Suppression of macrophage infiltration into the conjunctiva by clodronate liposomes in experimental immune-mediated blepharoconjunctivitis

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Abstract

Macrophages infiltrate the conjunctiva in severe cases of allergic conjunctivitis (AC) such as atopic keratoconjunctivitis (AKC). We established experimental immune-mediated blepharoconjunctivitis (EC) in Brown Norway (BN) rats as a model for severe types of AC. We investigated whether macrophage infiltration in the conjunctiva in this EC model is inhibited by clodronate liposomes (CL₂MDP-lip). The numbers of ED1-positive but not ED2-positive macrophages in the conjunctivas were increased by the induction of EC. Subconjunctival injection of CL₂MDP-lip decreased the number of ED2-positive but not ED1-positive macrophages in the conjunctivas of naive rats. CL₂MDP-lip did not affect macrophages in the spleen. Subconjunctival injection of CL₂MDP-lip into EC-developing BN rats decreased the number of ED2-positive macrophages at all the time points. ED1-positive cell infiltration was inhibited when treatment was administered just prior to OVA challenge. Intravenous injection of CL₂MDP-lip decreased the number of ED2-positive cells in the conjunctiva. Thus, we conclude that CL₂MDP-lip inhibits infiltration of macrophages into the conjunctiva within 24 h of antigen challenge.

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1. Introduction

Macrophages infiltrate the conjunctiva of various acute and chronic conjunctival diseases, including ocular cicatricial pemphigoid (OCP) (Foster and Sainz De La Maza, 2004). Increased expression of macrophage migration inhibitory factor (MIF), which induces accumulation of macrophages in the tissue, was demonstrated in the conjunctiva of OCP (Razzaque et al.,

2004). In addition, CD68 positive macrophages produce chemokines in the conjunctiva of patients with active vernal keratoconjunctivitis (VKC) (El-Asrar et al., 2002). Thus, macrophages could play a role in maintaining the progression of conjunctival disease processes.

In allergic conjunctivitis (AC), eosinophils and mast cells (Church and McGill, 2002) are major effector cells. Macrophages have also been identified in the conjunctiva in severe kinds of AC, such as atopic keratoconjunctivitis (AKC) (Foster et al., 1991). Infiltrating macrophages reportedly produce growth factors (Leonardi et al., 2000), and are therefore probably involved in the formation of giant papillae. However, the function of conjunctival macrophages in AKC patients is still unclear.

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We established experimental immune-mediated blepharoconjunctivitis (EC) in Brown Norway (BN) rats as a model for severe types of AC (Iwamoto et al., 2000; Yoshida et al., 1999). Eosinophils, macrophages and T cells infiltrate the conjunctiva in this EC model (Fukushima et al., 2003). Macrophages and T cells are abundant in the conjunctivas of patients with AKC (Foster et al., 1991), and the conjunctival cytokine profile (IFN- γ > IL-4) in the EC model (Fukushima et al., 2003) is quite similar to that in AKC patients (Broekhuysen et al., 1997). Thus, this EC model appears to be an appropriate model for AKC (Fukushima et al., 2003).

Clodronate liposomes (CL₂MDP-lip) have recently been shown to deplete macrophages in macrophage-implicated experimental disease models (Metz et al., 1997; Polfliet et al., 2002; Sakurai et al., 2003). Here, we investigated the inhibitory effects of CL₂MDP-lip on infiltration of macrophages into the conjunctiva in EC, to elucidate the role of macrophages in the initiation and maintenance of conjunctival inflammation.

2. Materials and methods

2.1. Animals

Male 8–12-week-old BN rats (Clea Japan, Tokyo, Japan) were maintained in a pathogen-free animal facility of the Kochi Medical School. All animal procedures conformed to the ARVO Resolution on Use of Animals in Research.

