

# Novel transglutaminase inhibitors reverse the inflammation of allergic conjunctivitis

See the related Commentary beginning on page 19.

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Steroidal anti-inflammatory drugs induce proteins that inhibit phospholipase A<sub>2</sub> (PLA<sub>2</sub>), including uteroglobin and lipocortin-1 (annexin I). Uteroglobin and lipocortin-1 retain several conserved sequences. Based on these sequences, several nonapeptides (antiflammins) were synthesized. These nonapeptides were shown to have anti-inflammatory effects *in vitro* and *in vivo*, possibly by inhibiting PLA<sub>2</sub>. Subsequent research showed that PLA<sub>2</sub> is activated by transglutaminase 2 (TGase 2). We hypothesize here that TGase 2 inhibitors may increase the anti-inflammatory efficacy of inhibiting PLA<sub>2</sub> activity. To test this theory, we constructed recombinant peptides containing sequences from pro-elafin (for inhibition of TGase 2), and from lipocortin-1, lipocortin-5, and uteroglobin (for inhibition of PLA<sub>2</sub>). The recombinant peptides, which had dual inhibitory effects on purified TGase 2 and PLA<sub>2</sub>, reversed the inflammation of allergic conjunctivitis to ragweed in a guinea pig model. The present work suggests that novel recombinant peptides may be safe and effective agents for the treatment of various inflammatory diseases.

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## Introduction

Inflammation processes are complex biochemical phenomena that are characterized physiologically in tissues by edema, pain, and leukocyte infiltration (1). Currently, the most effective anti-inflammatory drugs are glucocorticoids. Glucocorticoids induce many proteins, such as lipocortins. Lipocortins are inhibitors of phospholipase A<sub>2</sub> (PLA<sub>2</sub>; International Union of Biochemistry and Molecular Biology enzyme no. 3.1.1.4 [EC 3.1.1.4]) (2, 3). PLA<sub>2</sub> plays a key role in the pathogenesis of allergic conjunctivitis (4). In the inflammation process, PLA<sub>2</sub> hydrolyzes the acyl-ester bond at the *sn*-2 position of phosphoglycerides (5). This is the first rate-limiting step in the eicosanoid cascade, generating arachidonic acid. Arachidonic acid is converted to prostaglandins and leukotrienes that cause inflammation (6). Therefore, an effective treatment of inflammatory diseases is inhibition of PLA<sub>2</sub>, blocking both cyclooxygenase and lipoxygenase pathways in the arachidonic acid cascade (6). Steroidal anti-inflammatory drugs inhibit PLA<sub>2</sub> by inducing PLA<sub>2</sub>-inhibitory proteins, including uteroglobins (a subfamily of secretoglobins) (7) and lipocortin-1 (annexin I) (3, 8).

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**Nonstandard abbreviations used:** phospholipase A<sub>2</sub> (PLA<sub>2</sub>); antiflammin (AF); transglutaminase 2 (TGase 2).

Miele et al. identified a region of sequence similarity between uteroglobin and lipocortin-1 (9). Further, they designed several synthetic peptides corresponding to the region of highest similarity between uteroglobin and lipocortin-1. The most effective anti-inflammatory nonapeptides, termed antiflammins (AFs) by Miele et al. (9), corresponded to uteroglobin residues 39–47 and lipocortin-1 residues 246–254. Both peptides were shown to be PLA<sub>2</sub> inhibitors *in vitro* and were effective in a classic model of acute inflammation in carrageenan-induced rat footpad edema (9).

Interestingly, treatment of purified PLA<sub>2</sub> with transglutaminase 2 (TGase 2; EC 2.3.2.13) strikingly increased PLA<sub>2</sub> activity *in vitro*. TGase 2-catalyzed conformational changes in PLA<sub>2</sub> can be brought about by formation of an intramolecular  $\epsilon$ -( $\gamma$ -glutamyl)-lysine cross-link (10) or by incorporation of polyamines (11). These observations suggest that TGase 2-mediated modification may activate PLA<sub>2</sub> *in vivo*, following an influx of calcium. TGase 2 is a calcium-dependent enzyme that catalyzes the covalent cross-linking of the  $\gamma$ -carboxamide groups of a peptide-bound glutamine residue with the  $\epsilon$ -amino group of a peptide-bound lysine residue (12). Several physiological roles for TGase 2 have been demonstrated, such as wound healing (13), fibrosis (14), apoptosis (15), and matrix formation (16). Increased TGase 2 expression has been reported to occur in many inflammatory diseases, such as celiac disease (17), Crohn disease (18), and sporadic inclusion-body myositis (19). Pathological roles of TGase 2 in those diseases might be associated with activation of PLA<sub>2</sub>.

Therefore we hypothesize here that blocking *both* TGase 2 and PLA<sub>2</sub> activities may ameliorate PLA<sub>2</sub>-medi-

ated inflammation. To test this hypothesis, a series of new recombinant peptides using sequences from AFs and pro-elafin (cementoin) were constructed as competitive inhibitors of PLA<sub>2</sub> and TGase 2, respectively. Cementoin is a good TGase 2 substrate for anchoring pro-elafin onto matrix proteins (20). Further, it has been demonstrated that sequences derived from pro-elafin serve as competitive reversible TGase inhibitors (21). Here we show that recombinant peptides inhibit PLA<sub>2</sub> as well as TGase 2. We also show that the recombinant peptides abolish the TGase 2-catalyzed activation of PLA<sub>2</sub> *in vitro* and have a pronounced anti-inflammatory effect on allergic conjunctivitis *in vivo*.

