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EXPERIMENTAL

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Antagonization of TNF attenuates systemic hemodynamic manifestations of envenomation in a rat model of *Vipera aspis* snakebite

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Abstract *Objectives:* Tumor necrosis factor (TNF) has been reported as a mediator of local tissue injury following snake envenomation in an intact rat model. We investigated whether systemic release of TNF occurs following *Vipera aspis* envenomation. We further analyzed the possible connection between envenomation-related hemodynamic depression and TNF antagonization (TNF antibodies or soluble TNF receptor).
Design: A prospective, randomized, controlled experimental study using a rat model for snake envenomation.
Settings: A medical university hospital research laboratory.
Intervention: Eighty rats (300–400 g) were divided into four groups ($n = 20$): control and three experimental groups. Intramuscular injection of *V. asis* 500 μg/kg was administered to the three experimental groups: venom only (group 1), venom and 40 μg anti-TNF antibodies (group 2), venom and 250 μg soluble TNF receptor (p55-R; group 3). Hemodynamic parameters were monitored up to 4 h following venom injection.
Measurements and results: A significant hemodynamic deterioration (reduction in heart rate and blood pressure) occurred 30 min following venom injection in group 1 compared to groups 2 and 3, where hemodynamic parameters remained stable throughout the 4 h observation period. Serum levels of TNF were detected 15 min after venom injection and peaked after 2 h at 485 ± 12 pg/ml.
Conclusions: The hemodynamic consequences of intramuscular injection of *V. aspis* venom can be blunted in a rat by systemic antagonization of TNF activity prior to venom injection. The poisonous hemodynamic effects of the *V. aspis* venom might be caused by systemic release of TNF.

Keywords Snake envenomatiom · *Vipera aspis* · TNF antibodies · p55-R · Metalloproteinases

Introduction

Snake envenomation is still considered as a major health threat in various parts of the world [1, 2]. The clinical manifestations of envenomation are usually progressive, from initial signs limited to the bite area to sometimes severe, systemic manifestations which can lead to rapid death from multiorgan failure [2]. Recently it has been suggested that the severe local tissue destruction, edema, and necrosis following snakebite is mediated via the processing of tumor necrosis factor (TNF) by venom metalloproteinases [3]. TNF is a potent proinflammatory compound that has a pivotal role in the course of the immune response to infection [4]. It can activate neutrophils [5] and lead to activation of the vascular endothelial cells, resulting in their in-

creased permeability [6]. The multiorgan edema that follows can lead to tissue ischemia and death [7].

Viper venoms are rich in zinc metalloproteinases [8] which have been shown capable of cleaving recombinant pro-TNF to the biologically active mature TNF [9, 10, 11]. This observation was the basis for our hypothesis on the possible role of TNF in inducing some of the systemic effects of snake envenomation. The specific aim of our study was to clarify whether antagonization of TNF abolishes hemodynamic deterioration following venom injection in a rat model of *Vipera aspis* snake envenomation.

Material and methods

Experimental protocol

All experiments were carried out in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Tel-Aviv Medical Center. Adult male Sprague-Dawley rats weighing 300–400 g anesthetized with intraperitoneal injection of 125 mg/kg ketamine chloride were randomized into four groups (control and three experimental, $n = 20$ each). Group 1 rats were given intramuscular (IM) venom only. Group 2 rats received intravenous 40 μg anti-TNF antibodies (TNF Ab) followed by venom injection 10 min later. Rats in group 3 were given IM a dose of 250 μg of soluble TNF receptor (p55-R) 15 min before the venom. Sham-operated control animals ($n = 20$) underwent an identical procedure but were not given the venom, anti-TNF, or the soluble TNF receptor. The femoral vein and artery were cannulated via a skin incision, after which either saline or 500 μg/kg *V. asis* venom was administrated IM. Anesthesia was maintained by repeated intraperitoneal injections of 60 mg/kg ketamine chloride throughout the experiment, and the animal's heart rate and blood pressure were determined every 30 min using an intra-arterial pressure transducer. After 4 h the rats were killed. Blood samples (0.5 ml each) were taken via the arterial line to determine TNF levels at the beginning, 15, 30, 45, 60, 90, 120, 150, and 180 min after venom injection, and at the end of the experiment (240 min) in the control and the venom only groups. Since TNF Ab and p55-R doses were well above those required for complete neutralization of the amount of TNF activity in the venom only group (see below), TNF levels were not measured in experimental groups 2 and 3. The blood volume was replaced 3:1 with 0.9 % sodium chloride solution. All samples were immediately centrifuged (15,000 RPM) for 3 min at 4 °C, and the serum was frozen at –70 °C until analyzed.

