Urokinase Plasminogen Activator, uPa Receptor, and Its Inhibitor in Vernal Keratoconjunctivitis

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Purpose. Plasminogen activators play a role, not only in fibrinolysis but also in events such as chemotaxis, collagen degradation, and cell spreading. The serine protease urokinase (uPA) is a potent chemoattractant for leukocytes that may be involved in the pathogenesis of severe forms of allergic conjunctivitis such as vernal keratoconjunctivitis (VKC).

Methods. Tear and peripheral blood samples were obtained from 20 patients with active VKC and from 19 normal subjects who formed the control group. Levels of plasminogen activity, uPA, tissue plasminogen activator (tPA), and their inhibitor, plasminogen activator inhibitor type-1 (PAI-1) were measured in tears and plasma of patients with VKC. The presence of tPA, uPA, and urokinase receptor (uPAR) in conjunctival tissues were evaluated by immunohistochemistry. uPA, uPAR, and PAI-1 expression and production were measured in conjunctival epithelial cell and fibroblast cultures treated with cytokines.

Results. Tear levels of uPA and tPA and tear plasminogen activity levels were significantly greater in patients with VKC than in control subjects. Increased staining for uPA and uPAR was found in VKC tissues compared with normal conjunctiva. Both conjunctival epithelial cells and fibroblasts demonstrated an increased expression of uPAR after exposure to IL-4 or -13, whereas uPA was highly expressed by epithelial cells exposed to IL-4. PAI-1 levels in culture medium were increased in IL-4-exposed epithelial cells compared to nonstimulated cells and were decreased in fibroblast culture.

Conclusions. Increased expression of fibrinolytic system components and imbalance between plasminogen activators and PAI may be involved in the pathogenesis of severe allergic conjunctivitis, thus contributing to inflammatory cell migration and tissue remodeling. (*Invest Ophthalmol Vis Sci.* 2005;46: 1364–1370) DOI:10.1167/iovs.04-1196

In addition to its antithrombotic function, plasminogen activators are believed to play a role in events such as chemotaxis, collagen degradation, angiogenesis, and tumor growth and spreading. Urokinase-type (uPA) and tissue type (tPA) plasminogen activators are serine proteases that activate plasminogen to plasmin, whose primary function is the degradation of fibrin clots in vivo. Although tPA exhibits high affinity for fibrin and contributes to fibrinolysis in circulation, uPA functions in cell adhesion, cell migration, and tissue remodeling.[1] The activation of the fibrinolytic system is also associated with tumor progression and metastasis.[2] Urokinase is also known as a potent chemoattractant for leukocytes and thus may be involved in the pathogenesis of cell migration and tissue infiltration in the course of the inflammatory process.[3–5]

Vernal keratoconjunctivitis (VKC) is a severe chronic ocular allergic disease characterized by an intense inflammation with predominance of eosinophils and Th2-type lymphocytes.[6,7] Eosinophils are the major effector cells in allergic inflammatory response. They are increased and activated in tears and tissues of all allergic eye diseases[8,9]; yet, the mechanisms of eosinophil recruitment to the ocular surface are not fully understood. Patients with either positive or negative serum- and tear-specific IgE have the same clinical outcome: an intense ocular inflammation, massive eosinophil infiltration and activation,[10,11] and subsequent conjunctival tissue remodeling.[12] Several cytokines, chemokines, mediators, and proteases, such as tryptase, chymase, and metalloproteases (MMPs) have been found to be overexpressed in tears and tissues of patients affected by VKC, confirming the complexity of mechanisms involved in the pathogenesis of inflammatory cell infiltration in this disease.[6]

The migration of inflammatory cells from the peripheral blood to the tissue involves many steps, including chemokinesis, chemotaxis, expression of adhesion molecules, endothelial adherence, and transmigration through the endothelial wall.[13] This final step involves many cellular functions, such as modification of the cell skeleton, diapedesis, and digestion of the extracellular matrix and basement membrane.[14] Candidates for these final steps are the MMP and the plasminogen–plasmin system.[1,15] Plasmin and uPA directly or indirectly hydrolyze extracellular matrix proteins by inducing a cascade of proteolytic events leading to MMP activation. The binding of uPA to its cell-membrane receptor (uPAR) not only increases the rate of plasminogen activation, but also allows this proteolytic process to occur in a focal and spatially oriented manner, as is necessary for cell migration.[4–5,16] This system is known to promote degradation of the extracellular matrix and has been reported in monocytes, T lymphocytes, neutrophils, and eosinophils.[17–21] MMP-9 and the plasminogen–plasmin complex are the two most well-known proteases expressed by eosinophils.[22,23] Increased tear levels of the potent selective eosinophil chemotactic factors, eotaxin-1 and -2, and MMP-9 have been recently described in patients with VKC.[24–27] These factors correlate with the percentage of eosinophils in tears, suggesting that they are involved in eosinophil chemotaxis and transmigration in VKC. In an in vitro model of eosinophil transmigration through a basement membrane component, eotaxin proved to promote eosinophil transmigration via plasminogen–plasmin activation.[28] In similar studies, the combination of two other eosinophil activators, platelet-activating fac-

