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Research paper

Immunological characterization of the equine airway epithelium and of a primary equine airway epithelial cell culture model

Ayshea M. Quintana[a,b], Gabriele A. Landolt[a,b], Kristina M. Annis[b], Gisela Soboll Hussey[b,*]

[a] Department of Clinical Sciences, Colorado State University, Fort Collins, CO, United States
[b] Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, United States

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ABSTRACT

Our understanding of innate immunity within the equine respiratory tract is limited despite growing evidence for its key role in both the immediate defense and the shaping of downstream adaptive immune responses to respiratory disease. As the first interface to undergo pathogen invasion, the respiratory epithelium is a key player in these early events and our goal was to examine the innate immune characteristics of equine respiratory epithelia and compare them to an *in vitro* equine respiratory epithelial cell model cultured at the air–fluid interface (AFI). Respiratory epithelial tissues, isolated epithelial cells, and four-week old cultured differentiated airway epithelial cells collected from five locations of the equine respiratory tract were examined for the expression of toll-like receptors (TLRs) and host defense peptides (HDPs) using conventional polymerase chain reaction (PCR). Cultured, differentiated, respiratory epithelial cells and freshly isolated respiratory epithelial cells were also examined for the expression of TLR3, TLR9 and major histocompatibility complex (MHC) class I and class II using fluorescence-activated cell sorting (FACS) analysis. In addition, cytokine and chemokine profiles from respiratory epithelial tissues, freshly isolated respiratory epithelial cells, and cultured, differentiated, epithelial cells from the upper respiratory tract were examined using real-time PCR. We found that respiratory epithelial tissues and isolated epithelial cells expressed TLRs 1–4 and 6–10 as well as HDPs, MxA, 2′5′ OAS, β-defensin-1, and lactoferrin. In contrast, epithelial cells cultured at the AFI expressed TLRs 1–4 and 6 and 7 as well as MxA, 2′5′ OAS, β-defensin-1, but had lost expression of TLRs 8–10 and lactoferrin. In addition, MHC-I and MHC-II surface expression decreased in epithelial cells cultured at the AFI compared to isolated epithelial cells whereas TLR3 and TLR9 were expressed at similar levels. Lastly, we found that equine respiratory epithelial cells express an array of pro-inflammatory, antiviral and regulatory cytokines and that after four weeks of *in vitro* growth conditions, equine respiratory epithelial cells cultured at the AFI retained expression of GM-CSF, IL-10, IL-8, TGF-β, TNF-α, and IL-6. In summary, we describe the development of an *in vitro* equine respiratory epithelial cell culture model that is morphologically similar to the equine airway epithelium and retains several key immunological properties. In the future this model will be a used to study equine respiratory viral pathogenesis and cell-to-cell interactions.

Abbreviations: AFI, air–fluid interface; FACS, fluorescence-activated cell sorting; TLR, toll-like receptor; HDP, host defense peptides; EIV, equine influenza virus; EHV-1, equine herpesvirus-1; EREC, equine respiratory epithelial cell; PRR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; TEER, transepithelial electrical resistance.

* Corresponding author at: Department of Clinical Sciences, Colorado, State University, 300 W Drake Rd, Fort Collins, CO 80523-1678, United States. Tel.: +1 970 297 4284; fax: +1 970 297 1275.
E-mail address: husseygs@colostate.edu (G.S. Hussey).

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1. Introduction

Respiratory pathogens such as equine influenza (EIV) and equine herpesvirus-1 (EHV-1) continue to have serious health and economic impacts on the equine industry (Barbic et al., 2009; Kydd et al., 2006), despite vaccination. While in recent years we have made progress understanding the adaptive immune response to these common respiratory pathogens (Kydd et al., 2006; Paillot et al., 2007), the innate immune response remains poorly characterized. Elucidating the innate immune response to these viruses is important, as the early events following infection not only determine whether the virus can establish infection but also set the stage for downstream adaptive immune responses. Initiation of innate immunity relies on the activation of germline-encoded pattern recognition receptors (PRRs) through the recognition of pathogen-associated molecular patterns (PAMPs) (Carpenter and O'Neill, 2007). Toll-like receptors (TLRs) are classic PRRs that are found on immune cells as well as mucosal epithelia exposed to invading pathogens. Thirteen mammalian TLRs have been identified and TLRs 2–4 and 7–9 have been identified in horses (Astakhova et al., 2009; Zhang et al., 2008; Carpenter and O'Neill, 2007; Singh Suri et al., 2006). These TLRs function to recognize various pathogen ligands including lipopolysaccharides, diacylated and triacylated lipopeptides, flagellin, single-stranded and double stranded RNA, and CpG motifs among others (Astakhova et al., 2009; Carpenter and O'Neill, 2007). Recognition of invading pathogens by PRRs results in a signaling cascade that leads to the production of cytokines, chemokines, and host defense peptides (HDPs). Cytokines and chemokines are key mediators that initiate immediate immune responses and ultimately shape the adaptive immune responses via chemoattraction and activation of immune cells, and up-regulation of co-stimulatory molecules for antigen presentation (Carpenter and O'Neill, 2007). Host defense peptides are small proteins with demonstrated antimicrobial and antiviral activity (Sun et al., 2005; Townes et al., 2009). Moreover, HDPs have been shown to activate monocyte-derived dendritic cells and modulate IFN-γ production in antigen-presenting cells suggesting they may also play a role in the shaping of adaptive immunity (Nijnik et al., 2009; Presicce et al., 2009).