TNF assay

TNF activity was measured using the commercially available enzyme-linked immunosorbent assay kit Cytoscreen rat kit TNF (Immunoassay kit, Biosource, Camarillo, Calif., USA).

Venom

V. aspis venom was obtained from the Unite des Venins, Institut Pasteur (Paris, France).

Antibodies

Polyclonal rabbit anti-rat TNF Ab was purchased from Innogenetics (Belgium). These antibodies neutralize rat TNF and have no cross-reactivity with intrerleukin-1 or other cytokines. A dose of 1 ng of this antibody completely neutralizes 60 pg rat TNF.

Human recombinant soluble TNF receptor

In a pilot study p55-R (TNF soluble receptor) at doses of 200 and 300 μg was found to be effective in septic rats (data not shown). Based on this pilot study and a previous study [12] a dose of 250 μg was used.

Statistics

Results are reported as mean ± SEM. Analysis of variance with repeated measures was performed. Multiple comparisons were performed using Dunett's procedure. Contrast analysis was performed to compare between successive time points. $p < 0.05$ was considered significant.

Results

Hemodynamics

In all groups an initial rise in heart rate was observed in the first 20 min of the experiment. In group 1 (IM venom only) within 60 min following the injection of the venom, there was a significant ($p < 0.05$) reduction in heart rate (117 ± 3 in the venom group and 155 ± 6 beat/min in the control group; Fig. 1) that persisted throughout the 4 h of the observation time. The mean arterial blood pressure (Fig. 2) was similar in the control and the experimental groups at the beginning of the experiment. However, 90 min following the envenomation the mean arterial blood pressure was significantly ($p < 0.05$) lower in group 1 (67 ± 2 mm/Hg) than the control group (87 ± 8 mm/Hg). This difference persisted throughout the rest of the experiment. An improvement in mean arterial blood pressure followed a decrease in TNF levels which was measured in the late period of the experiment. In the control animals both heart rate and mean arterial blood pressure were stable during the 4 h of the study protocol (Figs. 1, 2). Administration of anti-TNF Ab or soluble TNF receptor (P55-R) to the venom-injected rats (groups 2 and 3, respectively) completely blocked the toxic hemodynamic effects of the venom injection (Figs. 1, 2). There was no significant statistical difference in either heart rate or mean arterial blood pressure between animals in groups 2 or 3 and the control group.

TNF levels were detected 15 min after the venom injection (35.2 ± 1.5 pg/ml and reached a peak after 2 h (485 ± 12.4 pg/ml) in the venom-injected rats (Fig. 3) while no increase being detected in the control group.

