European Journal of Pharmacology 740 (2014) 627–633

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and inflammation

Proteinase-activated receptor 2 blockade impairs CCL11- or allergen-induced eosinophil recruitment in experimental pleurisy

Natália A. Matos [a], Josiane F. Silva [b], Karine A. Damasceno [c], Geovanni D. Cassali [c], Virginia S. Lemos [b], Igor D.G. Duarte [a], André Klein [a,*]

[a] Department of Pharmacology, Universidade Federal de Minas Gerais, Avenida Antônio Carlos, 6627 Pampulha, 31270-901 Belo Horizonte, MG, Brazil
[b] Department of Physiology and Biophysics, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil
[c] Department of General Pathology, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

ARTICLE INFO

Article history:
Received 10 April 2014
Received in revised form
12 June 2014
Accepted 17 June 2014
Available online 24 June 2014

Keywords:
Eosinophil
Eosinophil migration
CCL11
Proteinase-activated receptor-2
PAR-2

ABSTRACT

Although proteinase-activated receptor (PAR)-2 has been implicated in inflammatory diseases, its role in regulating eosinophil recruitment in response to chemoattractants remains unclear. Here, we investigated the role of PAR-2 and PAR-2-activating Mast Cell (MC) tryptase on chemokine C–C motif ligand (CCL)11- and antigen-induced eosinophil recruitment to the pleural cavity of BALB/c mice. The PAR-2-activating peptide H–Ser–Leu–Ile–Gly–Arg–Leu–NH_2 (SLIGRL–NH_2) induced eosinophil recruitment whereas PAR-2 blockade inhibited ovalbumin (OVA)- or CCL11-induced eosinophil recruitment. Moreover, OVA and CCL11 induced PAR-2 expression in pleural leukocytes, and the MC tryptase inhibitor APC 366 ([N-(1-hydroxy-2-napthoyl)-L-arginyl-L-prolinamide hydrochloride]) abolished CCL11-induced eosinophil recruitment. These results suggest a pro inflammatory effect of PAR-2 and support a role for MC tryptase mediating eosinophil migration via PAR-2 signaling. Taken together, our results suggest that PAR-2 activation through endogenous MC tryptase activity could be required, at least partially, to mediate CCL11-induced eosinophil migration.

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1. Introduction

Proteinase-activated receptors (PARs) are a unique family of G-protein-coupled receptors that are activated through the proteolytic cleavage of a specific site on the N-terminus of the receptor. This cleavage reveals the tethered ligand that binds to and activates the receptor (Hollenberg and Compton, 2002). PARs family comprises four receptors (PAR-1 to 4), whose activation has been implicated in the regulation of inflammation, including vascular endothelial cell activity, *in vivo* leukocyte recruitment (Hollenberg and Compton, 2002; Vergnolle et al. 2002; Braga et al., 2010; Gomides et al., 2012) and cytokine release (Asokananthan et al., 2002; Steinhoff et al., 2005). PAR-2 is the receptor for trypsin and mast cell (MC) tryptase (Macfarlane et al. 2001; Adams et al., 2011) and it is of particular interest because it has been implicated in eosinophil activation (Bolton et al. 2003) and *in vivo* eosinophil and neutrophil migration (Schmidlin et al., 2002; Matos et al., 2013).

One of the hallmarks of allergic diseases is cellular infiltration that is markedly characterized by the eosinohphil accumulation (Rothenberg and Hogan, 2006). Eosinophils are considered the effectors of allergic reactions, and they are recruited from blood vessels into allergy-inflamed tissue by the local production of chemokines, such as C–C motif ligand (CCL)11 (Pope et al., 2005; Teixeira et al., 1997; Faccioli et al., 1991), or lipid mediators, such as leukotriene (LT)D_4 and cysteinyl LTs as well as interleukin (IL)-5 (Cheraim et al., 2008; Faccioli et al., 1996; Diamant et al., 1997). These mediators are produced by a variety of cell types, including airway epithelial cells, macrophages, eosinophils and MCs (Cook et al., 1998; Hirata et al., 1990; Martin et al., 1984), and appear to act directly on the eosinophil surface to induce recruitment *in vivo* (Pope et al., 2005; Faccioli et al., 1991). CCL11 plays an essential role in the recruitment of eosinophils during experimental allergic diseases as well as in allergic pleurisy in response to antigen (Ag) challenge (Teixeira et al., 1997; Van de Rijn et al., 1998; Klein et al., 2001). Once at the tissue, eosinophils are a crucial source of cationic proteins, lipid mediators, oxygen-derived radicals, cytokines and chemokines, which play an important role in the pathogenesis and severity of allergic diseases (Rothenberg and Hogan, 2006).

