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Original article

Camel urine inhibits inflammatory angiogenesis in murine sponge implant angiogenesis model

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ABSTRACT

Camel urine has traditionally been used to treat cancer, but this practice awaits scientific scrutiny, in particular its role in tumor angiogenesis, the key step involved in tumor growth and metastasis. We aimed to investigate the effects of camel urine on key components of inflammatory angiogenesis in the murine cannulated sponge implant angiogenesis model. Polyester-polyurethane sponges, used as a framework for fibrovascular tissue growth, were implanted in Swiss albino mice and camel urine (25, 50 and 100 mg/kg/day) was administered for 14 days through installed cannula. The implants, collected at day 14 post-implantation, were processed for the assessment of hemoglobin (Hb), myeloperoxidase (MPO), *N*-acetylglucosaminidase (NAG) and collagen, which were used as indices for angiogenesis, neutrophil and macrophage accumulation and extracellular matrix deposition, respectively. Relevant inflammatory, angiogenic and fibrogenic cytokines were also determined. Camel urine treatment attenuated the main components of the fibrovascular tissue, wet weight, vascularization (Hb content), macrophage recruitment (NAG activity), collagen deposition and the levels of vascular endothelial growth factor (VEGF), interleukin (IL)-1β, IL-6, IL-17, tumor necrosis factor (TNF)-α and transforming growth factor (TGF-β). A regulatory function of camel urine on multiple parameters of the main components of inflammatory angiogenesis has been revealed giving insight into the potential therapeutic benefit underlying the anti-cancer actions of camel urine.

1. Introduction

Recent clinical data have affirmed that angiogenesis, a process necessary for solid tumor growth and dissemination, is a key clinical target that has its potential to improve therapeutic outcomes. In addition to angiogenesis, it has become increasingly clear that inflammation is a key component in cancer insurgence that, in turn, can promote tumor angiogenesis and that these are tightly linked processes [1]. At the turn of this century, the seminal "*hallmarks of cancer*" were identified as self-sufficiency in growth signals, insensitivity to anti-growth signals, invasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis [2]. These six hallmarks are paramount to the onset and progression of cancer, but have been expanded and refined in the last 10 years to include cancer-related inflammation and interaction with the immune system [3]. Additionally, the mechanisms of inflammatory angiogenesis provide new approaches to target, cure and prevent tumor angiogenesis by synthetic or natural agents with anti-inflammatory properties [1].

The camel has played a crucial role in desert dwellers for thousands of years, not only as a means of transportation and food, but also its milk and urine have been used traditionally for the maintenance of good health and in the treatment of diverse diseases [4–6]. The medicinal use of camel urine dates back to the time of the famous scholar known as Avicenna (980–1037), author of al-Qanoon (The Canon). Until recently, it is traditionally claimed that drinking camel urine has cured numerous cases of cancer, but this claim has never been exposed to scientific scrutiny and investigation. Preliminary studies suggested an anti-carcinogenic activity of camel urine [7,8]. Camel urine inhibited cell proliferation and induced of apoptosis via downregulation of Bcl-2 [9]. We previously reported that camel urine inhibited the induction of Cyp1a1, a cancer-activating gene in murine hepatoma (Hepa 1c1c7) cells [10]. Since angiogenesis and inflammation co-exist in a variety of pathological conditions therefore, we hypothesized that camel urine might prevent inflammatory angiogenesis. To our knowledge, there is no study exploring the effect of camel on inflammatory

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angiogenesis. The present study, therefore, is testing the hypothesis that the camel urine may prevent inflammatory angiogenesis in the sponge implant model by determining its effect on neovascularization, granulation together with relevant pro-angiogenic and pro-inflammatory cytokines.

2. Materials and methods

Male Swiss albino mice ($n = 10$) aged 5 to 6 weeks and weighing 20–30 g body weight were used and were provided by the Central Animal Facility at Colleges of Medicine, King Saud University, Riyadh, Saudi Arabia. The animals were housed individually in plastic cages and allowed access to a normal diet and water *ad libitum*, with a light/dark cycle of 12:12 h. Measures were taken to avoid all unnecessary distress to the animals. Housing, anesthesia and postoperative care concurred with the guidelines established by our Institutional Animal Ethics Committee.

3. Camel urine sample collections

Urine was collected aseptically from female healthy domestic camels (*Camelus dromedaries*). The urine was collected from farm and desert living animals. The collection of urine was usually conducted during the feeding time and was performed by experienced attendants. Urine was allowed to flow directly into sterile stainless steel containers and then transferred to glass vials. Urine samples were lyophilized and stored at −80 °C.