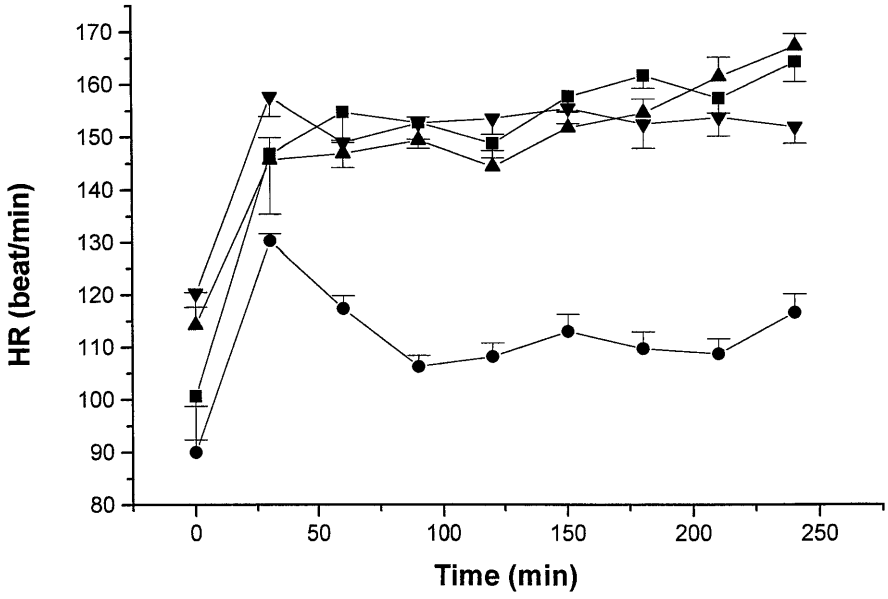

Fig. 1 Heart rate over time after the venom injection in all four groups. A significantly lower heart rate was observed in group 1 (venom-only injected group, ●) than in group 2 (anti-TNF antibodies, ▲), group 3 (p55-R, ▼), and the control group (■)

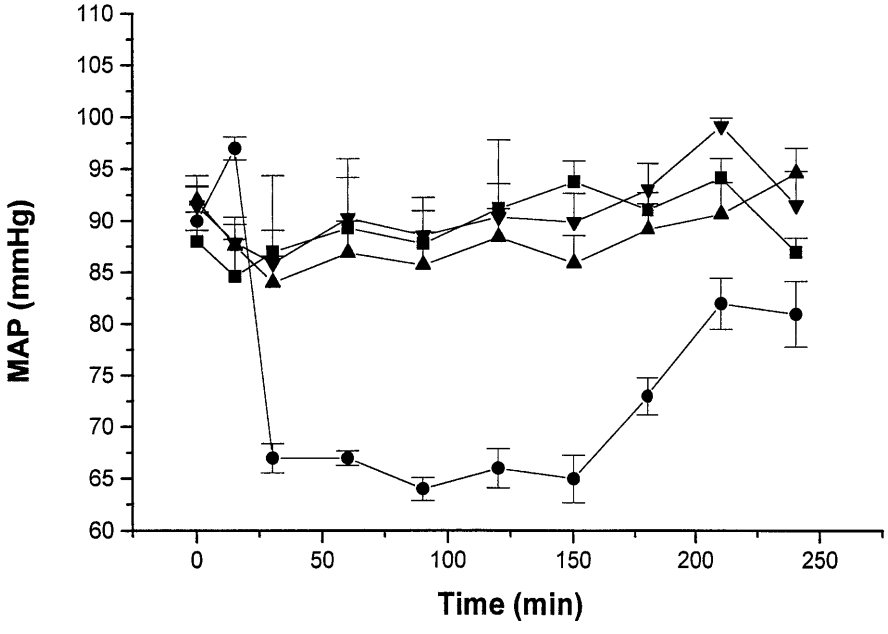

Fig. 2 Mean arterial blood pressure over time. A significantly lower arterial blood pressure in group 1 (venom-only injected group, ●) than in group 2 (anti-TNF antibodies, ▲), group 3 (p55-R, ▼), and the control group (■)

Discussion

This study is the first to show a significant increase in serum levels of TNF with parallel depressive hemodynamic effects (bradycardia and hypotension) after IM injection of *V. aspis* venom in a rat model of snake envenomation. These deleterious hemodynamic effects were completely blocked by the preenvenomation administration of anti-TNF Ab or of TNF surface receptor (p55-R) that mediate the effects of TNF on cell function [13, 14]. The p55-R is the main mediator of the TNF signaling to the cells. Two receptors (p55-R and p75-R) exist in soluble forms [13, 15, 16] and are probably derived by proteolytic cleavage from the cell surface. These soluble receptors can compete for TNF with the cell surface receptors and thus block its bioavailability, attenuating its activity and potential harmful effects [13, 14, 15, 16]. By pretreating the animals with p55-R the hypotensive effects of the venom were partially reversed. These effects of both anti-TNF Ab and the TNF soluble receptor indicate that TNF was responsible for the observed hemodynamic alternations.

