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C5a receptor is cleaved by metalloproteases induced by sphingomyelinase D from *Loxosceles* spider venom

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ABSTRACT

Neutrophils are involved in numerous pathologies and are considered to be major contributors to the establishment of cutaneous loxoscelism after envenomation by the *Loxosceles* spider. Neutrophils are attracted to the site of envenomation by locally generated C5a and contribute to the tissue destruction. We have investigated the effects of this spider venom on the receptor for C5a: C5aR/CD88, a seven transmembrane G-protein coupled receptor. We show here that the *Loxosceles* venom induces the cleavage of the C5aR at two sites, resulting in the release of the extracellular N-terminus, while retaining part of the transmembrane regions. Using specific inhibitors, it was shown that the cleavage was induced by activation of an endogenous metalloprotease of the adamalysin (ADAM) family, which was activated by the sphingomyelinase D in the venom. Although it resulted in the near complete loss of the N-terminus, C5a was still able to induce a small increase in intracellular calcium and increase secretion of IL-8. The cleavage of the C5aR may well be a protective response after envenomation, rather than contributing to the pathology of *Loxosceles* envenomation and may represent a general mechanism for how the body protects itself against excess C5a generation in pathological circumstances such as sepsis.

Introduction

The C5a Receptor (C5aR/CD88) is the facilitator of one of the main activation products of the Complement system. C5a is a pro-inflammatory agent, which can induce a plethora of events including chemotaxis, anaphylaxis, cytokine secretion, production of reactive oxygen species and release of proteolytic enzymes (reviewed in Monk et al. 2007; Klos et al. 2009; Ward 2009). The C5aR is a seven transmembrane G-protein coupled receptor (GPCR), highly expressed on cells of myeloid origin including neutrophils, monocytes, and has also been described on non-myeloid cells. It is a major chemotactic receptor on neutrophils and neutrophils migrate in response to C5a, generated in a variety of pathological conditions to the site of injury (reviewed in: Guo and Ward 2005; Klos et al. 2009). Neutrophils are implicated in a variety of pathological conditions, one of which is cutaneous loxoscelism (Smith and Micks 1970; Tambourgi et al. 2010).

Cutaneous loxoscelism is a consequence of envenomation by the spider of the genus *Loxosceles*, which can cause serious dermonecrosis (reviewed in Swanson and Vetter 2006; Tambourgi et al. 2010), and is characterised by abundant infiltration of neutrophils (Smith and Micks 1970; Futrell 1992). Depletion of neutrophils has been one of the suggested therapeutic approaches to reduce the lesion size. Previously, we showed that, in a rabbit model, depletion of the Complement system resulted in a large reduction of neutrophil infiltration and reduction in the lesion, suggesting that C5a was the main chemoattractant for the neutrophils and contributing to the pathology (Tambourgi et al. 2005).

C5a has been implicated in a variety of medical conditions including sepsis (Guo and Ward 2005; Klos et al. 2009). C5aR expression is reduced on human neutrophils during sepsis (Seely et al. 2002) and in mice blockade of C5aR was shown to be protective to the lethality of sepsis (Riedemann et al. 2002). The mechanism of these alterations in expression is not known. Systemic loxoscelism resembles some aspects of endotoxemic shock, such as increase in cytokine and nitric oxide production and lethality (Tambourgi et al. 1998a,b).

Considering the importance of neutrophils and the Complement system in various aspects of loxoscelism, our aim was to investigate the effects of *Loxosceles* venom on the expression and function of the C5aR.

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Materials and methods

Reagents (there is more than antibodies and inhibitors here)

Antibodies were from the following sources: monoclonal anti-C5aR (S5/1) and rabbit polyclonal anti-C5aR (SC-25774): Santa Cruz (Wembley, UK); monoclonal anti-β2microglobulin (β2m-01): V. Horejsi (Prague, CZ); monoclonal anti-CD11b, anti-CD43, Goat anti-mouse IgG FITC (GAM-FITC): Dako (Ely, UK); monoclonal anti-MCP (GB24): J. Atkinson (St Louis, USA); monoclonal anti-CD16 (3G8): ATCC (Teddington, UK); Rabbit anti-mouse IgG Horseradish Peroxidase (RAM-HRPO) and Goat anti-rabbit IgG Horseradish Peroxidase (GAR-HRPO): Stratech (Soham, UK); and IL-8 Elisa: Invitrogen (Paisley, UK). GM6001, TAPI-1: Merck Chemicals Ltd (Nottingham, UK); Human recombinant C5a, PhenylMethylSulFonamide (PMSF), 1,10 Phenanthroline: Sigma (Poole, UK). Buffers: Phosphate Buffered Saline (PBS): 145 mM NaCl, 3 mM Na_2HPO_4 and 2.5 mM Na_2HPO_4, pH 7.4; FACS buffer: 1% Bovine Serum albumin (BSA), 0.1% NaN_3 in PBS; Veronal Buffered Saline (VBS^{2+}): 3.7 mM Barbitone, 0.3 mM $CaCl_2$ and 0.8 mM $MgCl_2$, 145.5 mM NaCl, pH 7.2; Krebs/Hepes buffer: 25 mM Hepes, 120 mM NaCl, 4.8 mM KC1, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, pH 7.4.

