluorescein staining at 24 hours (top right, photo; bottom right, fluorescein staining), but not before adding eosinophils and only medium (top left, photo; bottom left, fluorescein staining).

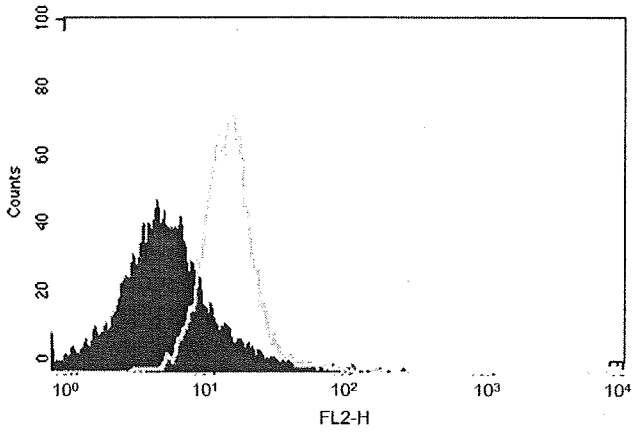

FIGURE 3. CRTH2 expression on eosinophils determined by flow cytometry (FACS). FACS analysis showed expression of CRTH2 on eosinophils. Background fluorescence reactivity was determined with control rat IgG.

activated T_H2 cells including allergen-responsive T_H2 cells, suggesting a pivotal role for this receptor in ongoing T_H2-type immune reactions. Furthermore, it has been reported that PGD_2 causes accumulation of eosinophils in the lumen of dog trachea.[17] Narumiya et al[18] have recently shown that CRTH2 but not DP receptor (DP) is present on human eosinophils and that PGD_2 activation of CRTH2 stimulates eosinophil migration. On the other hand, Gervais et al[19] reported that both DP and CRTH2 are detectable on circulating eosinophils, but DP is very low. Thus, we hypothesized that PGD_2 in conjunction with CRTH2 might control eosinophil function and have an important role in allergic diseases.

In our experiments, PGD_2 induced eosinophil chemotaxis in cells obtained from normal subjects. PGD_2 is capable of binding to CRTH2 as well as DP receptors, and stimulation

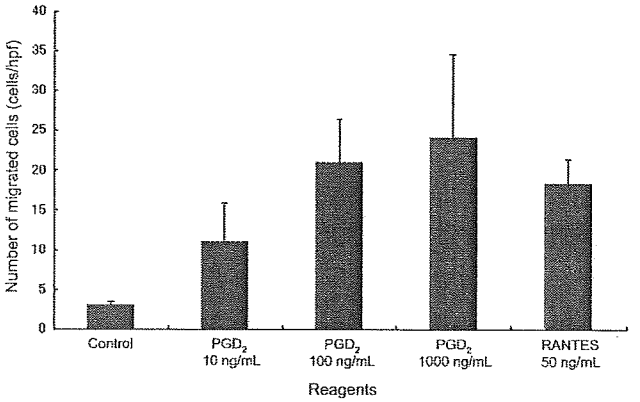

FIGURE 4. Chemotaxis of eosinophils in response to prostaglandin (PG) D_2. Chemotactic effects were evaluated by a Boyden chamber assay. Eosinophils migrating through a nitrocellulose filter were stained and counted. Vertical axis indicates the number of migrated eosinophils (cells/hpf). Eosinophils were induced by PGD_2 and RANTES. PGD_2 stimulated chemotaxis of eosinophils in a dose-dependent manner.

signals confirmed through CRTH2 stimulate chemotaxis. Hirai et al[20] reported that DK-PGD_2 induced eosinophil chemotaxis in cells from normal volunteers. The mechanism of increased CRTH2 expression is still unknown; however, studying expression patterns of CRTH2 on eosinophils induced by PGD_2 may contribute to clarifying the pathogenesis of eosinophil-associated allergic disease. Tsuda et al[21] showed that CRTH2 is expressed on human peripheral and decidual $CD4^+$ and $CD8^+$ T cells during the early stage of pregnancy. Subjects with atopic dermatitis show significant increases of $CRTH2^+$ cells in both the $CD4^+$ and the $CD8^+$ T-cell subsets. These results support the concept that CRTH2 is a reliable marker for detection of human T_H2 cells in both health and disease.[22] Anti-CRTH2 drugs, which could down-regulate expression of CRTH2, might be helpful for the treatment of allergic patients in future.

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REFERENCES

1. Fujishima H, Saito I, Takeuchi T, et al. Immunological characteristics of patients with vernal keratoconjunctivitis. *Jpn J Ophthalmol.* 2002;46:244–248.
2. Takano Y, Fukagawa K, Shimmura S, et al. IL-4 regulates chemokine production induced by TNF-alpha in keratocytes and corneal epithelial cells. *Br J Ophthalmol.* 1999;83:1074–1076.
3. Fukagawa K, Tsubota K, Shimmura S, et al. Chemokine production in conjunctival epithelial cells. *Adv Exp Med Biol.* 1998;438:471–478.
4. Lewis RA, Austen KF. Mediation of local homeostasis and inflammation by leukotrienes and other mast cell-dependent compounds. *Nature.* 1981; 293:103–108.
5. Matsuoka T, Hirata M, Tanaka H, et al. Prostaglandin D_2 as a mediator of allergic asthma. *Science.* 2000;287:2013–2017.
6. Proud D, Sweet J, Stein P, et al. Inflammatory mediator release on conjunctival provocation of allergic subjects with allergen. *J Allergy Clin Immunol.* 1990;85:896–905.
7. Nagata K, Tanaka K, Ogawa K, et al. Selective expression of a novel surface molecule by human Th2 cells in vivo. *J Immunol.* 1999;162:1278–1286.
8. Cubitt CL, Tang Q, Monteiro CA, et al. IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes. *Invest Ophthalmol Vis Sci.* 1993;34:3199–3206.
9. Ebato B, Friend J, Thoft RA. Comparison of limbal and peripheral human corneal epithelium in tissue culture. *Invest Ophthalmol Vis Sci.* 1988;29: 1533–1537.
10. Jumblatt MM, Neufeld AH. Beta-adrenergic and serotonergic responsiveness of rabbit corneal epithelial cells in culture. *Invest Ophthalmol Vis Sci.* 1983;24:1139–1143.
11. Hansel TT, De Vries IJ, Iff T, et al. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Methods.* 1991;145:105–110.
12. Rothenberg ME. Eosinophilia. *NEngl J Med.* 1998;338:1592–1600.
13. Fujishima H, Fukagawa K, Satake Y, et al. Combined medical and surgical treatment of severe vernal keratoconjunctivitis. *Jpn J Ophthalmol.* 2000; 44:511–515.
14. Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol.* 1999;17:255–281.
15. Miadonna A, Milazzo N, Gibelli S, et al. Nasal response to a single antigen challenge in patients with allergic rhinitis—inflammatory cell recruitment persists up to 48 hours. *Clin Exp Allergy.* 1999;29:941–949.