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TABLE 1. Clinical and Laboratory Data from 20 Patients with VKC

Gender/Age (y)	Type of VKC	Serum-Specific IgE/Prick	Tear uPA (ng/mL)	Tear tPA (ng/mL)	Subjective Clinical Score (0–10)	Corneal Clinical Score (0–4)	Papillae/Limbus Score (0–4)
F/12	L	G, D, Tr, Cat	.8	4.2	8	2	3
F/7	L	Neg.	1.2	6.9	8	0	3
M/9	T	C, Alt	.7	.7	4	0	3
M/19	T	G, Tr, D, Alt	1.5	6.0	8	1	4
M/8	L	G	1.2	14.4	7	3	2
F/17	T	Neg.	.5	5.8	6	2	3
M/17	T	G, D, Alt	3.8	3.8	8	0	2
M/10	T	D, P, G	.7	11	6	4	4
F/10	T	D, G, P, Tr	1.4	7.2	8	1	2
M/7	T	Neg.	1.1	3.7	9	4	3
M/14	L	G, D, P, Tr	.6	5.3	5	1	2
M/18	L	D	1.7	6.5	6	0	1
M/12	T	Neg.	12.2	9.8	8	2	3
F/14	L	P, D, Alt, Cat	2.6	2.2	7	2	2
F/12	T	D, Tr, C, G	7.1	57.1	9	2	3
M/13	T	Neg.	6.7	16.3	7	2	2
M/12	L	Neg.	7.4	4.7	7	1	2
M/6	T	G, D, Alt	10.6	4.4	8	1	3
M/10	T	Neg.	5.9	1.6	5	0	2
M/5	T	Alt, Tr	2.1	2	4	0	2

F, female; M, male; L, limbal VKC; T, tarsal VKC; D, *Dermatophagoides*; G, *Graminaceae*; P, *Parietariae*; C, *Compositae*; Alt, *Alternaria alternata*; Tr, tree pollens; Cat, cat dander; Neg., negative results.

tors (PAFs) and IL-5, induced eosinophil transmigration by activation of MMP-9 and serine proteases.[15,29]

Urokinase is a normal component of tear fluid[30] that originates from conjunctival and corneal epithelial cells. Levels and activity of uPA have been found increased in corneal ulceration[31] and after photorefractive keratectomy,[32] suggesting that the fibrinolytic system plays a role in corneal wound healing. Expression of these components by conjunctival tissues may be regulated under physiological conditions. In a severe allergic inflammatory condition such as VKC, proinflammatory cytokines may modulate the expression of these factors, enhancing inflammatory cell adherence and migration and tissue remodeling.

The purpose of this study was to investigate the role of the fibrinolytic system in the pathogenesis of VKC. Levels and expression of plasminogen, uPA, tPA, and plasminogen activator inhibitor (PAI)-1 were evaluated in tears, plasma, and human conjunctival tissues, and in conjunctival epithelial cells and fibroblasts in culture.

MATERIALS AND METHODS

Patients and Tear Samples

After clinical evaluation, tear samples, and peripheral blood were obtained from 20 patients with active VKC (mean age, 11.6 ± 4; range, 5–18; 14 males, 6 females) and 19 normal subjects who comprised the age-matched (mean age, 14.9 ± 4.4, range, 8–22) control group of 13 males and 6 females. None of the subjects in the control group used contact lenses or had any inflammatory signs and symptoms. The research adhered to the tenets of the Declaration of Helsinki. A written informed consent was obtained from all subjects or their parents before collecting samples. Diagnosis of VKC was based on clinical history and evaluation of signs and symptoms. Clinical scores (0–4) for each ocular symptom (itching, tearing, photophobia, and foreign body sensation) and each sign (conjunctival erythema, mucous discharge, papillae, limbal infiltrates, and corneal epithelial disease) were assigned at the time of the visit. Patients included in the study were free of topical medication for at least 3 days. All patients were in an active inflammatory phase of the disease with active limbal infiltrates (limbal VKC) or tarsal giant papillae (tarsal VKC; Table 1) and with at least three positive symptoms. A subjective clinical score (0–10) was given to each patient by one of the investigators (AL) to evaluate the overall severity of the disease. Patients with VKC were tested for skin test reactivity and the presence of specific IgE in serum for common environmental allergens (CAP System; Pharmacia & Upjohn, Uppsala, Sweden).

Tear samples were collected from the outer canthus with a microcapillary tube, and centrifuged for 10 minutes at 1000 rpm to separate the cells from the tear fluid. Tear volume ranged from 50 to 200 μL. Supernatants were stored at −80°C until assaying.

Cell pellets were resuspended in 2 μL aliquots. The percentage of eosinophils, neutrophils, and lymphocytes present in five microscopic fields of 0.15 mm^2 were counted on precolored slides (Testsimplet; Roche, Mannheim, Germany) using a microscope at high-power (400×) magnification (Carl Zeiss Meditec, Dublin, CA).