2.2. Preparation of CL₂MDP-lip

The method for preparation of CL₂MDP is published elsewhere (Van Rooijen and Sanders, 1994). CL₂MDP was a gift from Roche Diagnostics GmbH, Mannheim, Germany. Phosphatidylcholine (LIPOID E PC) was obtained from Lipoid GmbH, Ludwigshafen, Germany. Cholesterol was purchased from Sigma Chemical Co. (USA). CL₂MDP-lip was prepared by dissolving 1,2 dioleoyl-*sn*-glycero-3-phosphocholine and cholesterol in a mixture of methanol and chloroform (1:1). After low-vacuum rotary evaporation at 37 °C to remove the organic phase, the lipids were mixed with clodronic acid (1.9 g) dissolved in phosphate-buffered saline (0.6 M). The lipids sat at room temperature for 2 h in a nitrogen-purged chamber to induce liposome swelling. Liposomes were resuspended with water by sonication at room temperature for 3 min. The resultant liposomes were washed at 10,000 × *g* to 100,000 × *g* in an ultracentrifuge twice for 30 min at 16 °C. The milky liposomes were removed gently with a pipette, resuspended in 4 ml of sterilized PBS, and stored in a nitrogen chamber for up

to 1 week. Lipids were mixed with sterilized PBS to generate the PBS-liposomes.

2.3. Antibodies for immunohistochemistry

Anti-rat RT1B (OX-6) Ab was purchased from BD Pharmingen, San Diego, CA, USA. The target cells for OX-6 are MHC class II positive cells. Mouse anti-rat CD68 (ED1) (Dijkstra et al., 1985; van Rees et al., 1985) was purchased from Serotec, Kidlington, Oxford, UK. ED2 (Dijkstra et al., 1985; van Rees et al., 1985), which reacts with a membrane antigen (175, 160, and 95 kDa) on resident rat macrophages, was purchased from BMA Biomedicals AG, Augst, Switzerland. Anti-major basic protein (MBP) was obtained from Biodesign Int., Saco, ME, USA. MBP, which is released from eosinophils, is a molecule which induces tissue damage. Biotinylated rabbit anti-mouse immunoglobulins (RAMG) were obtained from Dako, Copenhagen, Denmark.

2.4. Establishment of OVA-specific T-cell lines

The detailed method is described elsewhere (Fukushima et al., 2003), but briefly, ovalbumin (OVA grade V, Seikagaku Co., Tokyo, Japan) primed lymph node cells from BN rats immunized with OVA in complete Freund's adjuvant were stimulated repeatedly in vitro with OVA. Three days after the second stimulation, living cells were harvested by gradient centrifugation (Lymphocyte Separation Medium, Organon Teknika Corporation, West Chester, PA) and used for transfer. Flow cytometric analysis confirmed that the cells were CD3⁺, CD4⁺ and CD8[−] (data not shown).

2.5. Induction and evaluation of EC

EC was induced by adoptive transfer of T cell line cells (Fukushima et al., 2003). In brief, OVA-specific T cells (5 × 10⁶/rat) were injected intravenously into syngeneic recipient rats. Four days later, they were challenged with OVA. The rats were sacrificed 24 h after OVA challenge and their eyes including conjunctivas were harvested for immunohistochemistry.

2.6. Treatment protocol with CL₂MDP-lip

CL₂MDP-lip or PBS as a control was injected either subconjunctivally or intravenously. Naive rats were injected with 20 μ l of CL₂MDP-lip or PBS subconjunctivally; 24, 48 and 72 h after injection, conjunctivas were harvested for immunohistochemistry (*n* = 6 per group). EC-developing rats were injected subconjunctivally 24 and 48 h before OVA challenge or just prior to OVA challenge with 20 μ l of CL₂MDP-lip or PBS (*n* = 12 per group). Intravenous injection of CL₂MDP-lip or PBS

Fig. 1. Subconjunctival injection of CL₂MDP-lip decreased conjunctival macrophages but not splenic macrophages in naive BN rats. Naive BN rats were subconjunctivally injected with CL₂MDP-lip. 24, 48 or 72 h after injection, conjunctivas were harvested for detection of ED1- and ED2-positive cells by immunohistochemistry. (A) Photographs of sections of conjunctiva. Injection of CL₂MDP-lip decreased ED2- but not ED1-positive cells in the conjunctiva at all the time points. $\times 240$. (B) Cell counting of conjunctival macrophages. The number of ED2-positive cells was significantly decreased by subconjunctival injection of CL₂MDP-lip. $**p < 0.01$. (C) Photographs of sections of spleen. Subconjunctival injection of CL₂MDP-lip did not affect splenic macrophages. $\times 240$. (D) Cell counting of splenic macrophages. The number of splenic macrophages did not differ significantly between the groups.

(2 ml of each) was performed at the time of Ag challenge ($n = 10$ per group).