## Methods

**Construction of synthetic peptides.** Four peptides (A1–A4) were synthesized based on published sequences of AFs from lipocortin-5 (A1), lipocortin-1 (A2), uteroglobins (A3), and the conserved region of AFs (A4) (9, 22). Three peptides (E1–E3) were synthesized based on the pro-elafin sequences (21, 23). Two recombinant peptide sequences (R1 and R2) were constructed by combining AFs and pro-elafin sequences. E1–E3 and R1–R2 peptides encoded putative TGase 2 substrate domains that will compete with other TGase 2 substrates (21, 23). Two synthetic peptide inhibitors of PLA<sub>2</sub> (P1 and P2) were tested for their effectiveness as positive controls (24, 25). All the peptides were synthesized and purified by HPLC, and the compositions and concentrations were determined accurately by amino acid analysis (Peptron Co., Taejeon, Republic of Korea). All peptides were dissolved in a small volume of PBS (6.7 mM phosphate, 150 mM NaCl [pH 7.3]) and stored frozen until used.

**Inhibitory effect of synthetic peptides on PLA<sub>2</sub> *in vitro*.** Using an established assay, we determined the enzymatic activity of PLA<sub>2</sub> by measuring the release of [<sup>14</sup>C]arachidonic acid from 1-acyl-2-[1-<sup>14</sup>C]arachidonic acid-glycerophosphoethanolamine (26). To determine the inhibitory activity, 0.18 U of PLA<sub>2</sub> (bovine pancreas, EC 3.1.1.4; Sigma-Aldrich, St. Louis, Missouri) was preincubated for 15 minutes with the synthetic peptides (1 × 10<sup>-8</sup> M) at 37°C in 60 μl of assay buffer (75 mM Tris-Cl, pH 9.0, 5 mM CaCl<sub>2</sub>, 1 mg/ml fatty acid-free bovine serum albumin) before adding 1-acyl-2-[1-<sup>14</sup>C]arachidonic acid-glycerophosphoethanolamine. 1.0 U of PLA<sub>2</sub> will hydrolyze 1.0 μmol of L-α-phosphatidylcholine to L-α-lysophosphatidylcholine and a fatty acid per min. at pH 8.0 at 37°C. The control was PLA<sub>2</sub> preincubated with buffer. After preincubation, PLA<sub>2</sub> activity was assayed using sonicated liposomes, prepared as described (27). Forty microliters of 1-acyl-2-[1-<sup>14</sup>C]arachidonic acid-glycerophosphoethanolamine (about 2 × 10<sup>5</sup> disintegrations per minute) was added to the preincubated mixture and incubated for 1 hour at 37°C. The reaction was terminated by addition of 0.75 ml of Dole's reagent (78% [vol/vol] *n*-heptane, 20% [vol/vol] propan-2-ol, and 2% [vol/vol] 2 M aqueous H<sub>2</sub>SO<sub>4</sub>). To extract the

liberated [<sup>14</sup>C]arachidonic acid, 0.25 ml of H<sub>2</sub>O was added to the incubated mixture, vortexed, and centrifuged at 1,200 g for 5 minutes. The upper phase was then transferred to a tube that contained 100 mg of silica gel (230–400 mesh; Sigma-Aldrich) and 0.75 ml of *n*-heptane. The sample was vortexed and centrifuged at 1,200 g for 5 minutes. The supernatant was collected for scintillation counting. Values are the means of three determinations (SD < 10%). For studies of the inhibitory peptides, each value is the mean of three separate experiments (SD < 10%).

**Inhibitory effect of synthetic peptides on TGase 2 *in vitro*.** We determined TGase activity by measuring the incorporation of [1,4-<sup>14</sup>C]putrescine into succinylated casein (12). To determine the inhibitory effects of the peptides, we preincubated 1 × 10<sup>-3</sup> U guinea pig liver TGase 2 (EC 2.3.2.13; Sigma-Aldrich) – 1.0 U of which will catalyze the formation of 1.0 μmol of hydroxamate per minute from *N*-α-CBZ-Gln-Gly and hydroxylamine at pH 6.0 at 37°C – for 15 minutes with the synthetic peptides (1 × 10<sup>-8</sup> M) in 0.1 ml of buffer containing 0.1 M Tris-acetate (pH 7.5) at 37°C. We then added the TGase/peptide mixture to 0.5 ml of the substrate solution, containing 0.1 M Tris-acetate (pH 7.5), 1% (wt/vol) succinylated casein, 1 mM EDTA, 10 mM CaCl<sub>2</sub>, 0.5% (wt/vol) lubrol PX, 5 mM DTT, 0.15 M NaCl, and 0.5 mCi [1,4-<sup>14</sup>C] putrescine dihydrochloride (118 Ci/mol; NEN Life Science Product Inc., Boston, Massachusetts, USA). Following incubation at 37°C for 1 hour, the reaction was terminated by addition of 4.5 ml of cold 7.5% (wt/vol) trichloroacetic acid (TCA; 4°C). The TCA-insoluble precipitates were collected onto GF/A (Millipore, Bedford, Massachusetts, USA) glass fiber filters, washed with cold 5% (wt/vol) TCA, dried, and counted. The control was TGase preincubated with buffer only. Values are the means of three determinations.