Peripheral whole blood samples (5.4 mL) were collected from all patients with VKC and control subjects in a tube (Vacutainer; BD Biosciences, Franklin Lakes, NJ), containing 0.6 mL trisodium citrate (0.13 M) as an anticoagulant, and immediately put on ice. After centrifugation at 3000*g* for 15 minutes, plasma aliquots were stored at −40°C and tested within 1 month.

Measurement of uPA, tPA, PAI-1, and Plasminogen in Tears and Plasma

PAI-1, t-PA, and uPA antigens were measured by ELISA (TintElize PAI-1, TintElize t-PA, and TintElize uPA; Biopool, Umeå, Sweden) according to the manufacturer's instructions. Plasminogen activity was measured with a chromogenic substrate (Berichrom Plasminogen; Behring, Scoppito, AQ, Italy) according to the manufacturer's instructions. The sensitivities of the assays were as follows: uPA, 0.11 ng/mL; t-PA, 3.1 ng/mL; and PAI-1, 1.2 ng/mL. For plasminogen activity, the measurement range extended from 0% to 150%, and normal levels were considered to be >75%.

Immunohistochemistry

In subjects under local anesthesia, conjunctival biopsies were obtained from the upper tarsal conjunctiva of seven patients with active VKC and five additional normal subjects (mean age, 21 years) at the time of

surgery for strabismus. No subject in the control group had a history of contact lens wear or any inflammatory signs and symptoms. Bioptic tissues were snap frozen with OCT compound in liquid nitrogen and maintained at −70°C.

Serial 5-μm-thick cryosections were cut on an ultramicrotome, mounted on gelatin-covered slides, fixed in acetone, and processed for immunohistochemistry. The following anti-human antibodies were used: monoclonal mouse anti-uPA (Serotec, Oxford, UK); polyclonal goat anti-tPA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which recognizes both latent and activated forms; and monoclonal mouse anti-uPAR (Santa Cruz Biotechnology, Inc.).

Briefly, for all antibodies, unspecific binding was blocked by the addition of serum from the same animal species of the secondary antibody. The slides were washed twice in Tris-buffered saline (TBS: 10 mM Tris, 150 mM NaCl [pH 7.4]), and the appropriately diluted anti-human antibodies were placed on each tissue section and incubated in a moist chamber for 60 minutes. After the primary incubation, slides were washed in TBS, incubated for 30 minutes with the secondary antibodies (Link-Ab-Dako anti-mouse), and treated with an alkaline phosphatase complex (APAAP; Dako, Milan, Italy). The reaction was developed with fast red solution and counterstained with Mayer hematoxylin. Negative control sections were obtained by omitting the primary antibody.

The positive red reaction was analyzed in VKC and normal specimens and was classified as very intense (+++), intense (++), slight (+), or absent (−). A 10-mm^2 grid was used with a microscope (Carl Zeiss Meditec) for each assessment, and all quantifications were performed in a masked fashion.

Immunocytochemistry

From five of the patients with VKC, part of the tear cell pellets were allowed to adhere to poly-L-lysine-coated glass slides, air dried, fixed with 4% paraformaldehyde in PBS, and washed. After nonspecific binding sites were saturated with rabbit serum, cells were incubated with the anti-uPA antibody for 1 hour. Slides were then washed twice in TBS and incubated for 30 minutes with the secondary antibody (Link-Ab-Dako; rabbit anti-mouse). After the cells were rinsed, they were treated with an alkaline phosphatase complex (APAAP; Dako), and reacted with fast red solution. Counterstaining was performed with Mayer hematoxylin.

Cell Cultures

Conjunctival biopsies specimens from two normal subjects were washed, cut in small pieces, seeded in multiwell dishes (Nunclon Multidishes; NUNC, Roskilde, Denmark) containing 100 mL Ham's F12 medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich) and antibiotics (penicillin 100 U/mL, streptomycin 100 mg/mL, and L-glutamine 2 mM/L; Sigma-Aldrich), and incubated at 37°C in 5% carbon dioxide in a humidified air atmosphere. Fibroblasts were subcultured with 0.05% trypsin and replated into 24-well plates (>95% vitality). Cells were characterized morphologically and stained positively with vimentin and negatively with cytokeratins. Third-passage fibroblasts were used for experiments. Wong-Kilbourne–derived human conjunctival epithelial cells were obtained from ATCC (clone 1-5c-4; Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics.

After a 24-hour period in basal medium without FCS, the two cell cultures were treated with either 1 or 10 ng/mL IL-4, IL-13, TNFα (Chemicon International, Inc., Temecula, CA), or IFNγ (Bender MedSystem, Vienna, Austria) for 24 hours. Supernatants were then collected and stored at −80°C for further analysis and the cells processed for RNA isolation. Experiments were performed three times for each cell culture.

RNA Isolation and RT-PCR

Total RNA was extracted from cultured cells by using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol reagent; Invitrogen-Gibco, Gaithersburg, MD). Briefly, the cells were lysed by addition of 1.0 mL of the extraction reagent, and total RNA was subsequently isolated according to the manufacturer's instructions. Complementary DNA was synthesized from 500 ng of total RNA per sample with 50 minutes of incubation at 37°C, using Moloney murine leukemia virus reverse transcriptase (Invitrogen-Gibco) and oligo(dT) primer. Amplification was performed in a programmable thermal controller (PTC-100; MJ Research Inc., Watertown, MA) with recombinant *Taq*DNA polymerase (PerkinElmer, Wellesley, MA) and specific primer pairs. The parallel amplification of cDNA for the housekeeping enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control.