2.7. Immunohistochemistry

Sections for immunohistochemistry were prepared according to Kawamoto's method (Kawamoto and Shimizu, 2000). Briefly, the eyes were immediately frozen in hexane cooled with dry-ice and immersed in 3% carboxymethyl cellulose (CMC) gel, and then placed in cooled hexane again until the CMC gel was completely frozen. After trimming to the central horizontal section including the optic nerve, the surface of the CMC block was covered with a polyvinylidene chloride film (Asahikasei Kogyo, Tokyo, Japan) pre-coated with

synthetic rubber cement (Cryoglu type 1, Finetec Co., LTD, Tokyo, Japan). Sections 4- μ m thick were cut with a cryomicrotome. The films with the sections were mounted on cooled slide glasses with double-sided adhesive tape (Nitoms, Inc., Tokyo, Japan). All samples were fixed for 5 min in cooled methanol, and then washed with 0.01 M PBS, pH 7.4. Endogenous peroxidase activity was inhibited with 0.1% NaN₃ and 0.3% H₂O₂ in distilled water for 10 min at room temperature. Samples were first exposed to Abs for 30 min and then to biotinylated RAMG for 1 h. All slides were revealed using an Avidin-Biotin-Complex kit (Vector laboratories Inc., USA), and then developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO).

Fig. 1 (continued)

2.8. Cell count and statistical analysis

Infiltrating cells in the conjunctiva were counted throughout sections. ED1- and ED2-positive cells in the periaarteriolar lymphoid sheath close to the germinal center of the spleen were counted per field. Three different sections per sample were counted. The data are presented as averages of different samples. Statistical comparisons of the number of Ab-stained cells were performed with the Mann–Whitney *U*-test.

3. Results

3.1. Subconjunctival injection of CL₂MDP-lip decreases ED2-positive cells in the conjunctiva of naive BN rats

First, to examine the effects of CL₂MDP-lip on macrophages in the conjunctiva, we injected CL₂MDP-lip subconjunctivally into naive BN rats and harvested

eyes at different time points after injection. Up to 72 h after injection, the number of ED2-positive cells was significantly less in the conjunctiva of rats treated with CL₂MDP-lip compared to the control group (Fig. 1A,B). In contrast, the number of ED1-positive cells was similar in the conjunctiva of both groups (Fig. 1A,B). To examine whether subconjunctival injection of CL₂MDP-lip induces local or systemic effects, macrophage numbers in spleens were evaluated by immunohistochemistry. The number of macrophages in spleens did not differ in any groups at any of the time points (Fig. 1C,D). Thus, subconjunctival injection of CL₂MDP-lip selectively decreases the number of conjunctival macrophages, especially ED2-positive cells.

3.2. ED1- but not ED2-positive cells are increased in the conjunctiva by EC induction

To examine the phenotypes of infiltrating macrophages, we induced EC by transfer of OVA-specific T cells. EC induction increased the numbers of ED1- but

not ED2-positive cells compared to the number in naive rats (Fig. 2A). The EC-induced increase in ED1-positive cells was statistically significant, while the number of ED2-positive cells was not affected (Fig. 2B).

3.3. Subconjunctival injection of CL₂MDP-lip decreases ED2-positive cells in the conjunctiva of rats developing EC

To investigate whether CL₂MDP-lip decreases the number of macrophages in the conjunctiva of rats with EC, CL₂MDP-lip was subconjunctivally injected into EC-developing rats at different time points prior to Ag challenge. CL₂MDP-lip significantly decreased the number of ED2-positive cells in the conjunctiva compared to the control group at all time points (Fig. 3B,E). However, the number of infiltrating ED1-positive cells was inhibited only when CL₂MDP-lip was subconjunctivally injected just prior to Ag challenge (Fig. 3A,E). Similarly, the reduction in OX-6-positive cells was observed only when the treatment was

performed just prior to Ag challenge (Fig. 3C,E). The number of MBP-positive cells in the conjunctiva was increased when the treatment was performed 24 h before or just prior to OVA challenge (Fig. 3D,E). Effects of CL₂MDP-lip on mast cells were little, if any (data not shown). Infiltration of inflammatory cells was greater in rats injected just prior to OVA challenge (Fig. 3A–E), compared to injections performed 24 or 48 h prior to OVA challenge. We believe the high permeability of OVA in the conjunctival substantial tissues accounts for this observation.