**Inhibitory effect of synthetic peptides on the activation of PLA<sub>2</sub> by TGase 2 *in vitro*.** The synthetic peptides (1 × 10<sup>-8</sup> M) were preincubated with 1 × 10<sup>-3</sup> U of TGase 2 and 1.8 × 10<sup>-3</sup> U of PLA<sub>2</sub> in 60 μl of assay buffer for 15 minutes at 37°C. A negative control was incubated in the absence of TGase, and TGase incubated in the absence of peptides was used as a positive control. After preincubation, 40 μl of 1-acyl-2-[1-<sup>14</sup>C]arachidonic acid-glycerophosphoethanolamine (about 2 × 10<sup>5</sup> disintegrations per minute) was added to the preincubated mixture, and the assay mixture was incubated for 1 hour at 37°C. The reaction was terminated by addition of 0.75 ml of Dole's reagent, and the released arachidonic acid was determined as described above.

***In vivo* experimental model of allergic conjunctivitis.** Female guinea pigs of the Hartley strain were selected as in previous reports (e.g., ref. 28). Eighty-six female Hartley guinea pigs (200–250 g) were housed in the animal facilities of the Asan Institute for Life Sciences (Republic of Korea), given guinea pig chow and water *ad libitum*, and cared for in accordance with the Declaration of Helsinki and the NIH Guide for the Care and Use of Laboratory Animals (Republic of Korea).

**Table 1**

Scale for 0-to-4+ evaluation of clinical signs in experimental conjunctivitis

Clinical signs	None (0+)	Minimal (1+)	Mild (2+)	Moderate (3+)	Severe (4+)
Conjunctival redness	Absent	Minimal redness	Easily detectable redness	Obvious redness	Diffuse redness
Conjunctival edema	Absent	Minimal edema	Focal-area chemosis	Obvious chemosis	Severe edema

Giant ragweed pollen (*Ambrosia trifida*, 1.0–1.5 mg; Sigma-Aldrich) was delivered once a day into the nostrils and the inferior conjunctival fornices of 84 animals on days 1–5 and 8–12 with a micropipette calibrated to 10  $\mu$ l. On day 15, immunized guinea pigs were divided into 14 groups, with six in each group. Eleven synthetic peptides, including the new recombinants described here, dexamethasone eye drops (Maxidex, 0.1% dexamethasone; Alcon-Couvreur, Puurs, Belgium), and antihistamine eye drops (Livostin, levocabastine; Janssen Pharmaceutica, Beerse, Belgium), were used for 13 groups, and no treatment was used for one group. Normal controls were six nonimmunized, untreated guinea pigs. All immunized guinea pigs were challenged with 1.0–1.5 mg of ragweed-pollen powder delivered to the inferior conjunctival fornix on day 15. All the procedures conformed to the experimental model of allergic conjunctivitis described by Merayo-Lloves et al. (28). All synthetic peptides were dissolved in sterile saline (100  $\mu$ mol/40  $\mu$ l concentration). All peptides and dexamethasone eye solutions were administered 3 hours before the last pollen application on day 15, 10 minutes after pollen application, and four times thereafter at 3-hour intervals.

**Clinical evaluation for anti-inflammatory activity.** Conjunctival edema and redness were judged in room light and under an operating microscope (Carl Zeiss, Oberkochen, Germany). The evaluation was performed in a blinded fashion at 20 minutes after allergen challenge. Each clinical parameter was scored on a scale of 0 to 4+ (0 absent, 1+ minimal, 2+ mild, 3+ moderate, 4+ severe) as described previously (Table 1) (28). Clinical scores of each group (12 eyes per group) were analyzed.

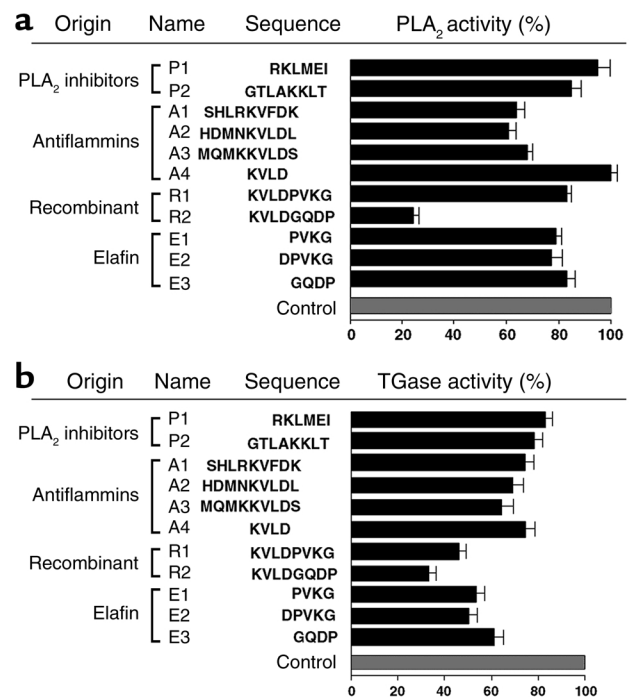
**Histology for anti-inflammatory activity.** The orbits were exenterated after the animals were killed with CO<sub>2</sub> asphyxia, and lower eyelids including inferior fornical conjunctiva were prepared for the light-microscopic examination. The central part of each specimen was cut sagittally and stained with hematoxylin and eosin. The eosinophils in standardized fields of conjunctival epithelium, immediate subepithelium, and stroma were counted in three nonconsecutive fields for each treatment in each experiment (magnification  $\times$ 400; 12 eyes per group).