To enable a semiquantitative comparison between samples, serial threefold dilutions of cDNA (corresponding to 100–1.2 ng of total) were subjected to increasing PCR cycles from 23 to 40 to define the linear amplification range for each primer set. Amplifications were performed at 40 cycles for PAI-1, 33 cycles for uPA and uPAR, and 24 cycles for GAPDH. Each cycle consisted of denaturation at 95°C for 15 seconds, annealing at 60°C for 5 seconds, and extension at 72°C for 18 seconds for amplification of PAI-1 cDNA; denaturation at 94°C for 15 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 10 seconds for amplification of uPA and uPAR cDNA; denaturation at 94°C for 1 minute, annealing at 59°C for1 minute, and extension at 72°C for 1 minute, for amplification of GAPDH cDNA. Each cycle was preceded by a denaturation step at 94°C for 10 minutes and terminated with an elongation step at 72°C for 5 minutes.

The sequences of primers used for each mRNA were as follows: PAI-1, forward 5′-TGC TGG TGA ATG CCC TCT ACT-3′ and reverse 5′-CGG TCA TTC CCA GGT TCT CTA-3′; uPA, forward 5′-ACT ACT ACG GCT CTG AAG TCA CCA-3′ and reverse 5′-GAA GTG TGA GAC TCT CGT GTA GAC-3′; uPAR, forward 5′-CTG GAG CTT GAA AAT CTG CCG-3′ and reverse 5′-GGT TTT TCG GTT CGT GAG TGC-3′; and GAPDH, forward GCCATCAACGACCCCTTCATT and reverse CGCCTGCTTCACCACCTTCTT.

The resultant bands were visualized on a 1% agarose gel stained with ethidium bromide and compared with a *Hin*dIII lambda DNA to confirm the predicted size. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment.

Statistics

Differences in the normally distributed fibrinolytic parameters and tear cytology between VKC and normal subjects were analyzed, using the unpaired Student's *t*-test. Differences in histologic staining scores between VKC and normal samples were analyzed using the nonparametric Mann-Whitney test, as is recommended for scored data. One-way analysis of variance (ANOVA) with a post hoc analysis (Fisher's protected least-significant difference [PLSD] test) was used to analyze the cytokines' effects on cell cultures. Spearman's rank correlation was also calculated to identify relationships between parameters. Results are given as the mean ± SD. The assigned statistical significance was $P \leq 0.05$.

Results

UPA, tPA, PAI-1, and Plasminogen in Tears and Plasma

In tear samples from control subjects, few epithelial cells and neutrophils were present. In all VKC samples, inflammatory cells were present in a high number. The percentages of eosinophils (41.1% ± 11%), neutrophils (43.8% ± 15%), and mononuclear cells (15.4% ± 7%) were significantly greater than those of normal subjects ($P < 0.01$), who had no inflammatory cells in their tears.

Tear levels of uPA (3.48 ± 3.5 ng/mL) were significantly greater in patients with VKC than in control subjects (0.8 ± 1.3

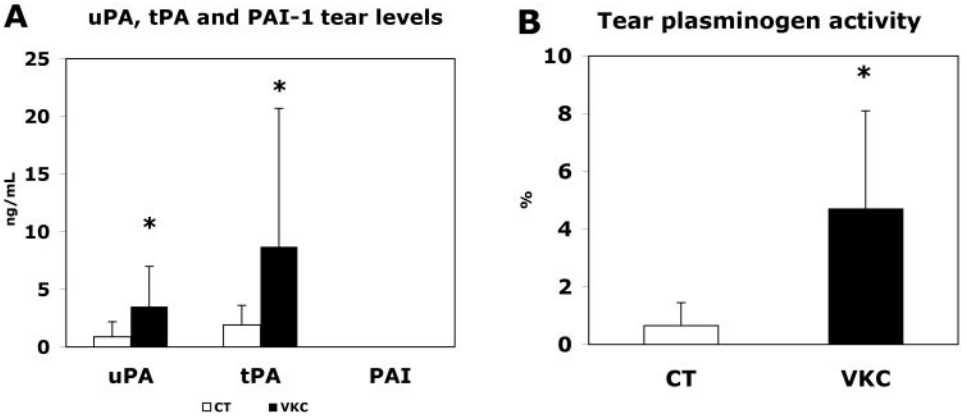

FIGURE 1. Tear levels of urokinase plasminogen activator (uPA), tissue plasminogen activator (tPA), and plasminogen activator inhibitor (PAI)-1, as well as plasminogen activity in 20 patients with VKC and 19 normal subjects (CT). Levels of uPA, tPA, and PAI-1 were analyzed in all subjects. Because of the limited quantity of sample available, plasminogen activity was analyzed in only 10 VKC and 10 CT subjects. Levels of uPA, tPA (**A**), and plasminogen (**B**) activity were significantly greater in VKC than in CT tears (*$P < 0.01$).

ng/mL; $P < 0.01$; Fig. 1A). Tear levels of tPA were also significantly greater in patients with VKC (8.68 ± 12 ng/mL) than in normal subjects (1.9 ± 1.7 ng/mL; $P < 0.01$). Conversely, levels of PAI-1 were not detectable. In addition to increased levels of plasminogen activators, plasminogen activity in tears was greater in patients with VKC ($n = 10$; 4.7% ± 3.4%) than in control tears ($n = 10$; 0.65% ± 0.83%; $P < 0.01$; Fig. 1B).