3.4. Systemic CL₂MDP-lip treatment decreases the number of infiltrating macrophages in the conjunctiva

Finally, we examined the effects of systemic CL₂MDP-lip treatment on macrophage infiltration into the conjunctiva. Intravenous injection of CL₂MDP-lip at the time of OVA challenge decreased the number of ED2-positive cells compared with the control (Fig. 4A,B). The numbers of ED1-positive and OX6-positive cells were lower, but not significantly, than in the control group (Fig. 4A,B). The number of MBP-positive cells was not affected by treatment (Fig. 4A,B). The number of ED1- and ED2-positive cells in the spleen was decreased by CL₂MDP-lip treatment (Fig. 4C,D).

Fig. 2. The number of ED1- but not ED2-positive cells increased in the conjunctiva after induction of EC. OVA-specific T cells were transferred into BN rats. Four days later, the rats were challenged with OVA. Conjunctivas were harvested for immunohistochemical analysis 24 h after OVA challenge and compared to conjunctivas from naive rats. (A) Photographs of ED1- and ED2-positive cells in the conjunctiva. $\times 240$. The number of ED1-positive cells was increased by the induction of EC. The number of ED2-positive cells was similar between the two groups. (B) Cell counting of ED1- and ED2-positive cells in the conjunctiva. EC induction significantly increased the number of ED1-positive cells but did not change the number of ED2-positive cells. ** $p < 0.01$.

4. Discussion

Mast cells and eosinophils are major effector cells in AC (Church and McGill, 2002), but infiltration of macrophages also occurs in the conjunctiva, especially in severe types of AC such as AKC (Foster et al., 1991; Leonardi et al., 2000). The role of macrophages in the development of AC has not yet been extensively investigated. In our previous experiments using EC as a model for AKC (Fukushima et al., 2003), we demonstrated that macrophages infiltrate into the conjunctiva and residential macrophages express MHC class II molecules (submitted for publication). Therefore, we speculate that macrophages might be important in the development of EC. In this study, we investigated first whether conjunctival macrophages are functional APC by depleting residential macrophages by injecting CL₂MDP-lip subconjunctivally 24 and 48 h prior to OVA challenge. Second, to determine if CL₂MDP-lip inhibits infiltration of macrophages into the conjunctiva, we injected CL₂MDP-lip subconjunctivally or intravenously just prior to OVA challenge.

The inhibitory effect of CL₂MDP-lip on ED2-positive cells (ED1-positive cells were unaffected) persisted in naive rats for at least 72 h in the conjunctiva

Fig. 3 (continued)

(Fig. 1). Treatment of EC-developing rats with CL₂MDP-lip at 24 or 48 h prior to Ag challenge did not decrease the number of ED1-positive cells, but decreased the number of ED2-positive cells in the conjunctiva (Fig. 3). These data suggest that ED2-positive cells might not serve as APC in the conjunctiva, although residual ED2-positive cells not depleted by CL₂MDP-lip might have induced an allergic response. It is still unknown whether ED1-positive cells are APC.

ED1-positive cells are reportedly the infiltrating macrophages, while ED2-positive cells appear to be the residential macrophages (Dijkstra et al., 1985; van Rees et al., 1985). In addition, the precursors which do not phagocytize (directly after leaving the bone marrow) or phagocytize little (shortly before differentiation into mature macrophages) are positive for ED1, but not ED2 (Angelov et al., 1996). Our data demonstrating that

numbers of ED1- but not ED2-positive cells in the conjunctiva are increased by the induction of EC (Fig. 2) suggest that ED1-positive cells infiltrated the conjunctiva, and ED2-positive cells are residential macrophages in the conjunctiva. Effects of CL₂MDP-lip on ED2-positive cells in the conjunctiva were noted up to 72 h after injection (Fig. 1), even when EC was induced (Fig. 3). In contrast, the number of ED1-positive cells decreased only when the treatment was performed just prior to OVA challenge. The reason why CL₂MDP-lip treatment just prior to OVA challenge inhibited the infiltration of ED1-positive cells is still unclear. One possible explanation is that effects of CL₂MDP-lip in the conjunctiva persist for less than 24 h and, therefore, its effective timeframe is restricted. In any case, CL₂MDP-lip could be useful therapeutically for inhibiting infiltrating macrophages within 24 h of antigen challenge.

