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Research paper

Ovis aries CR4 is involved in *Mannheimia haemolytica* leukotoxin-induced cytotoxicity

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ABSTRACT

Pneumonia caused by *Mannheimia haemolytica* is an important disease of domestic sheep (DS, *Ovis aries*) and cattle (BO). *M. haemolytica* is a normal commensal of the upper respiratory tract in ruminants, but during stress and viral infection it breaches the host innate mucosal defense and descents into lungs causing fibrinous pleuropneumonia. Leukotoxin (Lkt) produced by *M. haemolytica* is cytolytic to all subsets of ruminant leukocytes. Earlier, we and others have shown that DS and BO LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) can mediate Lkt-induced cytolysis. It is not clear whether CR4 (CD11c/CD18), which is involved in chemotaxis, phagocytosis and regulates host immune response can also mediate Lkt-induced cytolysis in ruminants. The host innate immune response to *M. haemolytica* is poorly understood and the involvement of CR4 in *M. haemolytica* pathogenesis is one of the most understudied. This problem is further compounded by the lack of *cd11c* genes from any ruminant species. Therefore, the objectives of this study were to clone *cd11c* and determine whether CR4 can serve as a receptor for Lkt. In this direction we cloned two alleles of *cd11c* gene from leukocytes isolated from DS blood by RT-PCR. Transfectants developed expressing functional DS CR4 were found to be cytotoxic to Lkt from four different isolates of *M. haemolytica*. This is the first report confirming the ability of a recombinant ovine CR4 to bind to *M. haemolytica* Lkt and mediate concentration-dependent lysis of host cells, thus, confirming their role in *M. haemolytica* pathogenesis. This is a critical step in understanding host innate immunity and the management of pneumonia in sheep.

1. Introduction

Mannheimia haemolytica is the principal bacterial pathogen involved in respiratory disease complex of ruminants (Clinkenbeard et al., 1989; Curtis et al., 1989; Frank, 1989; Griffin, 1997; Bowland and Shewen, 2000; Jeyaseelan et al., 2002; Poulsen et al., 2006). Although, *M. haemolytica* is a normal commensal of upper respiratory tract of ruminants, but during stress and in immunocompromised animals they migrate into lungs causing acute fibrinous pleuropneumonia or "shipping fever", which causes substantial losses to US sheep (54%) and cattle industry (>$1 billion) (Bowland and Shewen, 2000; NAHMS, 2002; Snowder et al., 2006). In sheep, *M. haemolytica* causes mainly pneumonia (Gilmour and Gilmour, 1989), but they have also been isolated from mastitis, myocardium and the brain of healthy animals (Angen et al., 1999). In addition, it causes disease in other ruminants, including goats, deer, bison, and wild sheep (Midwinter et al., 1985; Jaworski et al., 1998). *M. haemolytica* produces several virulence factors including leukotoxin (Lkt), lipopolysaccharide (LPS), adhesins, outer membrane proteins and neuraminidases (Frank and Tabatabai, 1981; Murphy et al., 1995, 1998; Breider

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et al., 1990; Highlander, 2001; Gioia et al., 2006). Lkt is cytolytic to all the leukocyte subsets of domestic sheep (DS) and other ruminants (Li et al., 1999; Jeyaseelan et al., 2000). Previously, we and others have identified β_2 integrins as the receptor for Lkt on ruminant leukocytes (Ambagala et al., 1999; Li et al., 1999; Dileepan et al., 2005; Dassanayake et al., 2007a). β_2 integrins, which are unique to leukocytes, have a common β subunit, CD18, that associates with three distinct α chains, CD11a, CD11b, and CD11c to give rise to three well-characterized β_2 integrins: CD11a/CD18 (LFA-1, lymphocyte function-associated antigen 1); CD11b/CD18 (Mac-1 or CR3, complement receptor 3) and CD11c/CD18 (CR4 or p150/95) (Springer et al., 1986).

Of the various functions performed by integrins, they are critical for cell–pathogen interactions and play a vital role in leukocyte trafficking, migration, immunological synapse formation, co-stimulation and phagocytosis (Springer, 1990). The integrin CR4 is predominantly expressed by cells of myeloid origin, also on monocytes, dendritic cells, natural killer cells and on the surface of stimulated T and B cells (Springer et al., 1979; Miller et al., 1986; Keizer et al., 1987; Vorup-Jensen et al., 2003). CR4 expression is induced by cell-differentiation promoting agents such as retinoic acid, phorbol 12-myristate 13-acetate (PMA) and vitamin D3 (Miller et al., 1986; Corbi et al., 1987; Duits et al., 1991). Ligand binding repertoire of CR4 includes fibrinogen, complement protein iC3b, and intercellular adhesion molecule (ICAM-1), lipopolysaccharide (Wright and Jong, 1986; Loike et al., 1991; Vorup-Jensen et al., 2003; Luo et al., 2007). CR4 in particular is involved in chemotaxis and phagocytosis, which in turn regulate host immune response (te Velde et al., 1987; Springer, 1990), and an integral part in understanding *Mannheimia* pathogenesis of sheep and other ruminants.