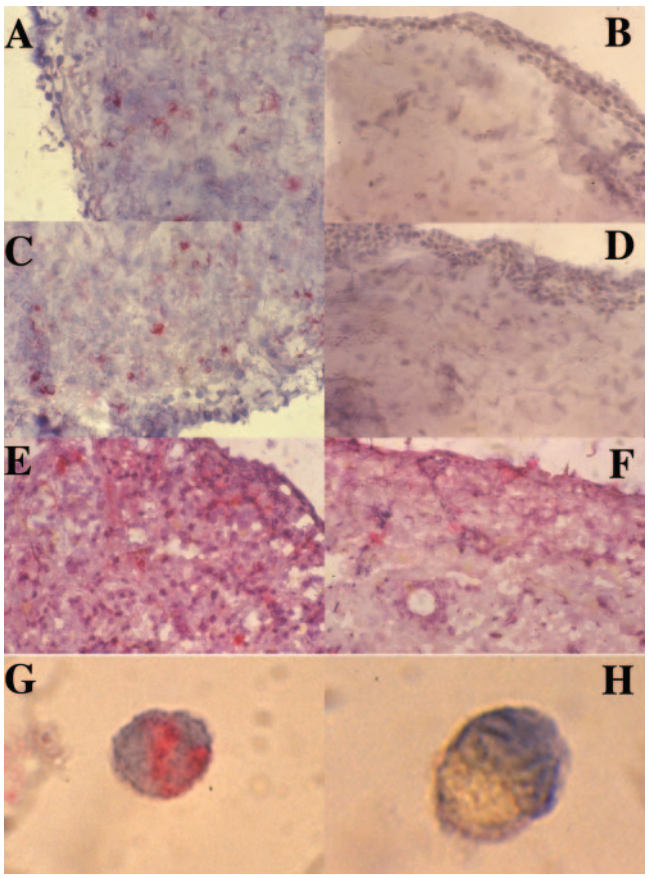

FIGURE 2. Immunostaining for uPA, uPAR, and tPA in samples from patients with VKC and normal subjects (CT). (**A**) *Red* staining shows uPA-positive cells in VKC conjunctiva; (**B**) negative staining for uPA in CT; (**C**) cell-membrane–positive staining (*red*) for uPAR in the VKC stromal conjunctiva; (**D**) negative staining for uPAR in CT; (**E**) positive staining for tPA in VKC conjunctiva; (**F**) slight positive staining for tPA in the CT conjunctiva; (**G**) a tear inflammatory cell with intracellular positive (*red*) immunoreactivity for uPA; and (**H**) a tear inflammatory cell negative for uPA. Original magnification: (**A–F**) ×40; (**G**, **H**) ×200.

Differentiating between tarsal and limbal VKC, corneal involvement was significantly more severe in tarsal patients. However, no differences in fibrinolytic components were found between tarsal and limbal VKC forms. Tear levels of uPA did not correlate with the subjective clinical score ($\rho = 0.336$), corneal score ($\rho = 0.111$), or giant papillae/limbal scores ($\rho = 0.142$). Conversely, tear tPA levels significantly correlated with the corneal score ($\rho = 0.466$, $P < 0.04$) but not with the subjective clinical score ($\rho = 0.361$) or giant papillae/limbal scores ($\rho = 0.285$). All other correlations with clinical symptoms and tear cytology were negative.

Plasma levels of tPA (2.33 ± 1.2 ng/mL) and plasminogen activity (91.0% ± 13.5%) were significantly lower in patients with VKC than in the control group (tPA 6.3 ± 3.2 ng/mL, $P < 0.01$; plasminogen 104.7% ± 14.9%, $P < 0.01$). However, all levels were still within the normal range. Plasma levels of uPA and PAI-1 were similar in the two groups.