Fig. 3. Subconjunctival injection of CL₂MDP-lip decreased the number of conjunctival macrophages in EC-developing BN rats. OVA-specific T cells were transferred into BN rats. Four days later, the rats were challenged with OVA. Conjunctivas were harvested for immunohistochemical analysis 24 h after OVA challenge. CL₂MDP-lip was subconjunctivally injected 24 or 48 h before or just prior to OVA challenge. (A) Photographs of ED1-positive cells in the conjunctiva. No apparent difference was noted between CL₂MDP-lip and PBS treated groups, although the number of ED1-positive cells was slightly decreased in the CL₂MDP-lip treated group at 0 h. $\times 240$. (B) Photographs of ED2-positive cells in the conjunctiva. CL₂MDP-lip treatment decreased the number of ED2-positive cells at all the treatment time points. $\times 240$. (C) Photographs of OX6-positive cells in the conjunctiva. Similar to the response of ED1-positive cells, CL₂MDP-lip slightly decreased the number of OX6-positive cells only at 0 h. $\times 240$. (D) Photographs of MBP-positive cells in the conjunctiva. The increase in MBP-positive cells induced by CL₂MDP-lip treatment was noted at -24 and 0 h. $\times 240$. (E) Cell counting of ED1-, ED2-, OX6- and MBP-positive cells. Cell counting confirmed the significant differences indicated as asterisks. * $p < 0.05$, ** $p < 0.01$.

Fig. 4. Intravenous injection of CL₂MDP-lip decreased the number of ED2-positive conjunctival macrophages in EC-developing BN rats. OVA-specific T cells were transferred into BN rats. Four days later, the rats were challenged with OVA. Conjunctivas were harvested for immunohistochemical analysis 24 h after OVA challenge. CL₂MDP-lip was injected intravenously at the time of OVA challenge. (A) Photographs of ED1-, ED2-, OX6- and MBP-positive cells in the conjunctiva. Intravenous injection of CL₂MDP-lip decreased the number of ED2-positive cells in the conjunctiva, whereas numbers of ED1-, OX6- or MBP-positive cells were not affected. $\times 240$. (B) Cell counting of ED1-, ED2-, OX6- and MBP-positive cells in the conjunctiva. Cell counting confirmed that only the number of ED2-positive cells differed significantly. $*p < 0.05$. (C) Photographs of ED1- and ED2-positive cells in the spleen. Numbers of both ED1- and ED2-positive cells were decreased by CL₂MDP-lip treatment. $\times 240$. (D) Cell counting of ED1- and ED2-positive cells in the spleen. Cell counting confirmed both ED1- and ED2-positive cells were significantly decreased by CL₂MDP-lip treatment. $**p < 0.01$.

Although the effects of CL₂MDP-lip on conjunctival macrophages have been partially elucidated, the regulating mechanisms of macrophage accumulation in the conjunctiva have not been examined. Several studies have demonstrated that molecules such as macrophage-colony-stimulating factor (Razzaque et al., 2002) and MIF (Razzaque et al., 2004) are involved in regulation of macrophage infiltration into the conjunctiva of OCP. Studying the expression of these molecules in the conjunctiva of EC is important to better understand how CL₂MDP-lip affects the infiltration of conjunctival macrophages. ED2-positive residential macrophages may also produce inflammatory molecules and, therefore, inhibition of ED2-positive cells by CL₂MDP-lip might be of value in suppressing inflammatory processes in the conjunctiva.

Systemic (intravenous administration) treatment with CL₂MDP-lip inhibited both ED1- and ED2-positive cells in the spleen, although not completely. Similarly, infiltration of both ED1- and ED2-positive cells into the conjunctiva was suppressed, but only the number of ED2-positive cells was significantly decreased. Thus, infiltrating macrophages in the conjunctiva originated from systemic circulating monocytes or macrophages.

An interesting but unresolved issue is that subconjunctival injection of CL₂MDP-lip increased the number of eosinophils in EC. This increase may have been due to the Th1/Th2 balance in the conjunctiva (Fukushima et al., 2003); macrophages produce IFN- γ and the lower relative concentration of IFN- γ due to the smaller number of macrophages following treatment with CL₂MDP-lip might have enhanced Th2 cytokines

Fig. 4 (continued)

(Gajewski et al., 1989; Sekine et al., 1997). Clarification is imperative because eosinophils are major effector cells in the development of AC.

In conclusion, CL₂MDP-lip suppresses infiltration of macrophages into the conjunctiva during the development of EC and may become a useful therapeutic tool in managing macrophage-mediated conjunctival diseases such as AKC.

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