Research Article

Induction of Mast-Cell Accumulation by Promutoxin, an Arg-49 Phospholipase A$_2$

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Received 23 July 2012; Accepted 11 September 2012
Academic Editor: Luis A. Ponce Soto

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Local inflammation is a prominent characteristic of snakebite wound, and snake-venom phospholipase A$_2$s (PLA$_2$s) are some of the main components that contribute to accumulation of inflammatory cells. However, the action of an R49 PLA$_2$, promutoxin from Protobothrops mucrosquamatus venom, on mast-cell accumulation has not been previously examined. Using a mouse peritoneal model, we found that promutoxin can induce approximately 6-fold increase in mast-cell accumulation, and the response lasts at least for 16 h. The promutoxin-induced mast cell accumulation was inhibited by cyproheptadine, terfenadine, and Ginkgolide B, indicating that histamine and platelet-activating factor (PAF) is likely to contribute to the mast-cells accumulation. Preinjection of antibodies against adhesion molecules ICAM-1, CD18, CD11a, and L-selectin showed that ICAM-1, and CD18, CD11a are key adhesion molecules of promutoxin-induced mast-cell accumulation. In conclusion, promutoxin can induce accumulation of mast cells, which may contribute to snake-venom wound.

1. Introduction

Snake-venom phospholipase A$_2$s (PLA$_2$s) are low-molecular-weight (13,000–14,000 Da), secretory phospholipases containing seven disulfide bonds. Usually, the PLA$_2$s from Crotalidae or Viperidae venom are divided into two major groups: the Asp-49 PLA$_2$s (D49 PLA$_2$s) and Lys-49 PLA$_2$s (K49 PLA$_2$s), and several minor groups: Ser-49 PLA$_2$s (S49 PLA$_2$s) [1–3], Asn-49 PLA$_2$s (N49 PLA$_2$s) [4, 5], and Arg-49 PLA$_2$s (R49 PLA$_2$s) [6–8]. Besides the digestive function, snake PLA$_2$s exhibit several other pharmacological properties including antiplatelet [9, 10], anticoagulant [11], hemolytic [9], neurotoxic (presynaptic) [12], and myotoxic [13–15] properties. They have also been involved in various inflammatory processes such as edema, inflammatory cell infiltration, and mast-cell activation [15–20]. Mast cells are primarily located in mucosal and perivascular areas of various tissues, which play an important role in body-defense processes. Mast cells can be activated by snake-venom and release carboxypeptidase A and possibly other proteases, which can degrade venom components [21, 22]. Several snake-venom PLA$_2$s were reported to be able to activate the rat mast cells and to induce microvascular leakage and inflammatory-cell accumulation at the sites of inflammation [15–20]. Our previous studies showed that TM-N49, an N49 PLA$_2$ purified from Protobothrops mucrosquamatus venom, induces skin edema and mast-cell activation and accumulation [23], and promutoxin, an R49 PLA$_2$ purified from the same snake, can activate mast cells and induce skin edema [24]. Both TM-N49 and promutoxin are devoid of catalytic activity and are thought to contribute to Protobothrops mucrosquamatus venom toxicity [5, 8]. Moreover, both TM-N49 and promutoxin are potent stimuli for induction of neutrophil, lymphocyte, macrophage, and eosinophil accumulation in the mouse peritoneum [25]. These observations suggested that the two novel subgroups of group II PLA$_2$s may contribute to the inflammatory process caused by snake-venom poisoning. However, the ability of R49 PLA$_2$ on induction of mast-cell accumulation has not yet been reported. In the present study, we investigated the mechanisms of promutoxin-induced mast-cell accumulation.
2. Materials and Methods

2.1. Reagents. Protobothrops mucrosquamatus crude venom was obtained from the stock of the Kunming Institute of Zoology, the Chinese Academy of Sciences. SP-Sephadex C-25, heparin sepharose (FF) and superdex 75 were obtained from LKB Pharmacia (Uppsala, Sweden). The following compounds were purchased from Sigma (St. Louis, USA): egg phosphatidyl choline, Triton X-100, trifluoroacetic acid, honey-bee venom phospholipase A2, platelet-activating factor (PAF), cyproheptadine, and ginkgolide B. Quinacrine was obtained from calbiochem (San Diego, CA, USA). Reagents for sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories Inc. (Hercules, USA). Coomassie Plus assay kit was purchased from Pierce Chemical Co. (Rockford, IL, USA). Fetal-calf serum (FCS) and minimum essential medium (MEM) containing 25 mM N-2-hydroxyethylpiperazine-N’-2-ethane sulphonic acid (HEPES) were purchased from Gibco (Paisley, Renfrewshire, UK). Rat monoclonal antibodies, anti-mouse CD11a lymphocyte function-associated antigen 1 (LFA-1) α chain, clone M17/4; anti-mouse CD62L (L-selectin), clone MEL-14; anti-mouse CD18 (integrin β2 chain), clone M18/2; rat IgG2a isotype standard, clone R35-95; hamster antimonoclonal antibody, clone 3E2; hamster IgG1 isotype standard, clone A19-3 were obtained from BD Biosciences Pharmigen (CA, USA). Hepes and all other chemicals were of analytical grade. BALB/c mice (20–25g) were bred and reared under strict ethical conditions according to international recommendation.