Immunohistochemistry and Immunocytochemistry

Of the seven tissue samples obtained in giant papillae biopsies, five showed a slight to intense positive staining for uPA, both in the epithelium and in the stroma, whereas none of the control conjunctival tissue was positive (Fig. 2, Table 2). Staining scores were significantly higher in VKC than in control tissues ($P < 0.05$). Of the seven VKC samples evaluated for uPAR expression, six showed positive staining scattered both in the epithelium and in the stroma, compared with the neg-

TABLE 2. Immunohistochemical Expression of uPA, uPAR, and tPA in Conjunctival Tissues of Normal Subjects and Patients with VKC

	Conjunctival Epithelium			Conjunctival Stroma		
Case	uPA	uPAR	tPA	uPA	uPAR	tPA
VKC 1	−	+	+	+	+	+
VKC 2	++	+	−	+	++	++
VKC 3	+	+	+	+	++	+
VKC 4	+	+	−	+	+	−
VKC 5	+	+	+	+	+	−
VKC 6	−	+	−	−	+	+
VKC 7	−	−	−	−	−	−
CT 1	−	−	+	−	−	+
CT 2	−	−	+	−	−	+
CT 3	−	−	−	−	−	−
CT 4	−	−	−	−	−	−
CT 5	−	−	+	−	−	+

Very intense (+++), intense (++), slight (+), or absent (−); CT, control subjects.

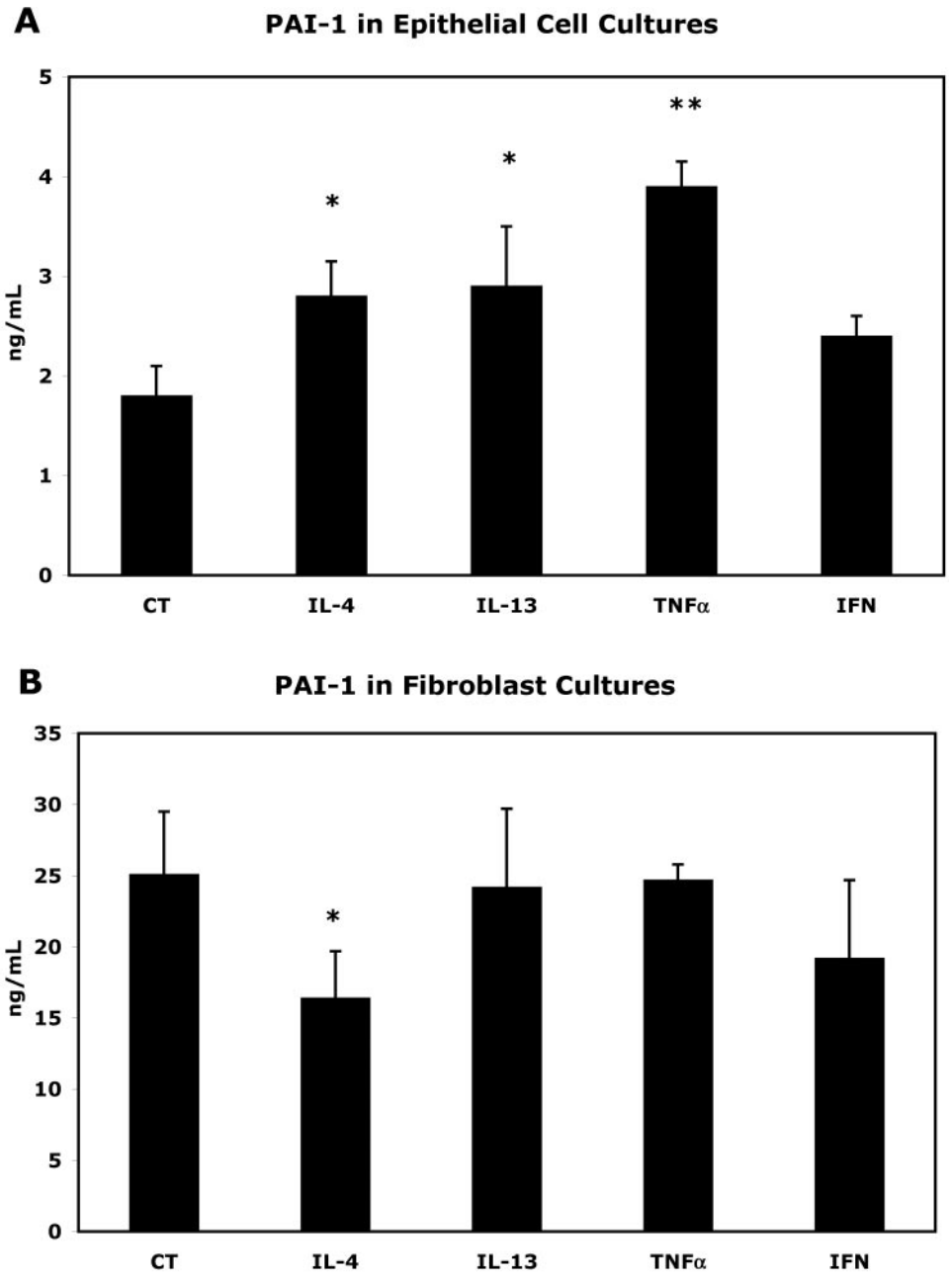

FIGURE 3. Levels of PAI-1 in culture medium of conjunctival epithelial cells and conjunctival fibroblasts exposed for 24 hours to 1 ng/mL of various cytokines. Data are reported as the mean results of three experiments performed on the epithelial cell line and as the mean results of three experiments performed in one of the two donor-derived fibroblast cultures. (**A**) In epithelial cell culture medium, PAI-1 levels were increased after exposure to IL-4 and -13 ($^{*}P < 0.05$) and TNFα ($^{**}P < 0.01$) compared with nontreated cells (CT); (**B**) in fibroblast culture medium, PAI-1 levels were decreased after exposure to IL-4 ($^{*}P < 0.05$) compared with nontreated cells (CT).

ative staining in all control specimens (Fig. 2, Table 2). Again, the staining score was significantly higher in conjunctival tissues of patients with VKC than in that of the control subjects ($P < 0.01$). Only a few samples showed positive staining for tPA, either in the stoma or in the epithelium, with no significant differences between VKC and control tissues (Fig. 2, Table 2).

