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Retinoids inhibit measles virus in vitro via nuclear retinoid receptor signaling pathways

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ABSTRACT

Measles virus (MV) infects 30 million children every year, resulting in more than half a million deaths. Vitamin A (retinol) treatment of acute measles can reduce measles-associated mortality by 50–80%. We sought to determine whether or not retinoids can act directly to limit MV output from infected cells. Physiologic concentrations of retinol were found to inhibit MV output in PBMC and a range of cell lines of epithelial and endothelial origin (40–50%). Near complete inhibition of viral output was achieved in some cells/lines treated with all-*trans* retinoic acid (ATRA) and 9-*cis* RA (9cRA). Important attenuation of the anti-MV effect of retinoids in R4 cells, a subclone of a retinoid-responsive cell line (NB4) deficient in RAR signaling, demonstrates that this effect is mediated at least in part by nuclear retinoid receptor signaling pathways. Inhibition of MV replication could not be fully explained as a result of retinoid effects on cell differentiation, proliferation or viability, particularly at low retinoid concentrations (1–10 nM). These data provide the first evidence that retinoids can directly inhibit MV in vitro, and raise the possibility that retinoids may have similar actions in vivo.

1. Introduction

Measles remains a major cause of mortality and morbidity in developing world children despite the availability of live attenuated vaccines for more than 30 years. It is estimated that MV infects 30 million individuals a year resulting in 0.5–1 million deaths (WHO, 2006). Measles has recently revisited several industrialized nations following spurious allegations of adverse events and decreased vaccine uptake. High dose retinol (vitamin A) is currently the therapy of choice for severe measles (Chabot and Ward, 2002; Joint WHO/UNICEF statement, 1987). Indirect effects of vitamin A on immune function and epithelial repair with prevention of secondary infections have long been assumed to underlie this beneficial effect. Although vitamin A has pleomorphic effects including actions on immune and epithelial cells (Semba, 1999; Stephensen, 2001), little is known about the molecular mechanisms that underlie the clinical effectiveness of vitamin A in measles infection.

Vitamin A is a fat-soluble vitamin obtained from the diet (meat, fish, eggs) that plays a critical role in vertebrate embryogenesis, cellular differentiation, activation, apoptosis, vision and the maintenance of epithelial integrity (Mark et al., 2006). Vitamin A (retinol or ROH) and its synthetic and natural derivatives such as all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9cRA) are collectively referred to as retinoids. Vitamin A is stored as retinyl esters in the liver and transported to target tissues as ROH where conversion to active metabolites occurs (e.g., ATRA, 9cRA) (Napoli, 1999). Although nuclear receptor-independent effects have been observed in the retina (Wolf, 2004) retinoids exert most of their known biological effects via nuclear retinoid receptor signaling. In this pathway, high affinity retinol metabolites such as ATRA and 9cRA bind directly to two families of nuclear receptors: retinoic acid receptors (RAR) and retinoid X receptors (RXR). Once bound by ligand, RAR/RXR heterodimers act as transcription factors for retinoid-responsive genes. The protein products of retinoid-responsive genes are responsible for exerting the effects of retinoids in the cell.

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Although peripheral blood mononuclear cells (PBMC) and a number of common human cell lines were included in this study, the NB4/R4 model was the principal tool used to investigate the role of retinoid receptors in regulating measles virus (MV) growth. NB4 are acute promyelocytic leukemia (APL) cells that contain reciprocal translocations between the RARα gene on chromosome 17 and the promyelocytic leukemia (PML) gene on chromosome 15, resulting in a PML–RARα fusion protein. The RARα component of the fusion protein retains its ligand and DNA-binding domains and can competently mediate signaling at higher concentrations of ATRA (Melnick and Licht, 1999). NB4-MR4 (R4) cells are a subclone of NB4 with a point mutation in the PML–RARα fusion molecule that confers a high level of resistance to retinoids (Shao et al., 1997). The mechanisms of response and resistance to retinoids in NB4 cells and its resistant subclones have been extensively characterized, making these cells a useful model to study retinoid effects on MV.

The experiments described in this manuscript test the hypotheses that retinoids can act against MV in vitro and that activation of nuclear retinoid receptors by their high affinity ligands contributes to the regulation of MV replication. We demonstrate that retinoids are active against MV in many cell lines and that this effect is mediated at least in part by nuclear retinoid receptor signaling pathways.

2. Materials and methods

2.1. Primary cells and cell lines

PBMC were isolated from healthy donors by discontinuous gradient centrifugation (Ficoll-Paque: GE Healthcare, Buckinghamshire, UK) and were activated with anti-CD3 (1 μg/mL). The large majority of PBMC (>80%) activated in this way and exposed to MV at a multiplicity of infection (MOI) of 1.0 express MV proteins within 48 h of infection (B.J. Ward, unpublished observation). Since wild-type MV targets the immune system and epithelial tissues throughout the body, we also assessed the anti-viral activity of retinoids in cell lines of immune (NB4/R4, U937, Jurkat), gastrointestinal (HCT8, T84, CaCo2) and respiratory origin (A549, BEAS-2B, Calu.1). Primary cells and cell lines were maintained in RPMI 1640 medium (Wisent, St-Bruno, QC) supplemented with 10% heat-inactivated fetal calf serum (FCS: Wisent: 56 °C for 30 min) and 10 mM Hepes buffer and 10 μg/mL gentamicin (cRPMI). All cultures were maintained at 37 °C in a 5% CO_2 humidified incubator.