16. Fukagawa K, Okada N, Fujishima H, et al. CC-chemokine receptor 3: a possible target in treatment of allergy-related corneal ulcer. *Invest Ophthalmol Vis Sci.* 2002;43:58–62.
17. Emery DL, Djokic TD, Graf PD, et al. Prostaglandin D2 causes accumulation of eosinophils in the lumen of the dog trachea. *J Appl Physiol.* 1989;67:959–962.
18. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev.* 1999;79:1193–1226.
19. Gervais FG, Cruz RP, Chateauneuf A, et al. Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD_2 receptors CRTH2 and DP. *J Allergy Clin Immunol.* 2001;108:982–988.
20. Hirai H, Tanaka K, Yoshie O, et al. Prostaglandin D_2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med.* 2001;193:255–261.
21. Tsuda H, Michimata T, Sakai M, et al. A novel surface molecule of Th2- and Tc2-type cells, CRTH2 expression on human peripheral and decidual $CD4^+$ and $CD8^+$ T cells during the early stage of pregnancy. *Clin Exp Immunol.* 2001;123:105–111.
22. Cosmi L, Annunziato F, Galli MIG, et al. CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. *Eur J Immunol.* 2000;30:2972–2979.

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Melanocytes in the corneal limbus interact with K19-positive basal epithelial cells

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Abstract

The human corneal limbus is identified by the distinct features of the palisades of Vogt (POV), which contain pigment granules that are aligned with the microplicae of the epithelium. Although it is presumed that pigments are produced by melanocytes, the characterization of melanocytes in the limbus has not been clearly documented. We examined human limbal tissues by whole mounts and serial histological sections to localize epithelial cells containing melanin granules. Most of the pigmented cells observed by immunohistochemistry were K19 (+) cells in the basal limbal epithelium. A superimposed image revealed that melanin granules were oriented towards the apex of each K19 (+) cell, acting as a pigmented cap facing the ocular surface. Melanocytes were identified by MART1, an antigen specific to melanocyte-lineage cells. Melanocytes were shown to exist as sporadic cells with dendritic processes that extend to surrounding epithelial cells. Melanocytes were also found in light-pigmented donor tissue when visualized by the tyrosinase assay using the enzyme substrate DOPA. Since tyrosinase activity was not found in epithelial cells, the production of melanin is exclusively the role of melanocytes that comprised 5·3±2·7% of the total cells in cytospin samples ($N=3$). Melanocytes and K19 (+) epithelial cells may form a functional network similar to the melanin unit of the skin.

Keywords: melanin; melanocyte; corneal epithelium; keratin; immunohistochemistry

1. Introduction

The palisades of Vogt (POV) aligning the peripheral cornea are often used to identify the limbal epithelium in a clinical setting. POV have a distinct vasculature with narrow, radially oriented hairpin loops (Goldberg and Bron, 1982), which moves centripetally with age as well as in contact lens users (Inoue et al., 2001). In addition to the characteristic rete ridges of the epithelial surface, varying levels of pigmentation are observed in humans (Davanger and Evensen, 1971) as well as other mammals such as the rabbit (Espana et al., 2003). Although it is presumed that the pigmentation is due to melanin granules, there are very few studies that have actually dealt with characterizing melanocytes in the human cornea.

Melanocytes have been studied extensively in the skin, where they reside within the basal layer of the epidermis and deliver melanin granules to surrounding keratinocytes (Mottaz and Zelickson, 1967). Although the amount of melanin granules in keratinocytes is what determines skin color, keratinocytes themselves do not express tyrosinase, and therefore the synthesis of melanin is exclusively the role of melanocytes (Seiji and Iwashita, 1965). The dendritic melanocyte interacts with a constant number of surrounding keratinocytes to form the 'melanin unit', which is approximately 36 keratinocytes for each single melanocyte (Hadley and Quevedo, 1966).

The significance of a pigmented limbus is not clear. Protection against UV rays may be an important function since the occurrence of pterygium is predominantly found at

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the nasal limbus, and demographic studies have shown a higher occurrence rate in individuals exposed to UV (Wong et al., 2001; Sliney, 2002). In a large-scale comparative study on the etiology of pterygia, darker skin complexion was shown to be a protective factor along with the use of sunglasses (Luthra et al., 2001). Melanin also has anti-oxidative activity (Prota, 1997), which may serve to quench UV-induced oxidant formation in the cornea epithelium (Shimmura et al., 1996). This study aims to clarify the anatomical distribution and characteristics of melanocytes in the limbus. By performing a functional assay of the melanin producing enzyme tyrosinase, we have confirmed that limbal epithelial cells themselves do not have the ability to produce pigments, and instead, dendritic melanocytes scattered in the basal epithelium undertake this role.

2. Material and methods

2.1. Antibodies

Mouse monoclonal antibodies (mAb) for MART1 and K19 were purchased from Calbiochem (A130, Merck KGaA, Darmstadt, Germany) and MP Biomedicals (RCK 108, Irvine, CA), respectively. FITC- or rhodamine-conjugated donkey anti-mouse IgG (secondary antibody) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.2. Preparation of corneal limbal tissue

Three donor corneas were obtained from the Northwest Eye Bank and preserved for experiments after central corneal buttons were used for transplantation. Demographics of donor tissue consisted of a 48-year-old Caucasian male (Donor 1), a 49-year-old African American male (Donor 2) and a 69-year-old Caucasian female (Donor 3). Limbal segments were embedded in Tissue Tech OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen. Frozen sections (5 μm thick) were used for DOPA reaction and for immunohistochemical staining. Paraffin sections were prepared according to the AMeX method (Sato et al., 1986). In brief, samples were fixed in acetone (Wako Pure Chemical Industries, Ltd. Osaka, Japan) at 4°C over night, immersed in methyl benzoate (Wako) at room temperature (RT) for 1 hr (×2), and immersed in xylene at RT for 1 hr (2×). After incubation in 60°C paraffin for 1·5 hr (×2) and over night once, samples were embedded into paraffin.

For cytospin samples, corneal limbal tissue were treated with 0·2% collagenase (Wako) for 90 min, and then with 0·25% Trypsin-0·02% EDTA −7 U/ml DNase (Invitrogen Corp., Carlsbad, CA) for 90 min at 37°C. Cells were harvested and washed twice with 5% FBS–HBSS (Invitrogen). After centrifugation at 780×g for 10 min, cells were resuspended in 2% FBS-10 mM HEPES (Invitrogen)-HBSS. To collect single cells, cell suspension was filtrated through a 40 μm Cell Strainer (BD Falcon, San Jose, CA). Cells for cytospin preparations ($5·0\times10^4$ cells/slide) were prepared by using auto smear CF-12D (Sakura Finetek, Tokyo, Japan).