4. Preparation of cannulated sponge discs and implantation

Polyester-polyurethane sponge discs, 5-mm thick and 1-cm diameter (Vitafoam Ltd., Manchester, UK), were used as the matrix for host tumour cells and to monitor angiogenesis [11–15]. One end of the polyvinyl tubing 1.2 cm long × 1.2 mm internal diameter (Portex Ltd., Hythe, Kent, UK) was secured to the centre of each disc with two 5/0 silk sutures (Ethicon Ltd., UK) so that the tube was perpendicular to the disc face. Sponges were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 minutes and irradiated with ultraviolet light for 20 minutes before implantation. Animals were anaesthetized by light ether anesthesia along with 2,2,2-tribromoethanol (1 mg kg/; *i.p.* Aldrich, USA). The dorsal hair was shaved and the skin wiped with 70% ethanol. The cannulated sponge discs were implanted aseptically into a subcutaneous (s.c.) pouch through a 1 cm long dorsal midline incision made with curved artery forceps. The cannula was exteriorized through a small incision in dorsal region. A 1.2 cm polyethylene cannula that was installed inside each sponge disc was exteriolized through needle puncture in the skin and secured in place by a 5-0 silk suture, and then plugged with a sterile polyethylene stopper. Postoperatively, the animals were monitored for any signs of infection at the operative site, discomfort or distress; any animal showing such signs was immediately sacrificed. Camel urine (25, 50 and 0.5 mg/kg/bw; 50 μL), including PBS, were administered daily 24 h post-sponge implantation through each of installed cannulas from day 1 to day 14. The control group of mice received vehicle (PBS).

5. Vascularization of implanted sponges

The extent of vascularization of sponge implants was assessed by the amount of hemoglobin detected in the tissue, using the Drabkin method [16,17]. At the 14th day post-implantation, groups of animals were euthanized and the sponge implants were excised carefully, released from the cannula, and weighed. Each implant was homogenized in 2 mL of Drabkin reagent and centrifuged at 12 000 × g for 20 minutes. The supernatants were filtered through a 0.22 mm Millipore filter. The hemoglobin concentration of the samples was determined spectrophotometrically by measuring absorbance at 540 nm using an ELISA plate reader and was compared against a standard hemoglobin curve. The content of hemoglobin in the implant was expressed as micrograms of Hb per milligram wet tissue.

6. Measurement of VEGF, TNF-α, IL-1β, IL-6, IL-10, TGF-β and MCP-1 production in the sponge implants

The implants were removed at day nine post implantation, homogenized in PBS pH 7.4 (2 mL) containing 0.05% Tween 20 and centrifuged at 10 000 × g for 30 minutes. The levels of cytokines VEGF, TNF-α, IL-1β, IL-6, IL-10, TGF-β and MCP-1 in the supernatant from each implant were measured in 50 μL of the supernatant using Bio-Plex Pro Mouse Cytokine 23-Plex Assay (Bio-Rad) according to the manufacturer's instructions. Cell-free supernatants (15 μL) and universal sample diluents in the kit (45 μL) were mixed and loaded onto a 96-well plate containing beads (each 50 μL). The raw data were first statistically compared by two-way analysis of variance (ANOVA). The results are expressed as ρg cytokine per milligram wet tissue.

7. Tissue extraction and determination of myeloperoxidase and *N*-acetylglucosaminidase activities

The extent of neutrophil accumulation in the implants was measured by assaying myeloperoxidase (MPO) activity as previously described [18,19]. After determining the hemoglobin concentration in the supernatant of the implants, a small part of the corresponding pellet was weighed, homogenized in 2 mL buffer pH 4.7 (0.1 M NaCl, 0.02 M NaH_2PO_4, 0.015 M Na-EDTA) and centrifuged at 12 000 × g for 10 minutes. The pellets were then resuspended in 0.05 M NaH_2PO_4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). MPO activity in the supernatant samples was assayed by the change in absorbance (optical density; OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). The reaction was terminated by the addition of 50 μL of H_2SO_4 (4 M). Results were expressed as change in OD per gram of wet tissue.

The infiltration of mononuclear cells into the implants was quantified by measuring the levels of the lysosomal enzyme *N*-acetylglucosaminidase (NAG), which is present in high levels in activated macrophages [15,16]. Part of the pellet that remained after the hemoglobin measurement was kept for this assay. These pellets were weighed, homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega), and centrifuged (3000 × g; 10 minutes at 4 °C). The resulting supernatant (100 μL) was incubated for 10 minutes with 100 μl of *P*-nitrophenyl-*N*-acetyl-beta-D-glucosaminide (Sigma) prepared in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO_4; pH 4.5) to yield a final concentration of 2.24 mM. The reaction was stopped by the addition of 100 μL of 0.2 M glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. The readings were interpolated on a standard curve constructed with *p*-nitrophenol (0–500 nmol/m) (SigmaAldrich). Data are reported as nanomole of products formed per milligram of wet tissue (implant).

8. Collagen measurement

Total soluble collagen was measured in whole tissue homogenates by the Sirius Red reagent based-assay [19,20]. The implants were homogenized in 1 mL of PBS and 50 mL of