For respiratory pathogens, including EHV-1 and EIV, the respiratory epithelium is the first to encounter and interact with invading pathogens and initiate early immune responses. In recent years it has become evident that as this first line of defense, epithelial cells are multifunctional and play an important role in immunity, in addition to providing a physical barrier (Smith et al., 1990). Epithelial cells have been shown to express TLRs, secrete host defense peptides, cytokines and chemokines, and even present antigen (Glanville et al., 1989; Schaefer et al., 2004; Smith et al., 1990). In addition, innate immune recognition by the epithelial cell barrier largely determines the functional properties of resident tissue macrophages and dendritic cells, thus driving the outcome of antigen-specific immunity (Mayer et al., 2008; Smith et al., 1990). While respiratory epithelia of many mammalian species have been studied recently and have been found to express TLRs, HDPs, cytokines and MHC class I and II molecules (Glanville et al., 1989; Lin et al., 2007; Smith et al., 1990), equine immunity at the respiratory epithelial cell barrier remains poorly described.

Investigation of this important area has been hindered by a lack of a suitable *in vitro* model. Conventional models of respiratory epithelial cell cultures were established by fully immersing cells in media (Goldman and Baseman, 1980; Harmon et al., 1977). One limitation to this culture method is the loss of respiratory epithelial cell differentiation over time (Heckman et al., 1978; Nevo et al., 1975). To address this limitation, a biphasic hamster airway epithelial cell culture model provided a powerful new technique to culture airway epithelial cells that maintained characteristics of cell differentiation (Whitcutt et al., 1988). Airway epithelial cell culture models grown at the air–fluid interface (AFI) have since been developed for other species as a tool to study allergy and infectious disease (Clark et al., 1995; Vandekerckhove et al., 2009; You et al., 2002). Primary equine airway epithelial cells have been successfully isolated using enzymatic digestion with trypsin and cultured submerged in liquid media (Shibeshi et al., 2008). In addition, a later study demonstrated the expression of cell surface beta-adrenergic receptors important in regulating inflammation in equine respiratory epithelial cells (Abraham et al., 2010). Moreover culture of differentiated equine bronchial epithelial cells cultured at the AFI has been described (Schwab et al., 2009). Morphologically, respiratory epithelial cells cultured at the AFI appear to mimic the equine airway epithelium as a heterogeneous population of ciliated cells, basal cells, and secretory cells generating a pseudo-stratified mucociliary epithelium, contrary to what is seen with respiratory epithelial cells cultured submerged (Rowe et al., 2004). In addition, secretion of mucin proteins has been characterized in differentiated respiratory epithelial cell cultures grown at the AFI (Rowe et al., 2004; Schwab et al., 2009). The formation of tight junctions, which is characteristic for epithelial cells and creates the physical barrier, can be demonstrated by transepithelial electrical resistance (TEER) measurement (Rowe et al., 2004). Finally, these cultures have been demonstrated to support viral infection and replication (Newby et al., 2006; Sajjan et al., 2008).

Despite evidence that respiratory epithelial cells grown at the AFI mimic the equine airway epithelium morphologically and submerged equine epithelial cells express functional beta-adrenergic receptors, little information exists as to how these cultures perform immunologically. So far, toll-like receptor and host defense peptides expression has only been described in human airway epithelial cells cultured at the AFI (Hertz et al., 2003). While constitutive cytokine expression has been demonstrated in respiratory epithelial tissues and conventional primary respiratory epithelial cell cultures (Smith et al., 1990), no such information is available for differentiated airway epithelial cells cultured at the AFI. The goals of this study were to establish baseline immunological characteristics in equine respiratory epithelial cells cultured at the AFI and compare them to the equine airway epithelium. In the future, this model will be used to study host immunity to