2.3. Immunohistochemistry

Frozen sections (Donor 3; MART1) and cytospin preparations (K19, MART1) were fixed for 5 min in cold acetone and washed in PBS for 5 min (×3). AMeX paraffin sections (Donor 2; K19) were dewaxed, and immersed in acetone to remove xylene, and rinsed in PBS. Paraffin sections were treated with protease XXV (Lab Vision Co., Fremont, CA) at 37°C for 5 min. Tissue sections and cytospin preparations were blocked by incubation with 10% normal donkey serum (Chemicon Int. Inc., Temecula, CA) with 1% bovine serum albumin (SIGMA, St. Louis, MO) for 1 hr at RT. Antibodies to MART1 (1:100) and K19 (1: 10) were applied and incubated for 90 min at RT, followed by incubation with FITC- or rhodamine-conjugated anti-mouse IgG secondary antibody. Isotype mouse IgG1 (DakoCytomation, Glostrup, Denmark) was used as control. After 3 washes with TBST, the sections were incubated with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Tokyo, Japan) at RT for 5 min. Finally, sections were washed three times in TBST and coverslipped using an antifading mounting medium (50 mM Tris buffer saline, 90% glycerin (Wako), 10% 1,4-diazabicyclo (2,2,2) octane (Wako).

2.4. DOPA reaction

Frozen sections (Donor 3) were washed in distilled water for 5 min (×3). The sections were incubated in 0·1% DL-β-(3,4-Dihydroxyphinyl)-alanine (DL-DOPA, Wako) in PBS (pH 7·4) at 37°C for 4 hr. Equivalent sections incubated in PBS were used as controls. Treatment with exogenous DL-DOPA induces the synthesis of melanin in melanocytes, and can then be identified as dark pigments by microscopy. Following the DOPA reaction, sections were rinsed in distilled water for 3 min, fixed in 10% formalin (Wako) and lightly stained with Mayer's hematoxylin and eosin (HE, Wako).

3. Results

3.1. Location of pigmented epithelial cells

In pigmented donor tissue, POV surrounding the peripheral cornea can easily be identified by the dark patches of pigment corresponding to individual ridges of epithelium. Histology sections show pigmented epithelial cells lining the basal layer of the limbus, with limited levels of pigmentation in the more superficial layers (Fig. 1C, D).

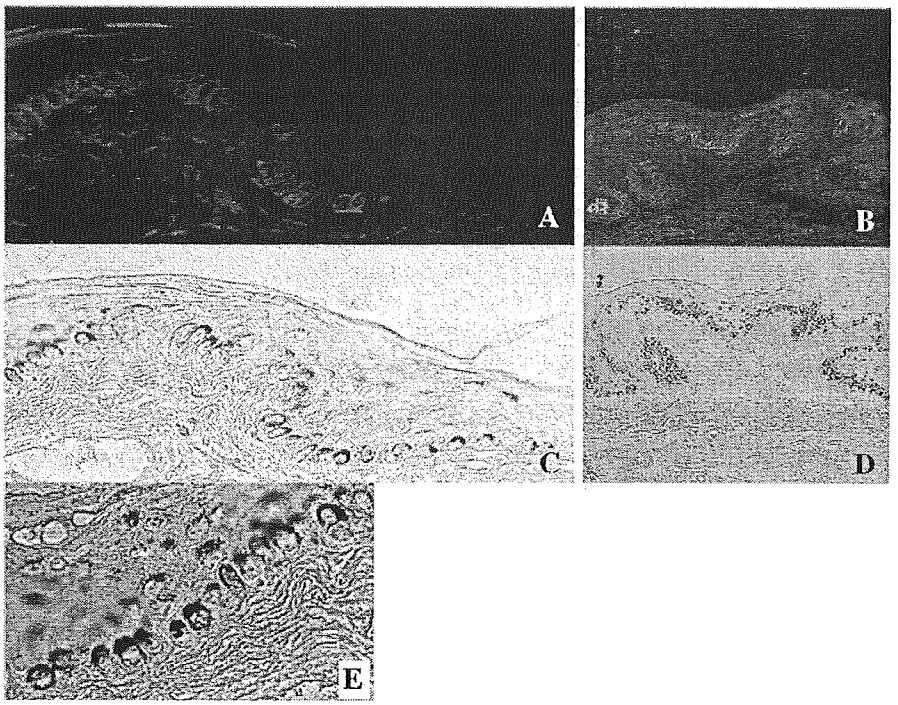

Fig. 1. Immunohistochemistry of the limbus shown in Fig. 1. Longitudinal (A, C) and horizontal (B, D) sections along the pigmented POV. K19 (+) epithelial cells are seen lining the basal epithelium (A, B). Bright field view of the same sections show that many of the pigmented cells are K19 (+) epithelial cells. An enlarged, superimposed image (E) clearly shows cap-like pigmentation (brown) in the apical end of each K19 (+) cell (green). Original magnification, 200×.

Immunohistochemistry with anti-K19 antibody shows that most limbal basal cells within the Palisades of Vogt express K19 (Fig. 1 A, B), and contain pigment in their apical domain facing the ocular surface (Fig. 1E).

3.2. Melanocyte distribution

Fig. 2 shows immunohistology of limbal tissue using an anti-MART1 antibody, which is a marker for cells of melanocyte lineage (Kawakami et al., 1994). Positive cells are found sporadically lining deeper layers of the limbal epithelium, and cytoplasmic extensions can be clearly identified extending to neighboring epithelial cells.

Melanin is produced by melanocytes through the action of tyrosinase, an enzyme that first converts tyrosine to DOPA, and further catalyses a complex chain of reaction to yield melanin (Prota, 1980). The DOPA assay makes use of the pigmented product to visualize enzyme activity. Fig. 3 shows the DOPA assay done in a light-pigmented Caucasian donor that does not exhibit any appreciable levels of pigmentation in the bright field and HE stains. Treatment of tissue with DOPA produces cells with melanin granules clearly visible on the HE stained sections. The digitally enlarged section in Fig. 3C shows melanocytes with pigmented processes surrounding neighboring epithelial cells. The DOPA assay is specific to tyrosinase (+) cells, and confirms that only melanocytes are capable of producing melanin in the limbal basal epithelium.

3.3. Phenotype of limbal cell suspensions

Melanocytes were found in the basal layers of the limbus in both pigmented and light-pigmented tissue. In order to investigate the density of melanocytes in the limbus, we performed immunocytology for melanocytes and K19 (+) epithelial cells in cyto-spin samples of digested limbal epithelium. Fig. 4 shows representative views of K19 (+) epithelial cells and MART1(+) melanocytes. An average of 3 fields of view from 3 different donors were calculated for each cell type. Of all cells isolated from the limbus, K19 (+) epithelial cells comprised an average of $48{\cdot}7 \pm 10{\cdot}4\%$, while melanocytes were found in the order of $5{\cdot}3 \pm 2{\cdot}7\%$.