**Inhibitory effect of synthetic peptides on TGase and PLA<sub>2</sub> activities in vivo.** Inferior fornical and palpebral conjunctiva including conjunctival epithelium and subepithelial stroma were dissected and homogenized by means of a Sonic Dismembrator (Fisher Scientific Co., Pittsburgh, Pennsylvania, USA) in a tenfold volume of ice-cold 0.1 M Tris-acetate (pH 7.5) and 1 mM

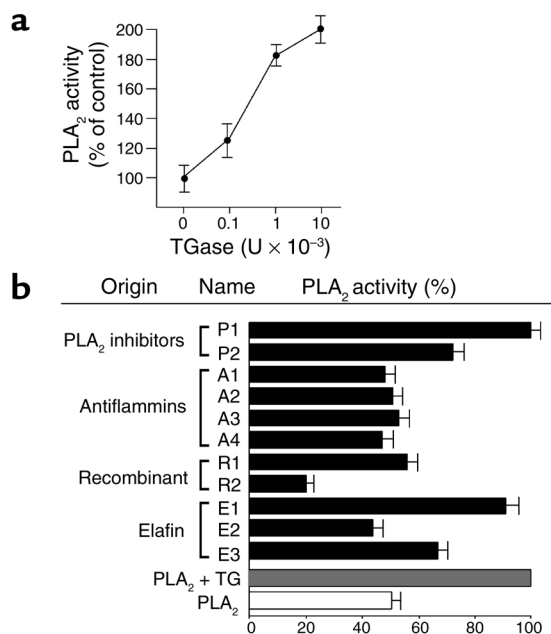
EDTA, containing 1% (vol/vol) Triton X-100 and protease cocktail. The homogenates were centrifuged at 10,000 *g* for 30 minutes at 4°C. The supernatant was used for analysis of TGase and PLA<sub>2</sub> activities. The assay conditions for TGase and PLA<sub>2</sub> were the same as those used for in vitro assays as described above.

## Results

The effect of the 11 synthesized peptides on PLA<sub>2</sub> activity is shown in Figure 1a. The PLA<sub>2</sub> activity in the absence of peptides was assigned a value of 100% (5.57 pmol arachidonyl per hour). AFs (A1–A3) inhibited PLA<sub>2</sub> activity by about 40%, while P1 and P2 did not significantly inhibit it (Figure 1a). Pro-elafin peptides (E1–E3) inhibited PLA<sub>2</sub> activity by as much as 25% (Figure 1a). The recombinant peptide (R2) designed

**Figure 1**

The effects of various peptides on (a) PLA<sub>2</sub> activity and (b) TGase 2 activity. (a) Purified PLA<sub>2</sub> was preincubated with the peptides for 15 minutes before assay. The recombinant peptide R2 showed the best inhibitory effect on PLA<sub>2</sub> activity, although most AFs (A1–A3) inhibited PLA<sub>2</sub> activity to some extent. (b) Purified TGase 2 was preincubated with the peptides for 15 minutes before assay. The recombinant peptide R2 showed the best inhibitory effect on TGase 2 activity, although all pro-elafin peptides (E1–E3) inhibited TGase 2 activity to some extent. Data are presented as mean  $\pm$  SD from three separate experiments.



