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Research paper

Hyaluronan mediates the adhesion of porcine peripheral blood mononuclear cells to poly (I:C)-treated intestinal cells and modulates their cytokine production

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ABSTRACT

Hyaluronan (HA), a major component of the extracellular matrix (ECM), has been increasingly recognized as a regulator of inflammation. Its role is complex since it has pro- and anti-inflammatory actions by modulating the expression of inflammatory genes, the recruitment of inflammatory cells and the production of inflammatory cytokines, but also by attenuating the course of inflammation and providing protection against tissue damage. Certain viruses and other inflammatory stimuli induce organization of HA into cable-like structures, which may be responsible for leukocyte recruitment and, on the other hand, low molecular weight fragments of HA have been shown to activate various inflammatory responses.

The aim of the present study was to analyze the effects of a simulated infection with the viral mimetic Poly (I:C) on HA deposition on different porcine intestinal cells (primary colonic muscular smooth muscle cells (SMC), and epithelial IPEC-J2 and IPI-2I cell lines) and on the recruitment of peripheral blood mononuclear cells (PBMC) to intestinal cell layers. We show that Poly (I:C) treatment induces the formation of an HA-based pericellular matrix coat in muscular SMC and in intestinal epithelial cells (IECs) and that, on differentiated IPEC-J2 cells, HA accumulates in the basolateral membrane. Porcine PBMCs bind to Poly (I:C)-treated cells and this binding is dependent on HA, since the increase in adhesion is abolished by hyaluronidase treatment of the cell layers. A second goal was to study the effect of different molecular weight HA forms on the production of pro-inflammatory cytokines and chemokines (TNF-α, IL-1β and IL-8) by porcine PBMCs. Low molecular weight HA fragments (100–150 kDa), in contrast to high molecular weight HA (2500 kDa), stimulate the release of these pro-inflammatory mediators by porcine PBMCs. Our results suggest that HA is involved in the inflammatory response against pathogenic insults to the porcine gut.

1. Introduction

Hyaluronan or hyaluronic acid (HA) is a glycosaminoglycan polymer composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, and it is a major constituent of the extracellular matrix (ECM). HA, which is secreted by many cell types, is assembled at the plasma membrane by HA synthases (HAS) and extruded into the extracellular space. HA degradation typically occurs at the cellular level by a mechanism involving the cell surface receptor CD44 and hyaluronidases (HYAL). However, HA may also be degraded at sites of inflammation by reactive oxygen species and secreted hyaluronidases of bacterial origin (Stern et al., 2007).

Beyond its physical properties as a structural ECM molecule, HA provides cellular cues to regulate inflammation and tissue repair (Petrey and de la Motte, 2014). The biological activity of HA is dependent upon its molecular mass. Large HA polymers support tissue structure and integrity due to their hygroscopic and viscoelastic properties and serve to suppress the inflammatory response. In response to injury and inflammation, HA undergoes increased synthesis through induction of HAS and degradation to smaller molecular mass fragments by hyaluronidases (Jiang et al., 2011). Overall, HA in its fragmented form is a reflection of tissue under stress and exert pro-angiogenic, inflammatory, and immunostimulatory effects mediated by binding to its receptors (Erickson and Stern, 2012). CD44 is the main cell surface HA receptor (Aruffo et al., 1990) and is expressed on the plasma membrane of most cells. In

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addition to binding to CD44 and other receptors, HA also binds to Toll-like receptor (TLR) 2 and 4 (TLR2 and TLR4), components of the innate immune system (Jiang et al., 2007). Thus, HA exists as both a pro- and anti-inflammatory molecule *in vivo*, these contradictory functions being determined by polymer length and the receptors HA engages.

Increased accumulation of HA, together with leukocyte recruitment is a consistent feature during inflammatory diseases (Petrey and de la Motte, 2014), including intestinal- affecting processes such as inflammatory bowel disease in humans (De la Motte et al., 2003) or experimental dextran sulfate sodium-induced colitis in rats (Kessler et al., 2008). Infection with respiratory syncytial virus (RSV) or measles virus also increase cell surface HA (De La Motte et al., 1999). In several cell types *in vitro*, cellular stress induced by virus or the double stranded RNA viral mimic polyinosinic:polycytidylic acid (Poly (I:C)), other ER stressors or hyperglycemia increase cell surface HA deposition and the formation of long cable-like structures that are important for leukocyte attachment since they have binding properties not featured by the normal HA found in the pericellular glycocalyx (De La Motte et al., 1999; Evanko et al., 2009; Jokela et al., 2008; Lauer et al., 2009; Majors et al., 2003; Stober et al., 2014; Potter-Perigo et al., 2010; Wang and Hascall, 2004). Adhesion of leukocytes to these HA forms is mediated by binding to the receptor CD44 (De La Motte et al., 1999; Mohamadzadeh et al., 1998). It is believed that these cable-like structures correspond *in vivo* to cross-linked forms of HA that would constitute a protective mechanism for tissues undergoing inflammation by keeping immune cells bound to these matrices in an inactivated state (Day and de la Motte, 2005).

On the other side, HA has been shown to undergo rapid degradation at sites of inflammation and low molecular weight degradation products are able to elicit various proinflammatory responses in immune cells and other cell types, at least in part through TLR4 (Termeer et al., 2002; Taylor et al., 2004; Yamawaki et al., 2009; Campo et al., 2010; Jiang et al., 2011).