4. Discussion

As expected, melanin-containing epithelial cells were observed aligning the basal layers of the limbus from a pigmented donor. Interestingly, the pigment granules often had polarity within the cell, with dense pigmentation observed towards the apex of the cell, the direction from which cells are exposed to UV radiation. Furthermore, pigments were observed in most of the K19 (+) cells, a cytokeratin known to be expressed by limbal basal cells, but not by central corneal basal cells (Kasper et al., 1988; Lindberg et al., 1993; Elder et al., 1997; Chen et al., 2004). From the merged image in Fig. 1E, we see the close association of melanin with K19 (+) cells, suggesting

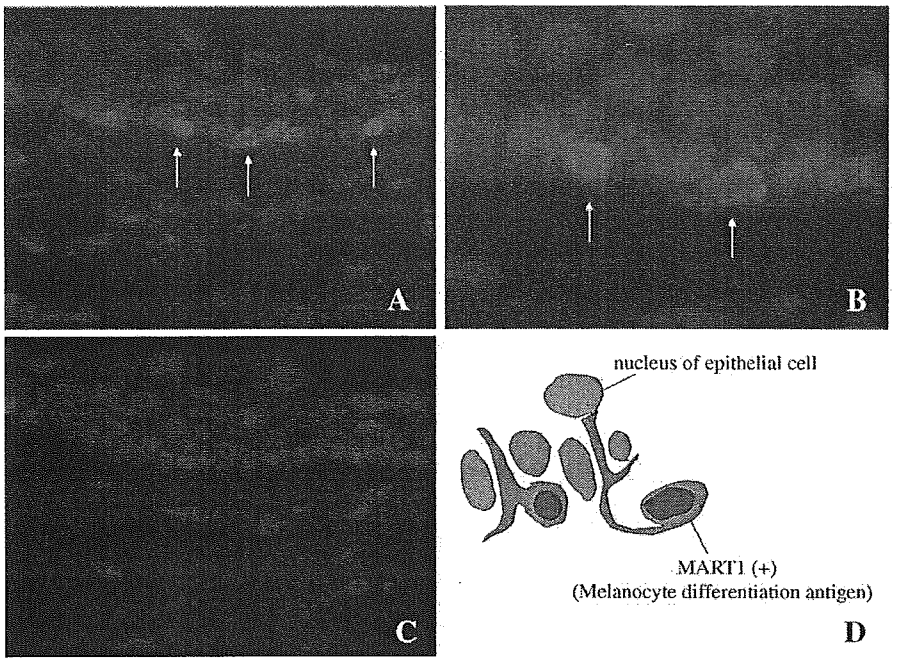

Fig. 2. (A) Immunohistochemistry of the limbus using an anti-MART1 antibody shows positive cells lining the limbal epithelium. An enlarged view (B), also shown as a schematic diagram (D), shows MART1 (+) melanocytes with cellular projections extending to surrounding basal epithelial cells. (C) Negative isotype control. Nuclear staining by DAPI shown in blue. Original magnification, 200×.

a possible role for these cells to act as a 'sun screen' for underlying structures. Similar supranuclear melanin caps reported in the skin were shown to reduce UV-induced DNA photoproducts in the epidermis (Kobayashi et al., 1998). UV also releases toxic reactive oxygen species within corneal epithelial cells at irradiation levels much lower than the lethal dose (Shimmura et al., 1996). Cellular functions such as inner mitochondrial membrane potential are disrupted, which leads to apoptosis (Shimmura et al., 2004) and decreased cell motility (Shimmura and Tsubota, 1997). Even more damaging to stem cells are UV induced mutations in genomic DNA, which can lead to neoplasmic transformation. Indeed, the occurrence of tumors in the ocular surface is highest in the limbal area (Waring, 1984). It is therefore reasonable that an anti-oxidant system exists in the limbus to protect stem cells that must survive an entire life span.

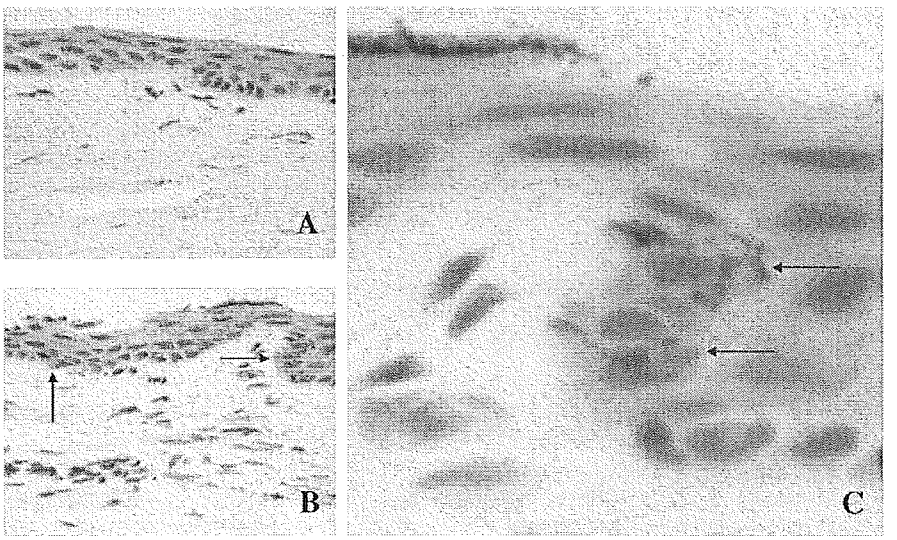

Fig. 3. (A) HE stain of a donor limbus with no visible pigmentation. (B) DOPA assay performed in the same donor produces dark pigments (arrow) in sporadic cells of the basal epithelium. (C) Digitally enlarged view of (B) shows that pigmented cells, corresponding to tyrosinase-positive melanocytes, have a dendritic morphology typical of melanocytes. Original magnification, 200×.

