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Anti-inflammatory action of angiotensin 1-7 in experimental colitis may be mediated through modulation of serum cytokines/chemokines and immune cell functions

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ABSTRACT

We recently demonstrated Ang 1–7 reduced inflammation in the dextran sulfate sodium (DSS) colitis model. In this study we examined the effect of Ang 1–7 on modulation of plasma levels of selected cytokines and chemokines and immune cell effector functions (apoptosis, chemotaxis and superoxide release) *in vitro*. The degree of neutrophil recruitment to the colon was assessed by immunofluorescence and myeloperoxidase activity. Daily Ang 1–7 treatment at 0.01 mg/kg dose which previously ameliorated colitis severity, showed a significant reduction in circulating levels of several cytokines and chemokines, and neutrophil recruitment to the colonic tissue. It also significantly enhanced immune cell apoptosis, and reduced neutrophil chemotaxis and superoxide release *in vitro*. In contrast, daily administration of the Ang 1–7R antagonist A779 which previously worsened colitis severity showed significant up-regulation of specific mediators. Our results demonstrate a novel anti-inflammatory action of Ang 1–7 through modulation of plasma levels of cytokines/chemokines and immune cell activity.

1. Introduction

There is a compelling body of evidence suggesting the importance of the renin-angiotensin-aldosterone system (RAAS) in the inflammatory response along with its already well-established role in the homeostatic control of cardiovascular, renal and adrenal functions (Fyhrquist and Saijonmaa, 2008; Geara et al., 2009). The angiotensin (Ang) 1–7/MAS receptor axis has received much attention in recent years due to its role in the control of blood pressure and cardiac function, and more importantly in view of the demonstration of its potent anti-inflammatory action in various animal models of inflammation such as diabetes (Yousif et al., 2014; Bossi et al., 2016), asthma (El-Hashim et al., 2012), atherosclerosis plaque (Dong et al., 2009) and arthritis (da Silveira et al., 2010). The anti-inflammatory properties of Ang 1–7 are thought to be mediated at least in part through a reduction in the level of several cytokines and chemokines (e.g. TNFα, IL-1β, MCP-1 and CXCL1), as well as the activity of key signaling molecules such as p38, ERK1/2 JNK, protein kinase C (PKC) and c-SRC kinase (Zhu et al., 2002; Tallant et al., 2005; Rebas et al., 2006; Su et al., 2006). We recently demonstrated that Ang 1–7 treatment (0.01–0.06 mg/kg) resulted in reduced colitis severity at gross and histological levels and that was associated with reduction in the colonic expression level of Ang II, and the activity of p38 MAPK, ERK 1/2 and Akt (the downstream target of PI3K). In addition, pharmacological inhibition of the endogenous effects of Ang 1–7 (by A779 treatment, at 1 mg/kg dose) aggravated colitis severity, highlighting the importance of this peptide in colitis pathogenesis (Khajah et al., 2016).

In the present study, we demonstrate that Ang 1–7 treatment (0.01 mg/kg) reduced while A779 treatment (1 mg/kg) aggravated colitis severity by modulating the plasma levels of various cytokines and chemokines, and neutrophil recruitment to the colonic tissue *in vivo*. Furthermore, Ang 1–7 treatment reduced neutrophil chemotaxis and superoxide release in response to WKYMVm (fMLP-peptide) stimulation, and increased spontaneous immune cell apoptosis *in vitro*.

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2. Materials and methods

2.1. Animals

BALB/c mice (6–10 weeks old, mean weight 20 g) were supplied by the Animal Resource Center of the Health Sciences Center at Kuwait University. All animals were kept under standard conditions including controlled temperature (25 °C), a 12-h light-dark cycle and had free access to food and drinking water *ad libitum*. All experimentations were approved by the Animal Care Committee at Kuwait University Health Sciences Center and conformed to their rules and regulations.

2.2. Induction of colitis and treatment protocols

Colitis was induced in mice as previously described (Khajah et al., 2016). Angiotensin fragment 1–7 acetate salt hydrate (Ang 1–7; m.wt 899; Sigma-Aldrich, St Louis, USA) was dissolved in 0.9% saline (vehicle) at 1 mg/ml and stored at −80 °C. A dose of 0.01 mg/kg [which previously demonstrated to exhibit anti-inflammatory effects (Khajah et al., 2016)] was freshly prepared from the stock each day of the experiment, and administered to mice by daily intra-peritoneal (i.p) injections in a volume of 500 μl per injection along with DSS treatment. A779 (MAS-1 R antagonist; m.wt 873; GenScript, USA) was similarly dissolved in distilled water at 1 mg/ml and stored at −80 °C. A freshly prepared dose of 1 mg/kg was administered to a second group of mice by daily i.p injections in a volume of 500 μl daily (for 4 days) along with DSS treatment. Plasma was separated by centrifugation and stored at −80 °C for subsequent analysis of various cytokines and chemokines (using Proteome Profile Kit from R&D Systems).