Infectious diseases are of great importance for pig industry due to increased medication costs and decreased food conversion or even death of the animals, all leading to high economic losses. The involvement of HA and other ECM components in the modulation of inflammation has become increasingly evident over the years, however, little is known about the role of HA as mediator of inflammation in porcine cells.

The objectives of the present study have been (a) to investigate the effect of the viral mimetic Poly (I:C) on HA deposition on different porcine intestinal cells (primary colonic muscular smooth muscle cells (SMCs), and intestinal epithelial IPEC-J2 and IPI-2I cells lines, and b) to study the effects of different molecular weight HA forms on the production of pro-inflammatory mediators (TNF-α, IL1-β, IL-6 and IL-8) by porcine PBMCs.

2. Materials and methods

2.1. Cell lines and cell culture

Porcine intestinal epithelial cell (IEC) lines IPI-2I and IPEC-J2 were obtained from Dr. Cristina Arce (Universidad de Córdoba, Spain) and Prof. Anthony Blikslager (North Carolina State University, Raleigh, NC, USA). The IPEC-J2 is a non-transformed cell line derived from the epithelium of a neonatal, unsuckled piglet jejunum (Rhoads et al., 1994). The IPI-2I is an SV40-immortalized cell line derived from the ileum of an adult boar (Kaeffer et al., 1993). IPEC-J2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (1:1) medium (Invitrogen TM Life Technologies, Carlsbad, CA, USA) supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (all from PAA Laboratories GMbH, Pasching, Austria). IPI-2I cells were cultured in DMEM/F-12 (1:1) medium supplemented with 10% FCS, 4 mM L-glutamine (PAA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown in a humidified atmosphere at 37 °C with 5% CO_2. IPEC-J2 cells were used between passages 65–85; IPI-2I cells were used between passages 40–65.

Porcine primary colon mucosal smooth muscle cells (SMCs) were prepared as described by De la Motte et al. (2003) with modifications. Colon samples were obtained from 6-month old Large White healthy pigs undergoing experimental surgery at the Facultat de Veterinària (UAB). Samples were thoroughly rinsed with sterile PBS in order to remove any remaining blood and lumen content. After cleaning, the muscle cell layer was removed, minced and digested in medium with 0.2 mg/ml type IA collagenase from *Clostridium histolyticum* (Sigma, St. Louis, MO), 0.1 mg/ml DNase (Roche, Manheim, Germany), 250 U/ml penicilin, 250 μg/ml streptomycin (PAA) and 0.625 μg/ml fungizone for 3 h at 37 °C under agitation. The supernatant was separated from the rest of non-digested tissue and centrifuged at 300*g* for 10 min. The resulting pellet was resuspended in DMEM/F-12 culture medium supplemented with 10% FCS, 250 U/ml penicilin, 250 μg/ml streptomycin and 0.625 μg/ml fungizone and seeded in 25 cm^2 culture flasks and cultured in a humidified atmosphere at 37 °C with 5% CO_2. All the procedure was performed under sterile conditions. The identity of SMC was confirmed by immunocytochemistry using an antibody against α-SMA. Primary cells were used between passages 2 and 4.

2.2. Treatment of cells with poly (I:C) and HA staining

Porcine primary colon mucosal SMCs and IEC lines (IPI-2I and IPEC-J2) were seeded in 24-well plates containing coverslips in the corresponding culture medium (2×10^4 cells/well and 5×10^4 cells/well respectively) and left overnight for attachment. Then the culture medium was removed and replaced by fresh culture medium containing 20 μg/ml of Poly (I:C) for 18 h. Control cells were incubated in parallel in culture medium without the viral mimic.

After treatment, cells were rinsed with phosphate-buffered saline (PBS) and fixed in methanol for 5 min at −20 °C. After rinsing with PBS, non-specific binding sites were blocked with 1% BSA in PBS for 30 min at room temperature. Cells were then incubated with 1 μg/ml of biotinylated hyaluronan-binding protein (HABPb) (Seikagaku, Tokyo, Japan) in PBS containing 0.1% BSA overnight at 4 °C. Subsequently, cells were washed in PBS and incubated with streptavidin Alexa-488 (Molecular Probes, Invitrogen, Eugene, OR) 1 in 1000 dilution in PBS. Nuclei were visualized by incubating samples with 10 μg/ml bisbenzimide trihydrochloride (Hoechst 33342, Sigma) for 3 min. Cells were washed and mounted in Fluorsave mounting medium (Calbiochem). Slides were examined using a confocal laser scanning microscope Olympus FluoView™ FW1000 (Olympus, Hamburg, Germany) with an objective Plan Apo 60× (NA 1.4, oil) using the Olympus FW10-ASW version 1.7.c software (Olympus). Alternatively, after incubation with the HABPb, cells were incubated with avidin-biotin-peroxidase complex (Immunopure ABC Standard Peroxidase Staining Kit, Pierce, Rockford, Illinois, USA). The reaction was developed using 3,3′-diaminobenzimide as peroxidase substrate (Liquid DAB + Substrate Chromogen System, Dako) and cells were counterstained with Mayer's haematoxylin (Merck, Darmstad, Germany). Cells were washed in tap water for 10 min, dehydrated in increasing concentrations of alcohols and mounted in DPX mounting medium (Sigma). Cells were examined with a Leica DM-6000 microscope and photographed with an integrated Leica DFC480 digital camera system using the Leica IM50, Version 4.0 Release 117 software (Leica Microsystems, Wetzlar, Germany).