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Small molecule inhibitors of the host cell COX/AREG/EGFR/ERK pathway attenuate cytomegalovirus-induced pathogenesis

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ABSTRACT

As with other herpesviruses, human cytomegalovirus (hCMV) has the ability to establish lifelong persistence and latent infection following primary exposure, salivary glands (SMGs) being the primary site of both. In the immunocompromised patient, hCMV is a common cause of opportunistic infections, and subsequent morbidity and mortality. Elucidating the molecular pathogenesis of CMV-induced disease is critical to the development of more effective and safer drug therapies. In the present study, we used a novel mouse postnatal SMG organ culture model of mCMV-induced dysplasia to investigate a candidate signaling network suggested by our prior studies (COX-2/AREG/EGFR/ERK). The objective was to employ small molecule inhibitors to target several key steps in the autocrine loop, and in this way ameliorate pathology. Our results indicate that upregulation of ERK phosphorylation is necessary for initial mCMV-induced pathogenesis, and that ErbB receptor family phosphorylation and downstream signaling are highly relevant targets for drug discovery.

Introduction

Nearly a century ago, pathologists reported that postmortem tissue examination of infants less than 1 year of age often revealed inclusion bodies in submandibular salivary glands (SMGs) and, less frequently, in liver, lung, kidney, and thyroid. The large cells ("cytomegalia") were found in acini and ducts of affected SMGs and the ducts were often dilated. By the 1950s, human cytomegalovirus (hCMV), a prototypical β-herpesvirus, was isolated and it became apparent that hCMV infection was common, 50–95% of adults being seropositive.

hCMV primary, recurrent and secondary infections are associated with variant adverse consequences, from asymptomatic viruria in immunocompetent hosts to serious congenital disorders (deafness, blindness, mental retardation) in newborns, infants, and toddlers. Further, hCMV is a common cause of frequent opportunistic infections in the immunocompromised patient (Kim et al., 2010; Mori and Kato, 2010), a significant contributing factor to morbidity and mortality. For example, in patients undergoing hematopoietic stem cell transplantation or receiving immunosuppressive chemotherapy, the risk of hCMV infection is 20–30% (Yahav et al., 2009).

As with other herpesviruses, hCMV has the ability to establish lifelong persistence and latent infection following primary exposure (Nichols and Boeckh, 2000), salivary glands being the primary site of both (Wagner et al., 1996; Nichols and Boeckh, 2000). hCMV shed in saliva from infected salivary glands are a key source for the etiology of oral and systematic disease in immunocompromised patients (Correia-Silva et al., 2007, 2010). Clearly, halting hCMV replication and survival in the salivary gland is key to eliminating hCMV oral infection and transmission. To date, there has been very limited success in developing an hCMV vaccine. Alternatively, four drugs have been licensed to treat adult hCMV infection, with intravenous ganciclovir being the treatment of choice (Schleiss and Choo, 2006; Andrei et al., 2008; Cheeran et al., 2009). Each of these compounds has significant toxicities that limit their use. Thus, there is an urgent need to develop new anti-CMV therapies.

Strict CMV species-specificity has hindered the study of hCMV in animal models. Nevertheless, since mouse CMV (mCMV) has many features in common with hCMV, and mCMV infection of mice resembles its human counterpart with respect to pathogenesis, the mouse animal model (in vitro and in vivo) has been extensively used to understand the pathogenesis of acute, latent, and recurrent infections (e.g. Reynolds et al., 1993; Lagenaur et al., 1994; Schmader et al., 1995; Krmpotic et al., 2003; Melnick et al., 2006; Pilgrim et al., 2007; Jaskoll et al., 2008a, 2008b; Bai et al., 2008; Kasman et al., 2009). As with humans, the SMG is the major target organ for mCMV replication in the infected mouse. Some insight into the cell and molecular pathogenesis of mCMV-infected SMGs has emerged from our study of mCMV-infected fetal SMGs (Melnick et al., 2006). CMV, in its intracellular habitat, exploits and subverts a variety of host cell

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factors for survival and growth in an otherwise hostile cellular environment (Melnick et al., 2006; Sanchez and Spector, 2008). Studies of mCMV-infected fetal SMGs suggest that prominent among these are receptor kinase pathways and activated NFκB target gene pathways (Melnick et al., 2006). These findings recommend a newly emerging drug discovery paradigm that identifies and targets hijacked host factors, in contrast to canonical pathogen-targeting strategies (Andrei et al., 2008; Schwegmann and Brombacher, 2008).

Though cellular signaling pathways may seem obvious targets for therapeutic intervention, such strategies are complicated by the fundamental problem of interrelating genomics, proteomics, and phenotype in complex disease. To approach this conundrum, we have recently developed a novel mouse postnatal SMG organ culture model of mCMV-induced pathology (Jaskoll et al., 2011). This CMV-induced "sentinel neoplasia" model provides an ideal system for investigating virally-induced dysregulation of multiple host cell signaling pathways, focusing on a network of interactions between genes and pathology. Moreover, since the three dimensional associations between acinar, ductal and stromal cells are maintained, this postnatal SMG organ culture permits delineation of the cell-specific localization of important molecules with progressive infection and identifies changes in pathway components in a variety of cell types, thus providing evidence for the physiologic relevance of those components.