By immunocytochemistry, tear cells obtained from tear pellets showed positive staining for uPA. These inflammatory cells (eosinophils and neutrophils) showed either intracytoplasmic or membrane-positive staining (Fig. 2).

Plasminogen Activators, Plasminogen Activity, and PAI-1 in Cell Cultures

In the epithelial cell culture medium, tPA and plasminogen activity did not change after exposure to various cytokines for 24 hours, whereas uPA was increased by exposure to 1 and 10 ng/mL IL-4 ($P < 0.05$). Furthermore, PAI-1 production increased significantly in stimulated epithelial cells compared with nonstimulated cells after 1 and 10 ng/mL IL-4, IL-13 ($P < 0.05$), and TNFα ($P < 0.01$) for 24 hours (Fig. 3A).

Culture medium from cytokine-treated fibroblasts showed no variations in levels of uPA and tPA and very low or undetectable plasminogen activity. Cells exposed to 1 and 10 ng/mL IL-4 produced significantly lower levels of PAI-1 than did unexposed cells ($P < 0.05$; Fig. 3B).

Expression of uPA, uPAR, and PAI-1 by Epithelial Cells and Fibroblasts

Conjunctival epithelial cells demonstrated a low basal expression of uPAR. After cytokine exposure for 24 hours, uPAR was slightly increased by IL-4 at 1 and 10 ng/mL, and by IL-13 at 10 ng/mL, but not by TNFα or IFNγ. PAI-1 expression increased twofold only after IL-4 exposure, whereas it was reduced by

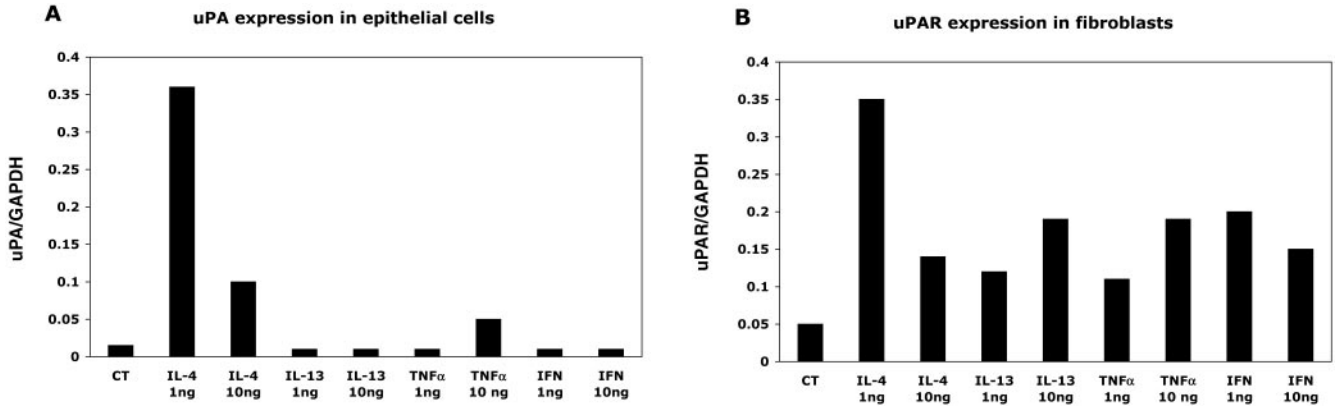

FIGURE 4. Effect of cytokines on fibrinolytic system components expression in conjunctival epithelial cells and fibroblasts. Cells were incubated for 24 hours in the absence (CT) or presence of various concentrations of cytokines before total mRNA extraction and RT-PCR. Results are from a single experiment representative of three separate experiments performed in each cell culture. In conjunctival epithelial cells, uPA expression was increased 20-fold after exposure to 1 ng/mL IL-4 (**A**). In conjunctival fibroblasts, uPAR was increased sevenfold after exposure to 1 ng/mL IL-4 (**B**).

other cytokines (not shown). Urokinase expression was increased 20-fold in conjunctival epithelial cells after exposure to 1 ng/mL IL-4 (Fig. 4A).

Conjunctival fibroblasts showed a very low or absent mRNA signal for uPA and PAI-1, both in nonstimulated and stimulated cultures (not shown). Conversely, uPAR expression increased sevenfold after exposure to 1 ng/mL IL-4 and fourfold after exposure to 10 ng/mL IL-13, 10 ng/mL TNFα, and 1 ng/mL IFNγ (Fig. 4B).