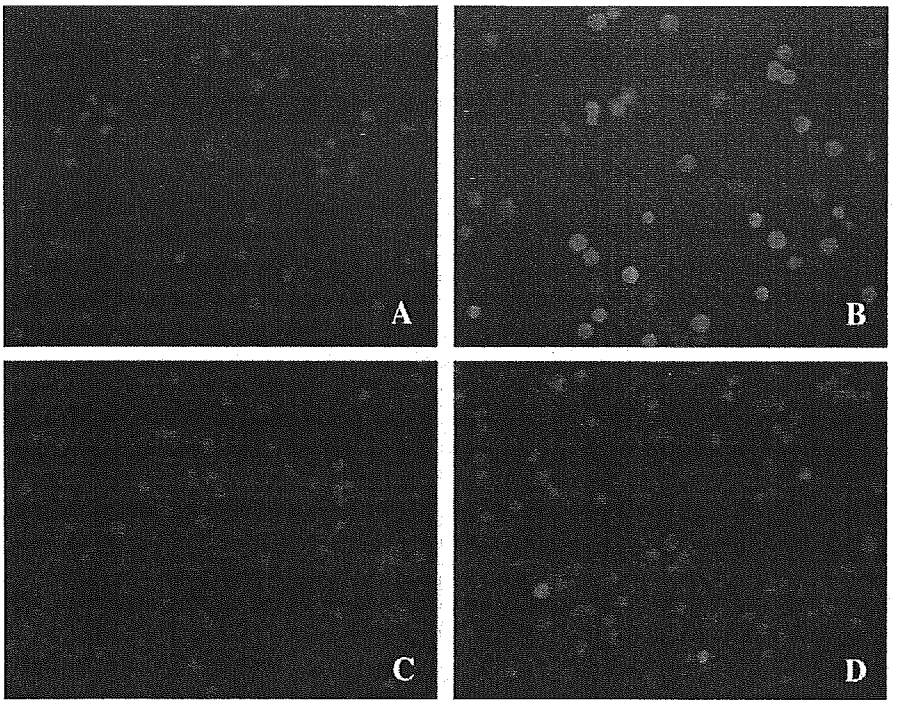

Fig. 4. Isotype control (A) and anti-K19 staining (B) of dissociated limbal epithelial cells. An average of 48·7±10·4% ($n=3$) of limbal cells were K19 (+). Isotype control (C) and anti-MART1 staining (D) of limbal cells from the same donor. An average of 5·3±2·7% cells were MART1 (+) melanocytes. Nuclear staining by DAPI shown in blue. Original magnification, 200×.

Melanocytes were identified in sections of limbal tissue using the melanocyte lineage-specific gene MART1, which is expressed by melanoma cells, melanocytes and retinal tissue (Kawakami et al., 1994). In the original report, MART1 was identified as a melanoma antigen recognized by HLA-A2 restricted tumor infiltrating lymphoctes. However, MART1 is also the predominant autoantigen in the pathogenesis of autoimmune vitiligo, a skin and hair disorder characterized by circumscribed depigmented lesions due to lack of melanocytes (Lang et al., 2001). Using a monoclonal anti-MART1 antibody, we were able to detect melanocytes scattered among K19 (+) basal epithelial cells, with multiple processes extending to surrounding cells. This configuration probably reflects a similar dendritic morphology observed in melanocytes of the skin. Limbal melanocytes also expressed vimentin (data not shown) (Commo et al., 2004), however, limbal Langerhans cells also express vimentin as was demonstrated in the conjunctiva (Baudouin et al., 1997) and skin (de Waal et al., 1984; Broekaert et al., 1988).

We counted the number of melanocytes in cytospin samples of the limbal epithelium following enzymatic digestion. Fig. 4 shows a typical cytospin sample where MART1 (+) melanocytes were found mixed among epithelial cells. In our sample, melanocytes consisted of approximately 5% of total cells, and the ratio of melanocyte to K19 (+) epithelial cell was approximately 1:10. This is approximately 3-fold higher than the average melanin unit of the skin (Hadley and Quevedo, 1966), where a single melanocyte produces and delivers melanin granules to more than 30 keratinocytes. The microstructure of the melanocyte-keratinocyte interaction is still not clear, however, the expression of E-cadherin seems to be required for the unit to function properly (Hsu et al., 2000).

In order to further observe melanin formation by limbal melanocytes, we performed the DOPA assay in limbal tissue from a light-pigmented donor. DOPA is an intermediate substrate of the melanocyte-specific enzyme, tyrosinase, which converts tyrosine to melanin. As shown in Fig. 3, pigmented cells were seen in this sample from a light-pigmented donor. The melanin (+) cells shown in the digitally enlarged view (Fig. 3C) demonstrate cellular processes compatible with the dendritic morphology of melanocytes. The difference in pigmentation observed among races is therefore probably due to different levels of melanin formation, rather than an absence of melanocytes in light-pigmented eyes.

The K19 (+), melanin (+) basal epithelial cells differ from the overlying epithelium in terms of both keratin expression and pigment retention. Although K19 has been proposed as a putative marker of the limbal stem cell (Chen et al., 2004; Kim et al., 2004), conjunctival epithelial cells also express K19 (Krenzer and Freddo, 1997; Ang et al., 2004). Although further studies are required to elucidate the origin of K19 (+) cells, it should be noted that pigmentation of POV is distinctly different from the occasional pigmentation in the conjunctiva. Collectively, our data show that the association of melanocytes with K19 (+) epithelial cells of

the POV may play a role in the microenvironment surrounding the limbal stem cell niche.

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References

Ang, L.P., Tan, D.T., Phan, T.T., Li, J., Beuerman, R., Lavker, R.M., 2004. The in vitro and in vivo proliferative capacity of serum-free cultivated human conjunctival epithelial cells. Curr. Eye Res. 28, 307–317.

Baudouin, C., Brignole, F., Pisella, P.J., Becquet, F., Philip, P.J., 1997. Immunophenotyping of human dendriform cells from the conjunctival epithelium. Curr. Eye Res. 16, 475–481.

Broekaert, D., Cornille, A., Eto, H., Leigh, I., Ramaekers, F., Van Muijen, G., Coucke, P., De Bersaques, J., Kluyskens, P., Gillis, E., 1988. A comparative immunohistochemical study of cytokeratin and vimentin expression in middle ear mucosa and cholesteatoma, and in epidermis. Virchows Arch. A Pathol. Anat. Histopathol. 413, 39–51.

Chen, Z., de Paiva, C.S., Luo, L., Kretzer, F.L., Pflugfelder, S.C., Li, D.Q., 2004. Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells 22, 355–366.

Commo, S., Gaillard, O., Bernard, B.A., 2004. Human hair greying is linked to a specific depletion of hair follicle melanocytes affecting both the bulb and the outer root sheath. Br. J. Dermatol. 150, 435–443.

Davanger, M., Evensen, A., 1971. Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 229, 560–561.

de Waal, R.M., Semeijn, J.T., Cornelissen, M.H., Ramaekers, F.C., 1984. Epidermal Langerhans cells contain intermediate-sized filaments of the vimentin type: an immunocytologic study. J. Invest. Dermatol. 82, 602–604.

Elder, M.J., Hiscott, P., Dart, J.K., 1997. Intermediate filament expression by normal and diseased human corneal epithelium. Hum. Pathol. 28, 1348–1354.

Espana, E.M., Kawakita, T., Romano, A., DiPascuale, M., Smiddy, R., Liu, C.Y., Tseng, S.C., 2003. Stromal niche controls the plasticity of limbal and corneal epithelial differentiation in a rabbit model of recombined tissue. Invest. Ophthalmol. Vis. Sci. 44, 5130–5135.