In the present study, we investigated a signaling network previously suggested in studies of CMV-induced fetal SMG dysplasia (Melnick et al., 2006), hypothesizing that this network would be highly relevant to postnatal CMV-induced tumorigenesis (Jaskoll et al., 2011). The objective of this study was to use small molecule inhibitors to target several key steps in the cognate COX-2/AREG/EGFR/ERK autocrine loop, and in this way ameliorate pathology. Our results strongly indicate that the upregulation of ERK phosphorylation is necessary for initial mCMV-induced postnatal SMG pathogenesis, and that ErbB-family phosphorylation and downstream signaling are highly relevant targets for drug therapy.

Materials and methods

Animals

Timed pregnant inbred C57/BL6 female mice (Charles River, Wilmington, MA) were purchased from Charles River (Wilmington, MA) [plug day = day 0 of gestation] and newborn mice were harvested as previously described (Melnick et al., 2006, 2009). All protocols involving mice were approved by the Institutional Animal Care and Use Committee (USC, Los Angeles, CA).

Organ culture

Newborn SMGs were dissected and cultured for 6 or 12 days using a modified Trowell method and BGJb medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 0.5 mg ascorbic acid/ml and 50 units/ml penicillin/streptomycin (Invitrogen Corporation), pH 7.2 as previously described (Melnick et al., 2006). For mCMV infection, SMGs were incubated with 1×10^{-5} plaque-forming units (PFU)/ml of *lacZ*-tagged mCMV RM427+ in BGJb on day 0 for 24 h and then cultured in virus-free media for a total of 6 or 12 days in culture; controls consisted of SMGs cultured in control medium for the entire period. SMGs were collected and processed for hematoxylin and eosin histology, qRT-PCR, Western blot analysis, immunolocalization, or cell proliferation (PCNA) analysis. For histology, immmunolocalization, and PCNA analysis, SMGs were fixed for 4 h in Carnoy's fixative at 4 °C or overnight in 10% neutral buffered formalin at room temperature, embedded in paraffin, serially-sectioned at 8 μm and stained as previously described (Melnick et al., 2006).

Cell proliferation assay

The cell-specific localization of PCNA (proliferating cell nuclear antigen) was determined using the Zymed mouse PCNA kit (Invitrogen Corporation) and counterstained with hematoxylin and eosin essentially as previously described (Melnick et al., 2006). In this set of experiments, the cytoplasm appears blue and PCNA-positive nuclei appear dark brown. For cell proliferation analysis, 3–8 SMGs per treatment per day were analyzed.

Quantitative RT-PCR

For analysis of gene expression, quantitative RT-PCR (qRT-PCR) was conducted as previously described (Melnick et al., 2006). We performed quantitative RT-PCR on NB + 6 control and mCMV-infected SMG samples; each sample consisted of 3–4 pooled explants. RNA was extracted and 1 μg RNA was reverse transcribed into first strand cDNA using ReactionReady™ First Strand cDNA Synthesis Kit: C-01 for reverse transcription (SABiosciences, Frederick, MD). The primer sets used were prevalidated to give single amplicons and purchased from SABiosciences (Frederick, MD): AREG (PPM02976A); Cox 2 (Ptgs2; Cat # PPM03647A); EGFR (PPM03714A); ERK1 (Mapk3; Cat. # PPM03585A); PCNA (Cat. # PPM03456A). Primers were used at a concentration of 0.4 μM. The cycling parameters were 95 °C, 15 min; 40 cycles of (95 °C, 15 s; 55 °C, 30–40 s and 72 °C, 30 s). Specificity of the reactions was determined by subsequent melting curve analysis. RT-PCRs of RNA (not reverse transcribed) were used as negative controls. GAPDH was used to control for equal cDNA inputs and the levels of PCR product were expressed as a function of GAPDH. The relative fold changes of gene expression between the gene of interest and GAPDH, or between the NB + 6 control and mCMV-infected SMGs, were calculated by the $2^{-\Delta\Delta CT}$ method.

Immunolocalization

Cultured SMGs were fixed for 4 h in Carnoy's fixative at 4 °C, embedded in low melting point paraplast, serially-sectioned at 8 μm and immunostaining was conducted as previously described (Melnick et al., 2006, 2009) using the following polyclonal rabbit antibodies: pERK1/2 (Thr202/Tyr204), pEGFR (Tyr1173), Amphiregulin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and Cox-2 (Cayman Chemicals, Ann Arbor, MI). Sections were incubated with biotin-labeled rabbit IgG (Fab fragment)(MP Biomedical, Aurora, OH) and then with Alexa-Fluor-labeled streptavidin (Invitrogen Corporation). Nuclei were counterstained with DAPI (Invitrogen Corporation). Negative controls were performed in parallel under identical conditions and consisted of sections incubated without primary antibodies. For each treatment group, 5–10 SMGs per day were analyzed.

Western blot analysis

NB + 6 uninfected (control), mCMV-infected, DCF-treated mCMV-infected, and GEF-treated mCMV-infected SMGs were collected; each independent sample consisted of 3–4 explants per group. Proteins (25–35 μg) were separated by SDS-PAGE gels and transferred to a PVDF membrane, and the membranes were subjected to chemiluminescence detection (ECL) according to the manufacturer's instructions (Thermo-Scientific, Rockford, IL) as previously described (Melnick et al., 2006). The following polyclonal antibodies were used: pERK1/2 (Thr202/Tyr204) (Cell Signaling Technology); Amphiregulin, Cox-2, and β-actin (Santa Cruz Biotechnology). Data was quantitated by using the ImageJ image analysis software (NIH) and normalized to the level of β-actin expression in each sample.