2.2. MV strains and infections

Three MV strains were used: Chicago-1 (CHI-1) is a tissue culture-adapted genotype D3 strain originally isolated in 1989 (courtesy of W Bellini, CDC, Atlanta, GA), Bilthoven (BILT) is a low passage, genotype C2 wild-type strain originally isolated in 1991 (courtesy A Osterhaus, Rotterdam, Netherlands) and Connaught (CN) is a Moraten-like, vaccine strain attenuated by growth in chick embryo fibroblasts (courtesy R Wittes, Connaught Laboratories, Willowdale, ON). Unless otherwise indicated, cells were infected with the Chicago-1 strain virus at an MOI of 1.0 for 90 min at 37 °C. Following infection, cells were washed and resuspended at a density of 1×10^6 cells/mL in cRPMI containing a reduced concentration of FCS (2%), in a 24 well plate (1 mL/well). Unless otherwise indicated, infected cells were incubated for a period of 72 h in the presence of retinoids or retinoid agonists/antagonists. Ethanol or DMSO diluted in cRPMI was used as the mock control in all experiments. Supernatant MV at 72 h reflects the production of new virions rather than residual input virus, since unattached virions are removed by washing after infection.

2.3. Retinoids and nuclear receptor agonists/antagonists treatments

ROH, ATRA, 9cRA and arotinoic acid (4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid or TTNPB), a selective pan-RAR agonist, were purchased (Sigma–Aldrich Fine Chemicals, ON). A potent RXRα agonist (rexinoid or LGD1305), a RARα agonist (LGD030593) and RARα antagonist (RO 41-5253) were obtained from Ligand Pharmaceuticals Inc. (San Diego, CA) and Roche Applied Science (Laval, QC) respectively. Retinoid stock solutions of 10^{-2} M were prepared in 100% ethanol or 100% DMSO and further dilutions were performed using Hanks buffered salt solution (HBSS) or RPMI. All retinoids were stored in opaque eppendorf tubes at −80 °C. MV-infected cells were treated with various concentrations of retinoids or agonists/antagonists (0.1–1000 nM).

2.4. Viral output by plaque assay

The principle outcome for most experiments was MV output from infected cultures (i.e., replication efficiency) measured by plaque assay. Briefly, Vero cells were seeded in 24-well plates (BD Biosciences, Mississauga, ON) to obtain 90% confluency. Vero monolayers were infected in duplicate with 100 μL of serial 10-fold dilutions of culture supernatants for 90 min at 37 °C. Viral suspensions were then removed, a 16% methylcellulose overlay in Liebovitz's L-15 media (Gibco/Life Technologies, Grand Island, NY) was applied and plates were incubated at 37 °C in 5% CO_2 for 4 days. Neutral red (4%) was added for an additional 24 h and monolayers were fixed with 3.7% formalin for 10 min. Visible plaques were counted to determine the number of plaque forming units (pfu)/mL.

2.5. Propidium iodide staining

Cells were infected at an MOI of 0.1 for 90 min at 37 °C. Following infection, cells were washed and resuspended at a density of 1×10^5 cells/mL in cRPMI containing a reduced concentration of FCS (2%). The infection was carried out for 48 h, after which the number of apoptotic cells was counted as previously described (Nicoletti et al., 1991; Riccardi and Nicoletti, 2006). Briefly, cells were washed in a PBS/5% FBS/0.01 M NaN_3 solution and resuspended in a 50 μg/mL propidium iodide (Sigma–Aldrich Fine Chemicals, ON)/0.1% sodium citrate/0.1% Triton X-100 solution for 20 min at 4 °C. Cells undergoing apoptosis (i.e., sub-G0 DNA content) were measured by flow cytometry and are expressed as a % of total cells.

2.6. Cell proliferation by thymidine uptake

Both retinoids and MV can also have anti-proliferative effects in many cells/cell lines. To assess the potential anti-proliferative effects of retinoid treatments ±MV infection, cells were infected and/or treated with retinoids and seeded in triplicate in 96-well plates (25,000 cells/well) for 6 h. The wells were then treated with 1 μCi of ^{3}H-thymidine/well (ICN, Costa Mesa, CA) for 18 h. Following one freeze-thaw cycle, DNA was harvested onto glass-fiber filters, and thymidine incorporation was measured by scintillation counter (Wallac, Microbeta, Finland). The results are shown as mean counts per minute (CPM) of triplicate samples (±standard deviation).

2.7. Real-time PCR for RARβ mRNA expression

The induction of RARβ was used as a test of intact RARα signaling. Total RNA was harvested from NB4 cells by phenol–chloroform extraction and 5 μg was transcribed into cDNA. The primer