Goldberg, M.F., Bron, A.J., 1982. Limbal palisades of Vogt. Trans. Am. Ophthalmol. Soc. 80, 155–171.

Hadley, M.E., Quevedo Jr., W.C., 1966. Vertebrate epidermal melanin unit. Nature 209, 1334–1335.

Hsu, M., Andl, T., Li, G., Meinkoth, J.L., Herlyn, M., 2000. Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. J. Cell Sci. 113, 1535–1542.

Inoue, T., Maeda, N., Young, L.S., Hamano, T., Shimomura, M.D., Tano, Y., Ohashi, Y., 2001. Epithelial pigment slide in contact lens wearers: a possible marker for contact lens-associated stress on corneal epithelium. Am. J. Ophthalmol. 131, 431–437.

Kasper, M., Moll, R., Stosiek, P., Karsten, U., 1988. Patterns of cytokeratin and vimentin expression in the human eye. Histochemistry 89, 369–377.

Kawakami, Y., Eliyahu, S., Delgado, C.H., Robbins, P.F., Rivoltini, L., Topalian, S.L., Miki, T., Rosenberg, S.A., 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc. Natl Acad. Sci. USA 91, 3515–3519.

Kim, H.S., Jun Song, X., de Paiva, C.S., Chen, Z., Pflugfelder, S.C., Li, D.Q., 2004. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. Exp. Eye Res. 79, 41–49.

Kobayashi, N., Nakagawa, A., Muramatsu, T., Yamashina, Y., Shirai, T., Hashimoto, M.W., Ishigaki, Y., Ohnishi, T., Mori, T., 1998. Supranuclear melanin caps reduce ultraviolet induced DNA photoproducts in human epidermis. J. Invest. Dermatol. 110, 806–810.

Krenzer, K.L., Freddo, T.F., 1997. Cytokeratin expression in normal human bulbar conjunctiva obtained by impression cytology. Invest. Ophthalmol. Vis. Sci. 38, 142–152.

Lang, K.S., Caroli, C.C., Muhm, A., Wernet, D., Moris, A., Schittek, B., Knauss-Scherwitz, E., Stevanovic, S., Rammensee, H.G., Garbe, C., 2001. HLA-A2 restricted, melanocyte-specific CD8(+) T lymphocytes detected in vitiligo patients are related to disease activity and are predominantly directed against MelanA/MART1. J. Invest. Dermatol. 116, 891–897.

Lindberg, K., Brown, M.E., Chaves, H.V., Kenyon, K.R., Rheinwald, J.G., 1993. In vitro propagation of human ocular surface epithelial cells for transplantation. Invest. Ophthalmol. Vis. Sci. 34, 2672–2679.

Luthra, R., Nemesure, B.B., Wu, S.Y., Xie, S.H., Leske, M.C., 2001. Frequency and risk factors for pterygium in the Barbados Eye Study. Arch. Ophthalmol. 119, 1827–1832.

Mottaz, J.H., Zelickson, A.S., 1967. Melanin transfer: a possible phagocytic process. J. Invest. Dermatol. 49, 605–610.

Prota, G., 1997. Pigment cell research: what directions?. Pigment Cell Res. 10, 5–11.

Prota, G., 1980. Recent advances in the chemistry of melanogenesis in mammals. J. Invest. Dermatol. 75, 122–127.

Sato, Y., Mukai, K., Watanabe, S., Goto, M., Shimosato, Y., 1986. The AMeX method. A simplified technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. Am. J. Pathol. 125, 431–435.

Seiji, M., Iwashita, S., 1965. Intracellular localization of tyrosinase and site of melanin formation in melanocyte. J. Invest. Dermatol. 45, 305–314.

Shimmura, S., Tsubota, K., 1997. Ultraviolet B-induced mitochondrial dysfunction is associated with decreased cell detachment of corneal epithelial cells in vitro. Invest. Ophthalmol. Vis. Sci. 38, 620–626.

Shimmura, S., Suematsu, M., Shimoyama, M., Tsubota, K., Oguchi, Y., Ishimura, Y., 1996. Subthreshold UV radiation-induced peroxide formation in cultured corneal epithelial cells: the protective effects of lactoferrin. Exp. Eye Res. 63, 519–526.

Shimmura, S., Tadano, K., Tsubota, K., 2004. UV dose-dependent caspase activation in a corneal epithelial cell line. Curr. Eye Res. 28, 85–92.

Sliney, D.H., 2002. Geometrical gradients in the distribution of temperature and absorbed ultraviolet radiation in ocular tissues. Dev. Ophthalmol. 35, 40–59.

Waring III., G.O., Roth, A.M., Ekins, M.B., 1984. Clinical and pathologic description of 17 cases of corneal intraepithelial neoplasia. Am. J. Ophthalmol. 97, 547–559.

Wong, T.Y., Foster, P.J., Johnson, G.J., Seah, S.K., Tan, D.T., 2001. The prevalence and risk factors for pterygium in an adult Chinese population in Singapore: the Tanjong Pagar survey. Am. J. Ophthalmol. 131, 176–183.

Hyaluronic Acid–CD44 Interaction Mediates the Adhesion of Lymphocytes by Amniotic Membrane Stroma

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Purpose: To demonstrate the role of intrinsic hyaluronic acid (HA) in the entrapment of inflammatory cells by amniotic membrane (AM) in vitro.

Methods: HA concentration in AM was analyzed by the sandwich protein binding assay, and the approximate molecular weight was measured by gel filtration chromatography. To localize HA in AM tissue, toluidine blue staining with and without hyaluronidase treatment was performed. Adhesion of the lymphocyte cell lines, Jurkat and Molt 4, and peripheral blood mononuclear cells (PBMC) to AM and HA-coated glass slides was analyzed in an in vitro binding assay. Flow cytometry was performed to quantify the expression of the HA receptor, CD44, in Jurkat, Molt 4, and PBMC.

Results: HA was present in high levels in the stroma of AM, also demonstrated by intense staining with toluidine blue. Staining was inhibited by both hyaluronidase treatment and acidic pH. Molt 4, which constitutively expressed CD44, bound to AM and HA-coated slides significantly more than Jurkat cells ($CD44^-$). Adhesion of Molt 4 was inhibited by pretreatment with both soluble HA and anti-CD44 antibody. LPS- or TNF-α-treated PBMC also bound to AM and HA-coated slides and was inhibited by pretreatment with an anti-CD44 antibody.

Conclusion: HA in AM stroma may play an important role in the entrapment of inflammatory cells including lymphocytes when used as a patch in ocular surface disease.