Venom collecting and sphingomyelinase purification

Loxosceles intermedia Mello-Leitão spiders were bred and maintained in house. Venom was obtained by electrostimulation (Tambourgi et al. 1995) and the recombinant Sphingomyelinase D (SMaseD) P1 from *L. intermedia* venom was obtained as described (Tambourgi et al. 2004). Venom from *Bothrops pirajai* was supplied by the Herpetology Laboratory (Butantan Institute, SP, Brazil). The permission to access the venoms from *Loxosceles* spider and *Bothrops* snake (permission no. 01/2009) was provided by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

Cells

Human neutrophils were isolated by standard methods using dextran sedimentation and ficoll gradient centrifugation. Cell lines, stably transfected with the C5aR, were as follows: U937 (from Eric Prossnitz (Kew et al. 1997) and RBL and CHO (from Monk et al. 1994).

Cell incubation with venom

Cells were harvested, washed and resuspended at 10^7 cells/ml in VBS^{2+} and incubated with *Loxosceles* venom or SMaseD at 10 μg/ml, (or as indicated) or VSB^{2+} as control, for 1 h at 37 °C. Cells were spun 3 min at 3000 rpm, and supernatants and cell pellets were used for western blotting or flow-cytometry, calcium signalling or IL-8 ELISA.

Flow-cytometry

Cells, resuspended at 5×10^6 cells/ml in FACS buffer, were incubated with specific mAb (5 μg/ml) for 30 min at 4 °C, washed and incubated for 30 min with the GAM-FITC. Cells were washed, fixed in 1% paraformaldehyde/PBS and analysed on a FACSCalibur. Results are expressed as median fluorescence intensity ± SD. Results are representative for at least three experiments carried out in triplicate.

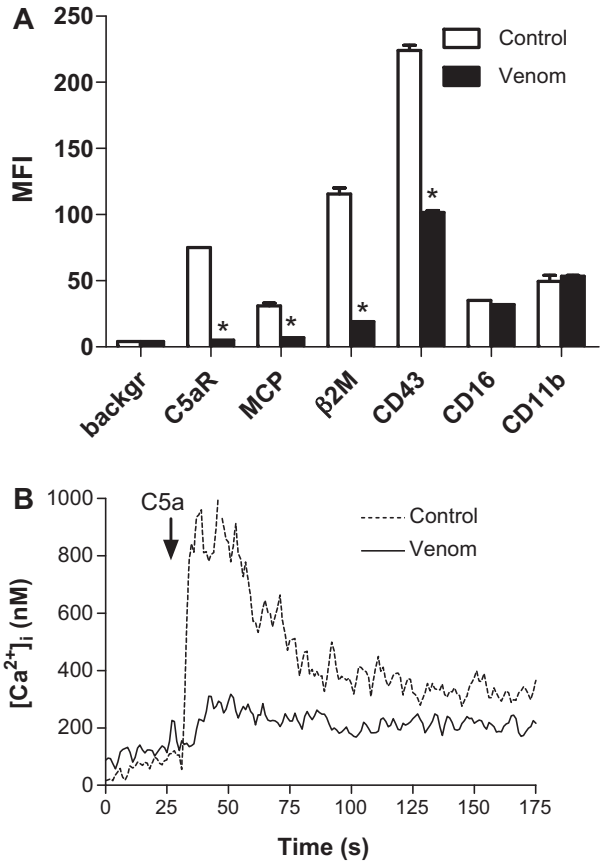

Fig. 1. *Loxosceles intermedia* venom reduces cell surface expression of the C5aR on neutrophils and the release of intracellular calcium in response to C5a. Neutrophils incubated with the spider venom or control buffer were analysed by A: flow-cytometry for cell surface molecules or B: loaded with Fura-2 and stimulated with C5a and intracellular calcium release was measured. * indicates $p < 0.001$ compared to cells not incubated with venom.

SDS-PAGE and western blotting

For western blotting, cell supernatants were mixed 1:1 with sample buffer, while cell pellets were resuspended at 10^7 cells/ml in sample buffer and run on 10% SDS-polyacrylamide gels under non-reducing conditions and blotted onto Hybond Nitrocellulose (GE Healthcare UK, Little Chalfont, UK). Membranes were blocked with 5% milk/PBS, incubated overnight with primary antibody (1/200). Membranes were washed, incubated for 1 h in the secondary antibody (1/1000), washed and developed using Supersignal West Pico substrate (Pierce, Cramlington, UK) and ECL Hyperfilm (GE Healthcare, Amersham, UK). Precision Plus All Blue standards (Biorad, Hemel Hempstead, UK) were used to calculate the molecular weights of the bands.

Calcium measurements

Cells (at 10^7 c/ml) were loaded with 2 μM Fura-2-AM (Anaspec, Cambridge Bioscience, Cambridge, UK) for 30 min at room temperature. Cells were washed and resuspended in Krebs/Hepes buffer and 200 μl of Fura-2-1oaded cells (5×10^6 ml) were added to a pre-warmed microtitre plate and stimulated with 5 nM C5a. Simultaneous excitation Fura-2 at 340 and 380 nm and fluorescence emission was monitored at 505 nm (Fluostar plate reader; BMGlabtech, Offenburg, Germany) at 37 °C. The average $[Ca^{2+}]$ in the cell population was calculated from the ratio of