In order to elucidate the interaction of *M. haemolytica* Lkt and other virulence factors with the different β_2 integrins, we had earlier cloned and characterized HEK-293 cells expressing recombinant DS LFA-1 and Mac-1 (Lawrence et al., 2007, 2008). Since native leukocytes express all three β_2 integrins which modulate host–pathogen interaction, it is important to understand the role of CR4 in *Mannheimia* pathogenesis without interference from the other two (LFA-1 and Mac-1) receptors in mediating innate immune response. To understand this molecular mechanism it was necessary to express functional DS (*Ovis aries*) CR4 receptor in a heterologous system devoid of Lkt receptors. Further, the lack of *cd11c* gene (s) (IGTAX) from any ruminant species prompted us to first clone this gene and characterize this gene from DS. Towards this goal, we cloned two alleles of *cd11c* gene (FJ601824 and FJ601825) from leukocytes isolated from DS blood by RT-PCR. Transfectants were developed expressing functional DS CR4 and were found to be cytotoxic to Lkt from *M. haemolytica*, serotypes A2 (bovine and ovine origin), A6 (ovine isolate) and A1 (bovine isolate). This is the first report confirming the ability of a recombinant ovine CR4 to bind to *M. haemolytica* Lkt and mediate concentration-dependent lysis of host cells, thus confirming their role in *M. haemolytica* pathogenesis.

2. Materials and methods

2.1. Cell lines and growth conditions

The human embryonic kidney cell line, HEK-293 (ATCC® Number: CRL-1573™) was cultured in complete culture medium (DMEM medium [Invitrogen] supplemented with 10% [v/v] heat-inactivated fetal bovine serum along with L-glutamine 4 mM and gentamicin 50 mg ml^{-1} [Sigma]). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. HEK-293 cells stably transfected with DS CR4 (CD11c/CD18) were selected and maintained in complete culture medium containing selection antibiotics; geneticin, 800 μg/ml (G418; Invitrogen) and blasticidin, 30 μg/ml (InvivoGen).

2.2. Monoclonal antibodies and leukotoxin

The monoclonal antibodies (MAbs) specific for ruminant CD11c (BAQ153A, IgM) (Stone et al., 1997) and human CD18 (HUH82A, IgG2a) which cross-reacts DS CD18 (Saalmuller et al., 2005), were obtained from Washington State University Monoclonal Antibody Center. Fluorescein isothiocyanate (FITC) conjugated Lkt non-neutralizing MAb MM605 (IgG2a) developed earlier in our laboratory (Gentry and Srikumaran, 1991) was used to demonstrate Lkt binding to CR4 receptor. The MAbs BAQ 111A (IgM) specific for bovine CD8 (Howard and Naessens, 1993) purchased from VMRD, and MM113 (IgG2a) specific for bovine herpesvirus 1 (Srikumaran et al., 1990) obtained from the Department of Veterinary and Biomedical Sciences at the University of Nebraska-Lincoln were used as isotype-match controls. The Lkt from *M. haemolytica* (serotypes; A1, A2 and A6) was prepared as described earlier (Gentry and Srikumaran, 1991). The culture supernatant containing Lkt was filter-sterilized and stored at −20 °C in aliquots until needed. Same batch of Lkt was used in all the experiments.

2.3. Ovis aries cd11c cloning

DS *cd11c* gene was cloned from total RNA isolated from leukocytes separated from peripheral blood (Deshpande et al., 2002). Overlapping primers were designed and cDNA cloning was performed by the Center for Functional Genomics, University at Albany, NY. Briefly, total RNA was isolated using TRIzol® (Invitrogen) and cDNA synthesized using Ambion Retroscript kit containing MMLV-reverse transcriptase. Series of overlapping primers were synthesized based on horse (*Equus caballus*) *cd11c* homology (Espino-Solis et al., 2008). The 5′ and 3′ clones were joined to make full length coding sequence at Bst XI restriction site. The two alleles were cloned into plasmid vector, selected on 100 μg/ml ampicillin plates (named as, pITGAXallele1 and allele2). The two alleles encoding DS *cd11c* (GenBank accession no. FJ601824 and FJ601825) were cloned into mammalian expression vector, pcDNA6.2/GW/D-TOPO (Invitrogen) by PCR using flanking primers. Briefly, 10 ng plasmid DNA from pITGAXallele1 and pITGAXallele2 was used as template and amplified using PfuUltra™ II Fusion HS DNA polymerase (Strata-