Key Words: antiinflammatory, cornea wound healing, amniotic membrane, hyaluronic acid, CD44, extracellular matrix adhesion

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Amniotic membrane (AM) has a thick collagen layer and an overlying basement membrane supporting a single layer of epithelium.[1] The use of AM as a surgical material dates as far back as 1913, when Stern reported the use of AM in surgical procedures on the skin.[2] In regard to application of fetal membrane to ocular surface disorders, De Rotth first reported the treatment of conjunctival defects after symblepharon repair in 1940,[3] followed by several similar reports in the next decade.[4,5] More recently, Kim and Tseng reported the use of AM for ocular surface reconstruction in the rabbit,[6] followed by several groups who have reported clinical use of AM in the treatment of ocular surface disease.[7,8] Results of such studies show that AM has the ability to suppress both inflammation and fibrosis.[7,9,10] Kim et al reported that the patching of AM on epithelial defects of the cornea resulted in rapid epithelialization and decreased infiltration of inflammatory cells.[11] Data have also been presented showing that AM can suppress proteinase and matrix metalloproteinase activation.[11] However, the precise mechanisms involved in the antiinflammatory effects of AM still remain unclear.

We previously found inflammatory cells trapped in the amniotic stromal tissue after AM patching in patients with various ocular surface diseases.[12] To elucidate the mechanisms involved, we conducted an in vitro study to determine the ability of AM to bind inflammatory cells, with emphasis on hyaluronic acid (HA), a high-molecular-weight glycosaminoglycan distributed widely in the body. HA is a major carbohydrate component of the extracellular matrix that can be found in skin, joints, and Wharton jelly in the umbilical cord.[13] It is also involved in cell-to-matrix adhesion and plays a central role in scarless wound healing in the fetus.[14] HA has high water retention capacity and participates in the proliferation and differentiation of cells.[14–18] In this study, we hypothesized that HA found in AM acts as a ligand for CD44 expressed on inflammatory cells and thereby traps such cells that have infiltrated to the ocular surface. The entrapment of inflammatory cells may serve to reduce damage to ocular tissue.

MATERIAL AND METHODS

Amniotic membranes were donated by mothers seronegative for human immunodeficiency virus and hepatitis B and C

viruses at the time of cesarian sections. AM was stored with 15% dimethylsulfoxide (Sigma, St Louis, MO) with PBS at −80°C until use. Written informed consent was obtained from all donors before surgery.

Detection of HA in Human AM

HA was extracted from AM with 0.5 N sodium hydroxide at 4°C for 20 hours and neutralized with hydrochloric acid. HA concentration was measured using the sandwich binding protein method, using plates bound with immobilized HA and peroxidase-labeled HA binding protein (Chugai Pharmaceutical Co, Ltd, Tokyo, Japan). The molecular weight distribution of HA was obtained by gel filtration chromatography with an Asahipack GS-620 column (Asahi Kasei Co, Tokyo, Japan).

AM stroma was also stained with toluidine blue, which specifically stains glycosaminoglycans containing HA. AM tissue was fixed in 10% buffered formalin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and embedded in paraffin. The sections were stained with 0.05% toluidine blue (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at a pH of 7.0 (0.1 M citric acid, 0.2 M disodium hydrogen phosphate 12-water).[19] Hyaluronidase-digested sections were prepared by treating paraffin-fixed samples with bovine testis hyaluronidase (Wako Pure Chemical Industries, Ltd, Osaka, Japan) in 0.1 M phosphate-buffered saline (pH 5.5) for 3 hours at 37°C.[20] To differentiate HA from other glycosaminoglycans, staining of toluidine blue was compared at 3 different pH values (7.0, 4.1, and 2.5) titrated with 0.1 M citric acid and 0.2 M disodium hydrogen phosphate 12-water.[19] Staining of HA by toluidine blue is weak under acidic conditions.

CD44 Expression by T-Lymphocyte Cell Lines and PBMC

T-cell lines used in the study (Jurkat and Molt 4) and human PBMC were analyzed by flow cytometry (Epics XL, Beckman Coulter, FL) to confirm the expression of CD44 on the cell surface. Jurkat, Molt 4, or human PBMC (1×10^6 cells) were incubated with either 33.3 μg/mL of FITC-labeled isotype IgG 1 (Immunotech, Marseilles, France) or a FITC-labeled anti-CD44 monoclonal antibody (Immunotech, Marseilles, France) containing 1.3% FCS and 0.1% sodium azide in PBS for 45 minutes at 4°C. Cells were washed twice with PBS before flow cytometry.

In Vitro Adhesion Assay

AM was fixed in Tissue-Tek OCT compound (Sakura Finetechnical Co, Ltd, Tokyo, Japan) and stored at −80°C until use. The cryostat sections were mounted on glass slides, air dried for 5 minutes, and fixed for 10 minutes at 4°C in 3% glutaraldehyde (TAAB, Berkshire, England) with PBS. The sections were washed in cold deionized water for 10 minutes. HA (0.1 mg/mL) (Biozyme Laboratories, CA) was applied to APS-coated (silanization) glass slides (Matsunami Glass Ind, Ltd, Osaka, Japan) and thoroughly air dried. Jurkat and Molt 4 (2.0×10^6 cells/mL) were suspended in RPMI 1640 (Gibco BRL, Grand Island, NY) containing 10% FCS.

PBMC from normal healthy donors were isolated by density centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). PBMC were cultured for 72 hours in RPMI 1640 medium supplemented with 10% vol/vol autologous serum at 37°C, 5% CO_2 in air. Supplements were used at the following concentrations: lipopolysaccharide (LPS) (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 1 μg/mL, TNF-α (Gibco BRL, Grand Island, NY) at 10 ng/mL. Cells were applied to HA-coated glass slides or fixed cryostat sections and then incubated for 10 minutes at 37°C. Samples were washed by dipping the glass slides in cold PBS and fixed for 10 minutes in 3% glutaraldehyde at 4°C. After washing with deionized water, slides were stained with hematoxylin and eosin stain (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

To inhibit the adhesion of cells, suspensions were preincubated with either 1 mg/mL HA for 30 minutes at 37°C or 20 μg/mL of anti-CD44 monoclonal antibody (Seikagaku Co, Tokyo, Japan) for 60 minutes at 37°C. The number of attached cells per square millimeter was analyzed by the NIH Image program (developed at the National Institutes of Health, Bethesda, MD). Statistical analysis was done by the Mann-Whitney *U* test using the StatView 5.0 program for the Macintosh (SAS Institute Inc, Cary, NC).

RESULTS

Detection of HA in Human AM

The HA content in AM was 140 ng/mg wet weight measured by the sandwich binding protein assay, with a high molecular weight in the range of 1670 kD. Figure 1 shows the distribution of glycosaminoglycans (GAGs), including HA, in AM when stained with toluidine blue. To differentiate staining of HA from other GAG members, treatment with 0.5% hyaluronidase markedly decreased toluidine blue staining of the stroma (Fig. 1B). This was also confirmed by toluidine blue stains of AM under various pH values: unlike other GAGs, HA does not stain at a pH of 2.5 (Fig. 1).