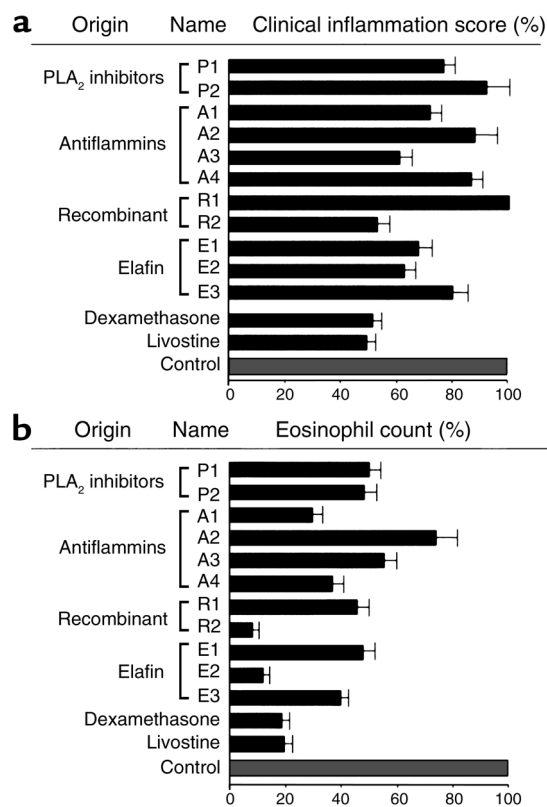
**Figure 2**  
The effects of various peptides on activation of PLA<sub>2</sub> by TGase 2. (a) Purified PLA<sub>2</sub> was preincubated with different amounts of TGase 2 for 15 minutes before assay. PLA<sub>2</sub> activity was increased by incubation with TGase 2 in a dose-dependent manner. Data are presented as mean ± SD from three repeats of each TGase 2 concentration. (b) Purified PLA<sub>2</sub> was preincubated with the peptides and 1 × 10<sup>-3</sup> U TGase 2 for 15 minutes before assay. The recombinant peptide R2 markedly decreased PLA<sub>2</sub> activity, although most AFs (A1–A3) and E3 inhibited PLA<sub>2</sub> activity. Data are presented as mean ± SD from three separate experiments.

from the core domains of AF and pro-elafin inhibited about 75% of PLA<sub>2</sub> activity (Figure 1a). To determine the inhibitory effect of the synthetic peptides on purified TGase 2, the enzyme was preincubated with synthetic peptides in vitro at room temperature for 15 minutes before the TGase assay was started. The activity of TGase 2 incubated without peptides was assigned a value of 100% (4.89 pmol putrescine per hour) (Figure 1b). Pro-elafin peptides (E1–E3) inhibited TGase 2 by 40–50% (Figure 1b). P1, P2, and A1–A4 inhibited TGase 2 by about 20–40% (Figure 1b). R2 inhibited TGase 2 activity by about 70% and PLA<sub>2</sub> activity by about 75%. R1 inhibited TGase 2 activity by about 55%, although it did not significantly inhibit PLA<sub>2</sub> activity (Figure 1a).

PLA<sub>2</sub> activity was increased by incubation with TGase 2 in vitro as previously reported (19). As the amount of TGase 2 in the mixture was increased, PLA<sub>2</sub> activity increased up to twofold (Figure 2a). The increased PLA<sub>2</sub> activity by TGase 2 was assigned a value of 100% (11.2 pmol arachidonyl per hour). R2 inhibited about 80% of the total PLA<sub>2</sub> activity increased by TGase 2, while AFs (A1–A4) inhibited about 50% of the total PLA<sub>2</sub> activity increased by TGase 2. The effect of R2 might be due to a combined inhibition of TGase 2 and PLA<sub>2</sub> activities (Figure 2b). As shown in Figures 1 and 2, R2 signifi-

cantly reduced the PLA<sub>2</sub> and TGase 2 activities in comparison with the peptides known to be PLA<sub>2</sub> inhibitors.

The effect of the synthetic peptides on the guinea pig allergic conjunctivitis model was determined. In addition to the same set of peptides used in Figures 1 and 2, dexamethasone and Livostin were employed as positive controls. Clinical evaluation of anti-inflammatory activity was based on the degree of conjunctival edema and redness (see Methods). AFs (A1–A4) and pro-elafin peptides (E1–E3) reduced the clinical inflammatory scores by 10–40% (Figure 3a). R2 reduced the clinical inflammation score by about 50%. This reduction is almost the same as that noted with dexamethasone and Livostin eye drops (Figure 3a; Table 2). We performed histological evaluation by counting eosinophils in the standardized fields of conjunctival epithelium and subepithelial stroma. Interestingly, most peptides, including AFs, Rs, and Es, reduced eosinophil infiltration (Figure 3b). R2 and E2 provided the best protection against inflammation (90% and 85% reduction, respectively) – even better than that shown by



**Figure 3**  
The effects of various peptides on the conjunctival allergic reaction in guinea pigs. (a) The recombinant peptide R2 reduced clinical allergic signs as much as dexamethasone and Livostin eye drops did. The data are the means of 12 eyes in each group. *P* values are in Table 2. (b) Eosinophils in the standardized fields of conjunctival epithelium and subepithelial stroma were counted. The recombinant peptide R2 and one peptide of the elafin series (E2) reduced eosinophil infiltration better than dexamethasone and Livostin did. Data are presented as mean of 12 eyes in each group. *P* values are in the Table 3.