2.2. Purification of Promutoxin. Promutoxin was isolated from Protobothrops mucrosquamatus crude venom following the procedures described previously [8]. Briefly, the lyophilized venom (1.5 g) was dissolved in 20 mL of 0.05 M sodium phosphate buffer (pH 5.8) before being loaded on an SP-Sephadex C-25 column. The absorbed proteins were eluted with a linear gradient (0–0.8 M NaCl). Peak 7 was collected and loaded on Superdex 75 column in 25 mM sodium phosphate buffer (pH 5.8 with 0.15 M NaCl). The main peak was collected, and then loaded on a reverse-phase C18 high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA, USA). The main peak fraction, representing the purified promutoxin, was pooled, lyophilized, and stored at −20°C.

Protein concentration was determined by using a Coomassie Plus assay kit with BSA as standard. The PLA2 activity was routinely assayed by a titration method using egg yolk as substrate [26] and by a colorimetric assay using L-phosphatidylcholine as substrate [27]. Honey-bee PLA2 was employed as positive control.

2.3. Induction of Mast-Cell Accumulation. Various doses of promutoxin, BSA or normal saline were injected in 0.5 mL volumes into the peritoneum of male BALB/c mice, whose abdominal skin was swabbed with 70% ethanol, a group of 6 mice for each dose. This model was adapted from that described by Thomas and colleagues [28], which complied with the European Community guidelines for use of experimental animals. At 10 min, 2h, 6h, or 16h following injection, animals were sacrificed by inhalation of carbon dioxide, and their peritoneal lavages were collected following a standardized procedure with 5 mL normal saline. After centrifugation at 500 g for 10 min at 4°C, supernatants were collected and stored at −40°C until use, and cells were resuspended in 1 mL of MEM. The total cell numbers were determined by enumerating them with an Improved Neubauer haemocytometer after being stained with 0.1% trypan blue. For the differential cell counting, cytocentrifuge preparations were made, air dried, and stained with modified Wright’s stain. Differential cell counts were performed for a minimum of 500 cells. The results were expressed as absolute numbers of each cell type per mouse peritoneum.

For the experiments investigating mast-cell-migration mechanisms, groups of mice were pretreated intravenously (tail vein injection) with monoclonal antibodies against the adhesion molecules L-selectin, CD11a, CD18, and ICAM-1 (all at a dose of 1 mg·kg−1) [29–31], respectively, for 30 min before intraperitoneal injection of 5 μg of promutoxin. Control animals received an equivalent dose of the corresponding normal rat or hamster IgG isotype control. At 6h following injection, the mice were sacrificed and their peritoneal lavages were processed as described above.

To investigate potential mechanisms involved in promutoxin-induced mast-cell accumulation, several anti-inflammatory compounds including cyproheptadine (2 mg·kg−1) [17], terfenadine (3 mg·kg−1) [32, 33], ginkgolide B (5 mg·kg−1) [34], and quinacrine (10 mg·kg−1) [35] were coinjected into the peritoneum of mice with promutoxin (5 μg per mouse). Control animals received an injection of drug alone. At 6h following injection, mice were sacrificed and their peritoneal lavages were processed as described above.

2.4. Statistical Analysis. Data are shown as mean ± SE for the number of experiments indicated, and ANOVA followed by Tukey’s tests were used for statistical comparison of the data. In all analyses, P < 0.05 was taken as statistically significant.

3. Results

3.1. Purification and Characterization of Promutoxin. Approximately 25 mg of promutoxin was obtained from 1.5 g Protobothrops mucrosquamatus crude venom following the procedures described above. The purity of the PLA2 was at least 98% as assessed by SDS-PAGE, HPLC, and mass spectrometry analysis [24].

3.2. Induction of Mast-Cell Accumulation by Promutoxin. As early as 10 min following injection, 5 μg of promutoxin was able to induce significant mast-cell accumulation in the peritoneum of mice. The mast-cell accumulation appeared to last for 16 h (Figure 1). Approximately, up to 6-fold increase
in mast-cell numbers was achieved at 16 h following injection (Figure 1).