2.3. Analysis of cytokines and chemokines

The level of 40 different cytokines and chemokines was measured using a Proteome Profiler™ Mouse Cytokine Array Kit (Cat # ARY006 from R&D Systems Minneapolis, USA) following the manufacturer's protocol, in plasma samples. Briefly, nitrocellulose membranes with duplicate spots of selected capture antibodies were incubated in 2 ml of Array buffer 6 in 4-well multi-dishes for 1 h. For each membrane, the plasma from two mice (30 μl from each), was pooled, diluted with 500 μl of Array buffer 4 and 940 μl of Array buffer 6, mixed with 15 μl of reconstituted cocktail of biotinylated detection antibodies, and incubated overnight with the array membrane. The cytokine and detection antibody complex was bound to its cognate antibody that is located and immobilized on the membrane. After incubation, membranes were washed 3 times (10 min each) with 1x washing buffer. Diluted streptavidin-HRP was added to each membrane, incubated for 30 min and signal detected using the standard ECL method. Sample spot intensities obtained from each membrane were quantified by densitometry and normalized (for any loading differences) using the mean density of three reference spots provided on each membrane.

2.4. Immunofluorescence

Colon sections (5 μm) were deparaffinized, rehydrated through a series of washes in graded ethanol and water, followed by an antigen retrieval step (by boiling the sections in 10 mM sodium citrate buffer, pH 6.0 for 20 min). Sections were then incubated in blocking solution (5% bovine serum albumin (BSA) + 0.3% Triton X-100 in PBS) for 1 h, followed by incubation overnight at 4 °C with primary antibody [Gr1 (1:50 dilution), Thermo Scientific] diluted in 1% blocking solution. On the following day, sections were washed and incubated with secondary antibody conjugated to Alexa Fluor 555 (Goat anti rabbit SFX kit; Life Technologies, USA, 1:400 dilution) for 2 h at room temperature) in the dark. After washes in PBS sections were stained with 4',6 diamidino-2- phenylindole and mounted. Images were captured on a ZIESS LSM 700 confocal microscope and fluorescence intensity estimated in defined fields using Image J software package. It should be noted that all figures have the same un-processed parameters (intensity, contrast, brightness, etc.) so any changes observed are real and not due to software-aided manipulation of these images. We preferred to analyse low magnification images to get a better more representative picture of large areas of the colon rather than trying to count individual cells with high magnification images that would be selective and also blurrier, introducing errors in imaging.

2.5. Myeloperoxidase activity (MPO)

MPO is an enzyme that is used as a biochemical marker for granulocyte (mainly neutrophil) infiltration into the tissue (Qi et al., 2005). Colonic tissue samples were weighed, and then homogenized for 15 s (using a polytron tissue homogenizer) in ice-cold potassium phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH 6) containing 5 mg/ml hexadecyltrimethylammonium bromide (Sigma-Aldrich, St Louis, MO, USA) at a ratio of 1 ml of buffer to 50 mg tissue. Homogenates were decanted into sterile eppendorf tubes and centrifuged at 8000 rpm for 4 min. Supernatants were transferred to new eppendorf tubes and 200 μl of the reaction mixture [containing 16.7 mg of o-diansidine (Sigma-Aldrich, St Louis, MO, USA0, 90 ml of dH_2O, 10 ml MPO buffer and 50 μl of 30% H_2O_2] added to individual wells of a standard 96-well plate that contained 15 μl of the supernatant of the samples, standards and blanks (in triplicate). Immediately after the addition of o-diansidine, readings were taken at 5 min intervals for 30 min at 450 nm on a kinetic microplate reader, MPO activity was expressed in units/mg tissue; the amount needed to degrade 1 μmol of hydrogen peroxide/min at 25 °C is considered as one unit of MPO activity.

2.6. Isolation of bone-marrow derived neutrophils

Neutrophils were isolated from naïve mouse bone marrow as described previously (Lieber et al., 2004; Khajah et al., 2011). Briefly, mice were euthanized and the femurs and tibias dissected from the animals and the ends of bones removed. Marrow cells were flushed from the bones with ice-cold PBS and collected by centrifugation at 1300 rpm for 6 min at 4 °C. After re-suspension in 3 ml of 52% Percoll (GE Healthcare), the marrow cells were layered on a 3-step Percoll gradient (72%, 64%, and 52% plus cells), and centrifuged at 2600 rpm for 30 min at 4 °C. Purified neutrophils were removed from the layer between the 64 and 72% Percoll and washed once with ice-cold PBS and then suspended in RPMI containing 20% fetal bovine serum (FBS) at 10^7 cells/ml. Cytospin of the cell suspension after Percoll gradient purification was performed to identify the cell population as > 95% neutrophils.

2.7. Isolation of spleen derived mononuclear cells

The spleen was removed from naïve mice after cervical dislocation and placed in petri dishes containing 3 ml RPMI media with 20% FBS. The organ was cut into small pieces using scissors and passed several times through a 19 G needle; the homogenate was then filtered using a 200 μm mesh nylon screen (R&D Systems). The filtrate was centrifuged at 1000 g at 4 °C for 10 min and the pellet was re-suspended in 5 ml RBS lysis buffer and kept at 20 °C for 5 min. The pellet was then washed with ice-cold PBS and re-