**Table 2**

Statistical evaluation of clinical scores shown in Figure 3a

Name	Sequence	P value compared with:		
		No treatment	Dexamethasone	Livostin
P1	RKLMEI	0.009	0.001	0.003
P2	GTLAKKLT	0.279	0.000	0.000
A1	SHLRKVFDK	0.003	0.007	0.009
A2	HDMNKVLDL	0.382	0.002	0.003
A3	MQMKKVLDS	0.000	0.089	0.043
A4	KVLD	0.082	0.000	0.000
R1	KVLDPVKG	0.965	0.000	0.000
R2	KVLDGQDP	0.000	0.796	0.515
E1	PVKG	0.001	0.017	0.012
E2	DPVKG	0.000	0.050	0.039
E3	GQDP	0.016	0.000	0.002
Dexamethasone		0.000		

n = 12; Mann-Whitney test.

dexamethasone and Livostin (Figure 3b; Table 3). Figure 4 shows the histochemical staining pattern of eosinophil infiltration and epithelial edema after treatment with R2, E2, dexamethasone, and Livostin. The inhibitory effect of synthetic peptides on TGase and PLA<sub>2</sub> activities in vivo is shown in Figure 5. The activities of TGase and PLA<sub>2</sub> in the control were 43.2 pmol/mg/h and 26.4 nmol/mg/h, respectively. After induction of allergy, PLA<sub>2</sub> activity increased fivefold, and TGase activity increased twofold. Treatment with recombinant peptides R2 and E2 reduced activities of TGase and PLA<sub>2</sub> (Figure 5, a and b). Dexamethasone and Livostin treatment resulted in a decrease of PLA<sub>2</sub> activity, but not of TGase activity. We have repeated the in vivo experiments (Figures 3–5) and have obtained the same pattern of results (data not shown).

## Discussion

Numerous studies have revealed that steroidal anti-inflammatory effects are mediated by the induction of lipocortins (3) and uteroglobins (8), which contain a conserved nonapeptide (AF). Although Hope et al. (29) reported that AFs have no inhibitory effect on PLA<sub>2</sub> and no anti-inflammatory activity in vivo, we confirmed the findings of others (9, 30, 31) that the AFs do indeed have inhibitory activity on PLA<sub>2</sub> in vitro and have anti-inflammatory effects on allergic conjunctivitis in vivo (Figure 1). We also confirmed the previous finding (10) that TGase 2 increases PLA<sub>2</sub> activity in vitro (Figure 2). Therefore, we hypothesized that blocking both TGase 2 and PLA<sub>2</sub> activities might provide maximal protection against inflammation. To test this hypothesis, we constructed recombinant peptides from human lipocortin-1 and pro-elafin as competitive reversible TGase inhibitors (21). These peptides contain Q and/or K residue substrates of the intact proteins. The recombinant peptide R2 showed the greatest inhibitory effect both on TGase 2 and on PLA<sub>2</sub> activities in vitro, accompanied by the best anti-inflammatory score in vivo.

Interestingly, AF-derived KVLD itself did not inhibit PLA<sub>2</sub> activity unless extra amino acids were added to the sequence (Figure 1a). Further, we found that, in addition to inhibiting PLA<sub>2</sub>, AFs also had some inhibitory activity on TGase 2 in vitro (Figure 1b). This inhibition might be due to the lysine residue of the AFs acting as an acyl acceptor during TGase 2 catalysis and thereby competing with TGase 2 substrates. Analysis of AF peptide sequences showed that AFs contain KVLD whereas pro-elafin contains a similar but reversed sequence (DPVK) (23). Cementoin (see below) is known to be a strong TGase substrate. Therefore, AFs containing the KVLD sequence may be dual-functional competitive inhibitors of TGase 2 and PLA<sub>2</sub> in vivo. R2 contains glutamine and lysine residues as acyl donor and acceptor for TGase catalysis, which might generate synergistic inhibitory effects on TGase 2. PLA<sub>2</sub> is a large superfamily of distinct enzymes whose products are important for signaling processes, eicosanoid and platelet-activating factor formation, membrane remodeling, and general lipid metabolism. The PLA<sub>2</sub> family is classified into ten groups, including calcium-dependent PLA<sub>2</sub> (sPLA<sub>2</sub>) and calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>), by, e.g., size, secretion characteristics, disulfide content, and molecular characteristics. It should be noted that we tested only bovine pancreas PLA<sub>2</sub> (sPLA<sub>2</sub> group 1) in the in vitro inhibition assay, as did others (9, 22). At present, we cannot predict what effects our peptides will have on other members of the PLA<sub>2</sub> family.

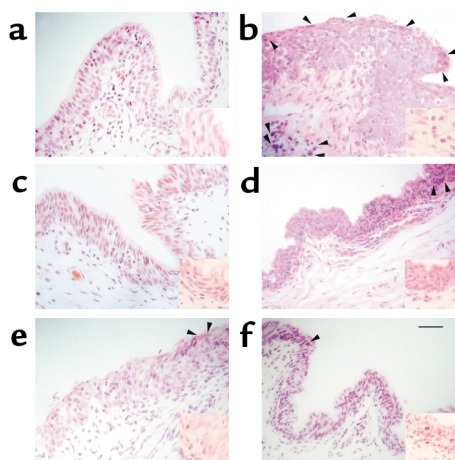
The structure of pro-elafin consists of two functionally unique domains; one is a TGase substrate (cementoin, amino acids 23–60) and the other is a proteinase inhibitor (amino acids 61–117). The cementoin domain consists of five repeats of VKGQDP that serve as both acyl donor (Q) and acceptor (K) for TGase. This repeat was confirmed to be a very good TGase substrate (19, 20). We found similar repeats, such as MKGQDS in guinea pig seminal vesicle protein (SVP-1) (32) and the reversed sequence of EPCQPKVP in human small proline-rich protein (SPR) (33), that are known to be