Detection of CD44 on Cell Lines of T-Lymphocytes

The T cell lines Jurkat and Molt 4 were examined for cell-surface CD44 expression by flow cytometry analysis. CD44, the receptor of HA, was expressed only in Molt 4 cells and not in Jurkat cells (Fig. 2A,B, solid peaks) when compared with the background values obtained with isotype IgG1 (Fig. 2A,B, open peaks).

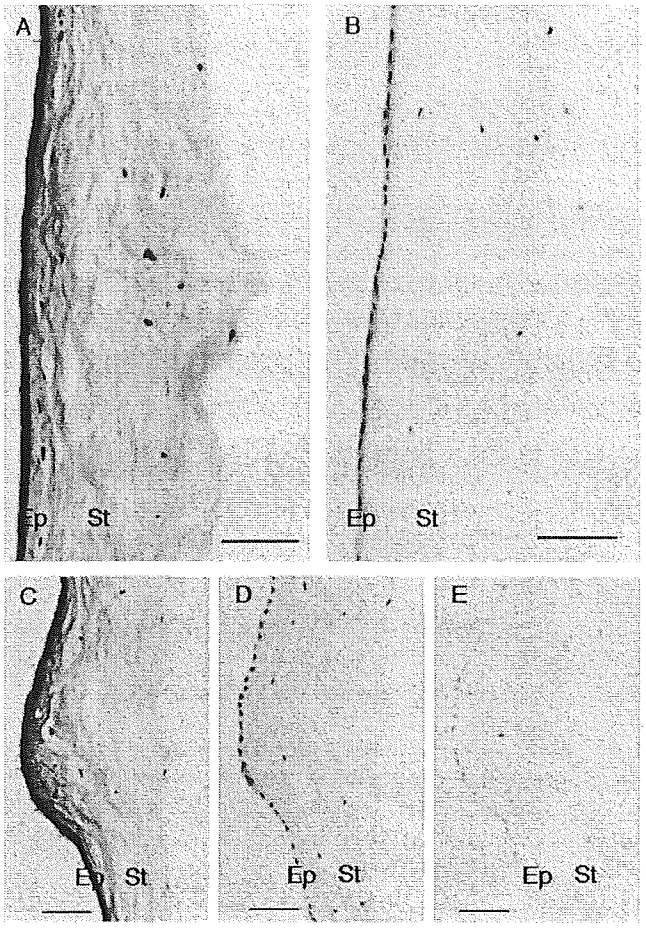

FIGURE 1. Paraffin sections of AM were treated with (B) or without (A) 0.05% bovine testicular hyaluronidase and stained with 0.05% toluidine blue (TB) (pH 7.0). Sections stained at pH 7.0 (C), 4.1 (D), and 2.5 (E) showed a gradual decrease in intensity, indicating that the glycosaminoglycan stained by TB was hyaluronic acid. Ep, amniotic epithelium; St, stroma of AM. The bar represents 50 μm.

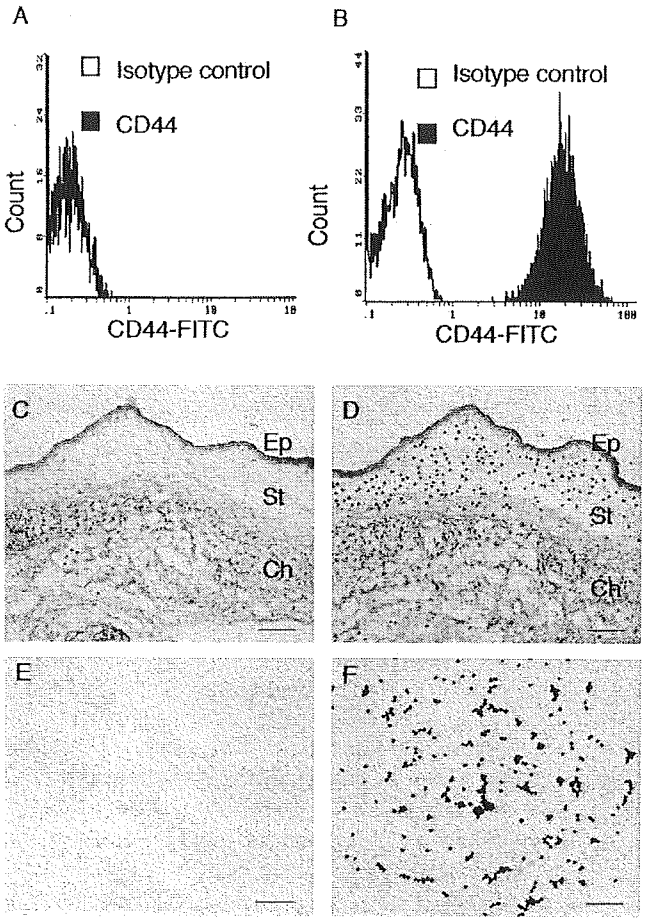

FIGURE 2. Flow cytometry of Jurkat (A) and Molt 4 (B) incubated with isotype control IgG1 (open peaks) or CD44 monoclonal antibody (solid peaks) confirmed the expression of CD44 on Molt 4. Adhesion assays of Jurkat (C, E) and Molt 4 (D, F) to cryostat sections of AM and immobilized HA both showed greater adhesion by Molt 4. E, amniotic epithelium; S, stroma of AM; C, chorion. The bar represents 100 μm.

Adhesion Assay of T-Lymphocytes Cell Lines

Adhesion assays of Molt 4 ($CD44^+$) and Jurkat ($CD44^-$) to AM revealed that only Molt 4 adhered to the AM stroma in areas with abundant HA and did not adhere to the epithelium and chorion (Fig. 2D). Adhesion of Jurkat cells was sparse (Fig. 2C), suggesting that HA-CD44 interaction is involved in lymphocyte adhesion to AM. Because AM contains other glycosaminoglycans and matrix components, Jurkat and Molt 4 binding assays were repeated using HA-coated glass slides. As with the AM adhesion assay, only Molt 4 bound to the immobilized HA (Fig. 2E,F). To confirm this, the same adhesion assay was done with Molt 4 preincubated with soluble HA or anti-CD44 mAb. Preincubation with soluble HA decreased the number of adhering Molt 4 cells to 6.5% of control (Fig. 3A–C, $P = 0.019$). Molt 4 adhesion was also blocked by anti-CD44 mAb to 11.7% of control (Fig. 3D–F, $P = 0.002$). Adhesion of Molt 4 to immobilized HA was blocked by anti-CD44 mAb (Fig. 3G,H).

Detection of CD44 on PBMC

PBMC were examined for cell-surface CD44 expression by flow cytometry analysis. To separate lymphocytes and monocytes in white blood cells, cells were analyzed by gating on forward and side scatter. CD44 was expressed on each cell type (Fig. 4A,B, solid peaks) when compared with the background values obtained with isotype IgG1 (Fig. 4A,B, open peaks).