The increasing evidence for a role of PAR-2 in eosinophil accumulation and the role played by these cells in the development of allergic inflammation suggest that understanding the mechanisms underlying PAR regulation in inflammation may help

* Corresponding author. Fax: +55 21 31 34092711.
E-mail addresses: klein@ufmg.br, farklein@hotmail.com (A. Klein).

http://dx.doi.org/10.1016/j.ejphar.2014.06.018
0014-2999/Published by Elsevier B.V.

in the development of novel strategies for the treatment of inflammatory diseases. In this work, we investigated the ability of PAR-2-activating peptide to induce eosinophil recruitment to the pleural cavity of mice. We also evaluated the effects of PAR-2 blockade on eosinophil recruitment in response to Ag challenge in sensitized mice, and to intra-pleural injection of CCL11.

2. Materials and methods

2.1. Animals

Female BALB/c mice (18–25 g) were used throughout the experiment. The mice were housed in a temperature-controlled room with free access to food and water. Throughout the experiments, the mice were managed in accordance with the principles and guidelines for the care of laboratory animals. All of the experimental procedures were subject to evaluation and were approved by the local Animal Ethics Committee (certificate number 193/2012).

2.2. Drugs and Reagents

Ovalbumin (OVA) and aluminum hydroxide gel were purchased from Sigma (SP, Brazil). An anti-PAR-2 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The PAR-2 antagonist ENMD-1068 (N^1-3-methylbutyryl-N^4-6-aminohexanoyl-piperazine) was purchased from Enzo Life Sciences (San Diego, CA, USA). PAR-2-activating peptide SLIGRL–NH_2 (H–Ser–Leu–Ile–Gly–Arg–Leu–NH_2) and the selective inhibitor of MC tryptase, APC 366 ([N-(1-hydroxy-2-napthoyl)-L-arginyl-L-prolinamide hydrochloride]), were purchased from Tocris Bioscience (Bristol, UK). ENMD 1068 and SLIGRL–NH_2 were dissolved in phosphate-buffered saline (PBS, pH 7.4), APC366 was diluted in 20% DMSO and 80% PBS, and all of the samples were stored at −20 °C until use. Recombinant murine CCL11 was obtained from Peprotech (Rock Hill, NJ, USA). CCL11 was dissolved in PBS containing 0.01% bovine serum albumin (BSA, Sigma, SP, Brazil) and stored at −20 °C until use.

2.3. Sensitization

Mice were immunized with OVA adsorbed to aluminum hydroxide gel, as previously described (Klein et al., 2001). Briefly, the OVA and aluminum hydroxide gel were injected subcutaneously (s.c.) on days 1 and 8 with 0.2 ml of a solution containing 100 μg of OVA and 70 μg of aluminum hydroxide.

2.4. Leukocyte migration into the pleural cavity induced by OVA or PAR-2-activating peptide

Sensitized animals were injected intrapleurally (i.pl.) with antigen (OVA,1.0 μg/0.1 ml/cavity) or vehicle, 8 days after the final immunization. PAR-2-activating peptide SLIGRL–NH_2 was injected into naïve mice. The mice were killed at 24, 48, or 72 h after the i.pl. injection of the stimuli and the cells present in the cavity were harvested by injecting 2 ml of PBS. Total cell counts were performed in a modified Neubauer chamber using Turk's stain, as previously described (Klein et al., 2001). Differential cell counts were performed on cytospin preparations stained with May-Grünwald using standard morphological criteria to identify cell types. The results are presented as the number of cells per cavity.