**Table 3**

Statistical evaluation of eosinophil count shown in Figure 3b

Name	Sequence	P value compared with:		
		No treatment	Dexamethasone	Livostin
P1	RKLMEI	0.002	0.000	0.000
P2	GTLAKKLT	0.004	0.000	0.000
A1	SHLRKVFDK	0.000	0.004	0.006
A2	HDMNKVLDL	0.161	0.000	0.000
A3	MQMKKVLDS	0.007	0.000	0.000
A4	KVLD	0.001	0.028	0.045
R1	KVLDPVKG	0.002	0.001	0.002
R2	KVLDGQDP	0.000	0.001 <sup>A</sup>	0.002 <sup>A</sup>
E1	PVKG	0.002	0.000	0.000
E2	DPVKG	0.000	0.028	0.060
E3	GQDP 0.001	0.007	0.000	
Dexamethasone		0.000		

n = 12; Mann-Whitney test. <sup>A</sup>Better than dexamethasone and Livostin.



**Figure 4**  
Hematoxylin-and-eosin staining of inferior palpebral and fornicial conjunctiva of guinea pigs. (a) Normal control. (b) Marked eosinophilic infiltration (arrowheads) and epithelial edema induced by ragweed pollen. (c) The recombinant peptide R2 markedly reduced eosinophilic infiltration and epithelial edema. (d) E2 showed mild epithelial edema with few eosinophils. (e and f) Dexamethasone and Livostin eye drops showed minimal epithelial edema with few eosinophils. Scale bar, 100  $\mu$ m; insets show doubled magnification.

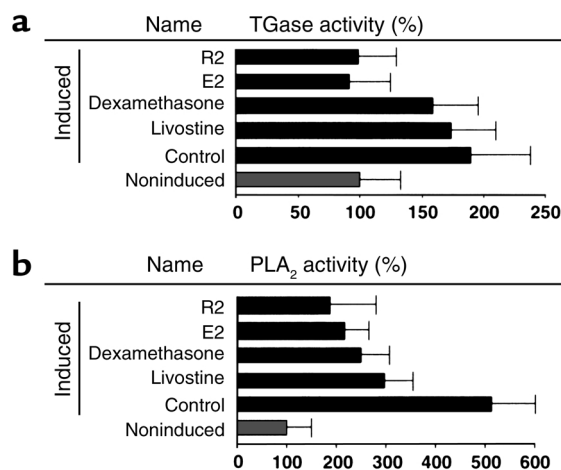
good TGase substrates. Therefore, we adopted the sequences of PVKG and GQDP from cementoin to construct recombinant peptides R1 and R2 using the KVLD core sequence of AFs. In accord with our hypothesis, the recombinant peptide containing GQDP (R2) resulted in the best TGase inhibition *in vitro* and the best anti-inflammatory score *in vivo*.

TGase 2 is involved in regulating the physiological roles of pro-elafin, uteroglobin, and lipocortin (34–40). Pro-elafin was first discovered in keratinocytes from hyperproliferative human epidermis, such as psoriatic epidermis. However, subsequent research revealed that pro-elafin is a cornified cell envelope component in normal skin (34). Once inflammation activates monocytes (polymorphonuclear cells), an active form of elafin (amino acids 61–117) is released by proteolysis and protects tissues from damage by elastase and proteinase-3 (23). The cementoin peptides remaining in the matrix may have anti-inflammatory effects via competing TGase substrates. The short peptides GQDP and PVKG, derived from cementoin, have previously been demonstrated to be inhibitors of cornified cell envelope formation (21). Uteroglobin was first discovered as a progesterone-induced protein that suppresses sperm antigenicity (35). TGase is involved as a key modulator in modifying the surface of spermatozoa with uteroglobin (36). Therefore, uteroglobin can compete with other TGase substrates, such as PLA<sub>2</sub>. Lipocortin-1 (annexin I) was discovered as a major glucocorticoid-induced protein (37). Most lipocortins behave as extrinsic membrane proteins, which bind reversibly to phospholipid membranes in a manner that depends on calcium ions. Also, during epithelial cell differentiation, annexin I (lipocortin-1) becomes

incorporated into the cornified cell envelope via cross-linking by TGase and cannot be extracted by SDS (38). In buccal epithelium, annexin I constitutes about 10% of the total barrier envelope proteins, as established by protein sequencing (39). A recent study showed that expression of annexin I is increased at the border between involved and noninvolved skin in psoriasis vulgaris (40). Thus, annexin I itself is involved in the barrier formation as a major building block and possibly also contains an anti-inflammatory function.