3.3. Effects of Anti-Inflammatory Compounds and Blocking Antibodies on Mast-Cell Accumulation. When coinjected, ginkgolide B, cyproheptadine and terfenadine inhibited 35.9, 71.3, and 92.6% promutoxin-induced mast-cell accumulation in the peritoneum of mice, respectively. However, quinacrine did not significantly alter the extent of promutoxin-induced mast-cell accumulation. At the dose tested, ginkgolide B, cyproheptadine, terfenadine, and quinacrine by themselves failed to induce mast-cell accumulation in the peritoneum of mice (Table 1).

Intravenous injection of monoclonal antibodies against CD18, ICAM-1, and CD11a 30 min prior to intraperitoneal injection of the PLA2-blocked promutoxin-induced mast-cell accumulation by 87.2, 76.7, and 53.8%, respectively. Monoclonal antibody against L-selectin failed to diminish promutoxin-induced mast-cell accumulation. Normal rat and hamster IgG-isotype controls tested had little effect on promutoxin-induced mast-cell accumulation (Table 2).

4. Discussion

It is found for the first time that promutoxin, a novel member of a minor subgroup (R49 PLA2) of snake-venom group II PLA2s, can induce mast-cell accumulation. The observation supports our previous finding that N49 PLA2, another minor subgroup of snake-venom group II PLA2s [23] can induce mast-cell accumulation. Obviously, promutoxin does not induce mast-cell accumulation in a concentration-dependent manner [24]. We previously found that promutoxin could activate mast-cells. The reduction of mast-cell number induced by promutoxin in higher doses may be due to the activation of accumulated mast-cells by promutoxin.

Ginkgolide B, a PAF antagonist, inhibited more than 35.9% promutoxin-induced mast-cell migration, indicating that PAF is likely involved in mast-cell migration by promutoxin. This result appears to agree with a previous work which found that PAF may contribute to mast-cell migration induced by TM-N49 [23]. Inhibition of 71.3 and 92.6% promutoxin-induced mast-cell accumulation by cyproheptadine, a histamine/5-HT antagonist, and terfenadine a selective histamine-H1-receptor antagonist, implies that histamine is likely to be involved in the above event through its H1 receptor. Indeed, we have found previously that promutoxin can activate mast cells to release histamine [24] and anticipate herein that released histamine then elicits mast-cell migration. The fact that terfenadine inhibited...
promutoxin-provoked mast-cell recruitment to a greater extent than cyproheptadine further implies the selectivity of histamine receptor involved. It was found that snake-venom promutoxin could induce mast-cell accumulation even at 10 min following injection, but the number of lymphocyte, macrophage, eosinophil, and neutrophil migration was not altered at 10 min following injection of promutoxin [25]. It seemed the accumulation of neutrophils, lymphocyte, macrophage, and eosinophil induced by promutoxin could be a secondary event, in which accumulated mast cells are activated by promutoxin and release their chemoattractant factors such as serine proteinases [36, 37], histamine, and PAF to recruit inflammatory cells.

ICAM-1 appears to be a key adhesion molecule of promutoxin-induced mast-cell accumulation as an antibody against ICAM-1 blocked more than 76.7% of the influence of promutoxin on the cell migration. ICAM-1 involved in lymphocyte [38], macrophage [39], eosinophil [40, 41] and mast-cell accumulation [23] has been reported previously, which may support our current observations. An antibody against CD11a (LFA-1) blocked more than 53.8% promutoxin-induced mast-cell accumulation, indicating that CD11a plays an important role in the migration of mast cells, which is consistent with previous reports that TMA-N49-induced mast-cell accumulation is mediated by CD18, CD11a, and ICAM-1 [23]. As expected, antibody against CD18 blocked more than 87.2% promutoxin-induced mast-cell accumulation in the present study. Though L-selectin is involved in the neutrophil and eosinophil accumulation provoked by promutoxin, it seemed that L-selectin is not involved in the promutoxin-induced mast-cell accumulation.

Promutoxin, as a novel member of minor subgroup of PLA$_2$, is an enzymatically inactive enzyme. It induced mast-cell accumulation via a PAF, and histamine H$_3$ receptor-dependent mechanism, and through a CD11a/CD18-and ICAM-1-associated adhesion pathway. Accumulated mast cells may contribute to snake-venom wound.

Acknowledgments
The authors are grateful to Miss Jie-lian Lin and Miss Qiu-Yu Chen for their assistance in the animal experiments. This paper was sponsored by the Grants from the National Natural Science Foundation of China (30972822, 81273274, 81001329, and 30972714), Jiangsu Province’s Key Provincial Talents Program (RC201170), the Key Allergy Laboratory Fund of Jiangsu Province, China (XK201115), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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