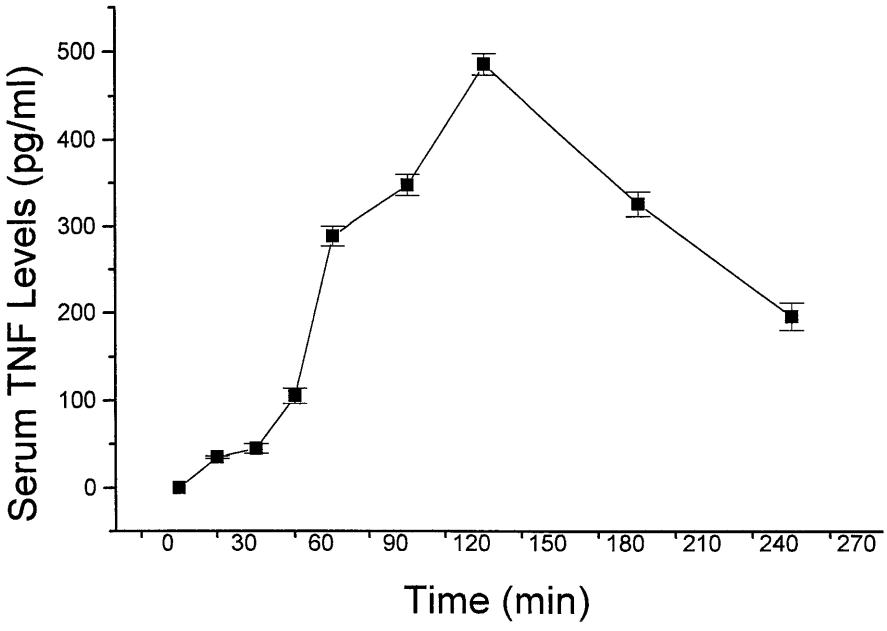

Fig. 3 Serum TNF levels over time. A rise in the TNF was detected within 15 min of the venom injection and peak after 2 h

In many other studies TNF levels were measured in various patient group. In a study of neonatal septicemia TNF levels were high as 739 ± 728 pg/ml [17], while in another study by Damas et al. [18] of adults with sepsis the TNF levels ranged from 100 to 5000 pg/ml with a mean of 701 ± 339 pg/ml. In a rat study using bowel ischemia and reperfusion as a model for severe systemic inflammatory response model TNF concentrations increased to 830 ± 66 pg/ml [12]. The differences in the protocols used in these studies make it impossible to compare them, but observed TNF levels in this study are within the range of TNF concentrations reported by others. The initial rise in heart rate in all groups and the small and nonsignificant rise in mean blood pressure in group 1 can be attributed to an increased sympathetic activity following induction of anesthesia by ketamine chloride [19]. The slower heart rate in the venom-injected group is compatible with the known direct negative chronotropic and depressive effect of the venom on the rat as well as the human heart [20, 21].

In this study the venom was administered IM, mimicking an actual snakebite. This route of envenomation likely resulted in systemic absorption of the venom that can generate a systemic inflammatory response. A similar model of IM administration of venom was used in two previous studies [22, 23] that described the pharmacokinetics of viper venom after experimental envenomation in rabbits. They were able to detect free venom antigens both in the plasma and urine of the study animals shortly after IM injection of the venom. In an attempt to characterize the host reactivity to *Bothrops asper* venom, Lomonte et al. [9] investigated inflammatory responses in a mouse footpad model. After subcutaneous injection of the venom, they observed a rapid increase in serum interleukin-6 concentration that peaked after 3–6 h. In contrast, serum TNF and interleukin-1 were not detectable at any time throughout their study period. The difference between our results and those of Lomonte et al. [9] seems to be due to the difference in study protocols. Subcutaneous injection into the footpad may induce local generation of TNF that results in necrosis but with no systemic absorption of the venom. In addition, differences in venom composition in various snake species can lead to differences in serum cytokine profile after envenomation. In accordance, our results are species-specific and applicable to *V. aspis* envenomation only.

In conclusion, IM injection of *V. aspis* venom in a rat model of snake envenomation can result in depressive cardiovascular effects that are partly mediated by TNF. As in this experimentation, the antagonization of TNF took place prior to the envenomation, further experimental studies are required to assess the possible therapeutic role of modifying TNF activity in *V. aspis* bitten patients.

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