DISCUSSION

Proteolytic mechanisms are thought to have a central role in the organization and remodeling of normal tissues, in wound healing, and in cell recruitment and migration. Cell recruitment and infiltration are multistep processes regulated by cytokines, chemokines, and growth factors through multiple signaling pathways coordinated among different types of cells. Recent evidence has demonstrated the role of the uPA system in cell recruitment and migration through integrated mechanisms involving proteolysis and cell adhesion. These functions hinge on uPA's interaction with its receptor, uPAR, and its inhibitor, PAI-1.[1] Inflammatory cells—in particular, neutrophils and eosinophils— use the plasminogen–plasmin system to transmigrate into tissues,[20,28] suggesting that this system is also involved in ocular inflammatory diseases characterized by an important eosinophil infiltration, such as VKC. In the present study, low levels of plasminogen activators were found in normal tears associated with undetectable levels of its inhibitor, PAI-1. uPA and tPA levels were higher in VKC than in normal tears; however, the uPA level did not correlate with disease severity. Only tear tPA level correlated with the severity of corneal injury in VKC, indicating an activation of the fibrinolytic system. The lack of detection of PAI-1 may be due to its short half-life and high affinity for binding with uPA. Plasma levels of tPA and plasminogen activity were lower in patients with VKC than in the control group. However, these levels are still considered to be within the normal range for the general population. Moreover, these data further support the hypothesis of a local extravascular activation of the fibrinolytic system in the inflammatory phase of VKC.

Immunohistochemistry demonstrated the expression of both uPA and uPAR in conjunctival epithelial and stromal cells of pathologic and non-normal tissues. These cells may be one source of the high levels of activators found in tears. Immunocytochemistry performed on cells collected from tear samples clearly showed that both eosinophils and neutrophils expressed uPA on the cell surface or in the cytoplasm. Similar evidence was found in isolated peripheral blood eosinophils and neutrophils.[20,21] In this last experiment, uPA staining was more evident in granules of quiescent cells, whereas a mild stimulation with PAF induced translocation of uPA from granules to the cell membrane,[21] suggesting that uPA is involved in eosinophil invasiveness.[28] The present study suggests that this system is also activated in ocular allergic inflammation. Inflammatory mediators involved in the allergic cascade[6] may increase the production and release of plasminogen activators and initiate the fibrinolytic process.

The main proteolytic enzyme at work in fibrinolysis is plasmin, which is responsible for the degradation of fibrin. This serine protease is formed from inactive plasminogen mainly by the endogenous activators tPA and uPA. Normal tear fluid contains plasmin at very low concentrations, which are probably needed for normal healing of epithelia.[30–32] Plasmin activity may increase after corneal injury, depending on the severity of the corneal involvement[33] and epithelial defect.[34] This activity was shown to be dependent primarily on a release of tPA and uPA from conjunctival tissues.[35] In an experimental rabbit model, repeated UBV irradiation evoked active uPA in the anterior segment.[36] More recently, uPA has been shown to be upregulated in corneal epithelial cells after mechanical wounding, thus contributing to epithelial cell migration.[37] Recently, the uPA–uPAR complex was shown to anchor to the actin cytoskeleton and to be involved in the migration of corneal fibroblasts.[38]

Regarding the possible role of conjunctival cells in expression and production of fibrinolytic system components during allergic inflammation, conjunctival epithelial cells demonstrated a substantial upregulation of uPA after IL-4 exposure, but only a slight increase of its inhibitor, PAI-1, whereas its receptor, uPAR, remained unchanged. Although statistically significant, the increased production of PAI-1 in response to different cytokines was at very low doses. This was not confirmed by the PCR results at 24 hours, suggesting that the expression of PAI-1 was probably only slightly induced at earlier time points and that this factor is poorly produced by these epithelial cells. In contrast, conjunctival fibroblasts exposed to the same cytokine showed a significant increase of uPAR but not of uPA or PAI-1 expression. Thus, conjunctival cell types may have diverse roles in the production, activation,

and modulation of fibrinolytic cascade components during the allergic reaction.

PAI-1 is thought to be the principal player in fibrinolysis inhibition by activator blockade. Active PAI-1 inactivates tPA and uPA, forming stable complexes with these compounds.[1] The absence of PAI-1 in tears and the finding that the cytokines typically present in allergy, and VKC in particular, slightly increased PAI-1 in epithelial cells and reduced its production in fibroblasts indicates an imbalance between plasminogen activation and inhibition. Reduced inhibition of uPA may result in increased cell proliferation and migration that contributes to the tissue remodeling typical of VKC tissues.[6] A possible activity of urokinase is the regulation of activity and/or the expression of metalloprotease enzymes, participants in extracellular matrix degradation that were shown to be activated in VKC.[25]

In conclusion, these results provide a direct link between the fibrinolytic machinery and the allergic inflammatory response. The key components of this system, uPA and uPAR, were shown to be active during a severe ocular allergic inflammation, to be expressed by conjunctival tissues and inflammatory cells, and to be expressed by conjunctival cells in vitro. Conversely, the chief inhibitor PAI-1 was negligibly expressed in conjunctival tissues. An imbalance of this system may be involved in the complex pathogenesis of VKC.

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