2.5. Effects of PAR-2 antagonist pretreatment on eosinophil recruitment induced by OVA or CCL11

To investigate the contribution of PAR-2 in OVA- or CCL11-induced eosinophil recruitment, the PAR-2 antagonist ENMD 1068 (3 μg/mice) was administered i.pl. 15 min prior to the i.pl. injection of OVA (1 μg/0.1 ml) or CCL11 (100 ng/0.1 ml) in immunized or naïve mice, respectively. The number of infiltrating eosinophils was assessed after 48 h as described above.

2.6. Effects of the MC tryptase inhibitor APC 366 on eosinophil recruitment induced by CCL11

To evaluate the contribution of MC tryptase in mediating eosinophil migration into the pleural cavity in CCL11-injected mice, the animals were pretreated s.c. with the irreversible MC tryptase inhibitor APC 366 (5 mg/kg) 1 h prior to i.pl. injection of CCL11 (100 ng/0.1 ml). The number of infiltrating eosinophils was assessed after 48 h.

2.7. Western blot analysis of leukocyte PAR-2 expression in OVA or CCL11-injected mice

To investigate the expression of PAR-2 in pleural fluid leukocytes, pleural fluid washes from OVA-treated mice (1 μg/0.1 ml) or CCL11-treated mice (100 ng/0.1 ml) were obtained 24 and 48 h after i.pl. injection of stimuli. Four animals were used for each time point. The fluid recovered from the animals in each group was pooled and centrifuged at 1.300 × *g*. Lysis buffer (0.3 ml; in millimoles per liter: 150 NaCl, 50 Tris–HCl, 5 ethylenediamine tetraacetic acid, 2 Na, 1 $MgCl_2$) containing 1% Triton X-100 and 0.5% sodium dodecyl sulfate (SDS) plus a cocktail of protease inhibitors (SigmaFAST) was added to the cell pellets. The lysate was centrifuged at 8000 × *g* for 8 min, and the protein concentration in the supernatant was determined spectrophotometrically using the Bradford method. Thirty micrograms of protein were separated using a denaturing SDS polyacrylamide gel (10%) and transferred to a nitrocellulose membrane (Millipore). The blots were blocked with 3% bovine serum albumin (BSA) in TBS and 0.1% Tween 20 at 4 °C overnight. The following primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used: goat anti-PAR-2 polyclonal Immunoglobulin (Ig)G (1:1000) and mouse monoclonal anti-β-actin (1:3000). The following secondary antibodies (Millipore) were used: goat anti-mouse IgG-horseradish peroxidase (HRP) (1:8000) and rabbit anti-goat IgG-HRP (1:8000). Immunocomplexes were detected using a chemoluminescent reaction (Luminata Western HRP Substrates—Millipore®) followed by densitometric analyses using the Image Quant software package. β-actin content was used to normalize for total protein content.

2.8. Immunohistochemical analysis of PAR-2 in leukocytes of OVA- or CCL11-injected mice

Pleural washes were obtained 48 h after the i.pl. injection of OVA (1 μg/0.1 ml) or CCL11 (100 ng/0.1 ml). The samples were centrifuged, and the resulting pellet was resuspended in PBS containing 3% BSA. Cytospins containing 5×10^4–10^5 cells were prepared. Slides were fixed in 70% alcohol until use. The slides were washed with PBS for 5 min and incubated in 10% hydrogen peroxide (30 vol.) and 90% methanol twice for 15 min each to block endogenous peroxidases. The slides were then rinsed with PBS twice for 5 min each. Ultra Block V (Thermo Scientific) was used to block endogenous biotin. Next, the slides were incubated with a rabbit anti-murine PAR-2 primary antibody (1:100; Santa Cruz Biotechnology, USA) for 1 h at 37 °C. A biotin-peroxidase