Many reports have shown an increase of TGase activity in diseases associated with inflammation. These diseases include celiac disease (17), Crohn disease (18), and sporadic inclusion-body myositis (19). An increase of TGase 2 activity in these diseases might be associated with important physiological regulation such as activation of PLA<sub>2</sub>. However, the mechanism for aberrant TGase 2 expression is not yet clear. In the inflammatory region, local cytokine levels are changed. Eosinophils are not only the source of cytotoxic and proinflammatory mediators but can also generate cytokines and growth factors, including IL-4, IL-5, and IL-10 as Th2 cytokines, and IFN- $\gamma$  as a Th1 cytokine (41). Inflammatory cytokines such as IFN- $\gamma$  can increase the expression of TGase 2 in small-intestinal cells (42). Consequently, an increase of TGase 2 may cause stimulation of PLA<sub>2</sub> activity. In addition to PLA<sub>2</sub> activation, TGase 2 is also involved in the adhesion and migration of monocytes (43), which suggests that TGase 2 may accelerate neutrophil adhesion to endothelial cells in the inflammatory lesion. Therefore, a TGase 2 inhibitor might have dual anti-inflammatory properties.

The mucous membrane of the conjunctiva in the ocular surface is continually exposed to a variety of antigens.



**Figure 5**  
The effects of synthetic peptides, dexamethasone, and Livostin on (a) TGase activity and (b) PLA<sub>2</sub> activity in the conjunctiva. After induction of allergy, PLA<sub>2</sub> activity is increased fivefold, and TGase activity is increased twofold. (a) TGase activity is decreased by treatment of R2 and E2. Data are presented as mean  $\pm$  SD from three samples. (b) R2 and E2 markedly decrease PLA<sub>2</sub> activity. These peptides are as effective as dexamethasone and Livostin. Data are presented as mean  $\pm$  SD from three samples.

In sensitized individuals, pollen, plant, house-dust mite, or animal-dander allergens dissolved in the tear film cross the conjunctival epithelium and activate substantia propria mast cells, leading to inflammation in conjunctiva, i.e., to allergic conjunctivitis (44, 45). The experimental guinea pig allergy model closely resembles human allergic conjunctivitis. The guinea pig conjunctiva is more similar to that of the human than to that of the rat or mouse (28). Short ragweed pollen was used as an antigen to develop conjunctivitis, because it is a “natural allergen” that induces hay fever conjunctivitis in susceptible humans. The pollen was delivered to the ocular and nasal mucosa in a powder form instead of as an injected solution to mimic more closely the human clinical picture found in hay fever rhinoconjunctivitis (28). Toxic eosinophil proteins are present in conjunctiva, cornea, tears, and contact lenses of patients suffering from ocular allergy, suggesting an effector role for the eosinophil in the ophthalmic manifestations of atopy. The present study showed the same trend, with clinical appearance and infiltration of eosinophils. Thus, histological evaluation provided a means for evaluating the effectiveness of the synthesized peptides (Figure 3). AFs and novel recombinant peptides showed antiallergic effects similar to the in vitro inhibition of TGase 2 and PLA<sub>2</sub> activity of those peptides. The recombinant peptide R2 was as effective as dexamethasone and antihistamine eye drops. Interestingly, R2 and E2 reduced eosinophil infiltration (Figures 3b and 4). This pattern was associated with inhibition of TGase 2 activity measured in vitro (Figure 1b) and in vivo (Figure 5). The in vivo results (Figure 5) are consistent with the in vivo clinical inflammation score and eosinophil count (Figure 3), as well as with the in vitro PLA<sub>2</sub> inhibitory effect of E2 and R2 (Figure 2b).

R2 was more effective than dexamethasone and antihistamine eye drops in lowering the histological scores, as judged by counting of infiltrated eosinophils. The eosinophil infiltration was almost completely blocked by the recombinant peptide R2 (i.e., the score was similar to that of untreated controls). The strong protective effect of R2 may be due to a synergistic inhibitory effect on PLA<sub>2</sub> and TGase 2. Recently other possible physiological actions of AFs in inflammation have been discussed. Topical pretreatment with AF-2 reduces plasma leakage, cell influx, edema, and leukotriene B<sub>4</sub> levels in acute inflammatory reaction induced by the topical application of phorbol ester (TPA, 12-O-tetradecanoylphorbol 13-acetate), but not in that induced by topical application of arachidonic acid. This suggests that AFs may affect arachidonic acid mobilization and/or arachidonic acid metabolism by 5-lipoxygenase (46). AFs have little effect on LPS-stimulated expression of E-selectin and ICAM-1 on human leukocytes and human coronary artery endothelial cells (HCAECs). Treatment of HCAEC cultures with AFs did not decrease neutrophil (polymorphonuclear cell) binding to endothelial cells. However, preincubation of polymorphonuclear cells

with AFs markedly decreased their adhesion to LPS-activated HCAECs. This suggests that AFs may block leukocyte trafficking in inflammation (47). AF-2 also reduced the adhesion of human promyelocytic leukemia cells to endothelial cells (48).

Although they have potent anti-inflammatory effects, steroids have harmful side effects when excessive doses are prescribed. Therefore, novel recombinant anti-inflammatory peptides theoretically have enormous possibilities as potent and safe antiallergic and anti-inflammatory drugs. In conclusion, we have shown that rationally designed recombinant peptides that coinhibit both PLA<sub>2</sub> and TGase 2 are potent anti-inflammatory agents, which in some cases are as effective as dexamethasone and Livostin in reducing symptoms of allergic conjunctivitis.

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