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The tyrosine kinase inhibitor genistein induces the detachment of rotavirus particles from the cell surface

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ABSTRACT

Group A rotaviruses are a major cause of severe gastroenteritis in young infants. In this work we evaluated the potential role of protein tyrosine kinases on rotavirus infectivity and viral progeny production. From the broad-spectrum inhibitors tested, only genistein, a flavonoid, inhibited rotavirus infectivity. The inhibition observed was dose and strain dependent, with more than 10-fold IC_{50} differences for some rotavirus strains, and the effect of the drug was shown to be dependent of their activity as a protein tyrosine kinase inhibitor, since the inactive analogue of genistein, daidzein, had no effect on virus infection. Investigation of the stage of virus replication blocked by the drug showed that it interferes with the early interactions of the virus with receptors and/or co-receptors, since treatment of the cells with genistein promoted the detachment of the virus from the cell surface.

1. Introduction

Group A rotaviruses are the leading cause of severe dehydrating diarrhea in infants and young children worldwide (Estes and Kapikian, 2007). Rotaviruses are formed by three concentric layers of protein; the outer layer of the virus is constituted by glycoprotein VP7, while spikes formed by protein VP4 project from the virus surface.

Rotavirus cell entry is a complex process with several receptors and co-receptors implicated in the early interactions of the virus with the host cell. The virus attaches to the cell surface through the VP8 domain of the virus spike protein VP4, using as receptor different types of glycans, depending on the virus strain (Arias et al., 2015). It has been reported that after cell binding rotavirus may interact with various integrins, the heat shock cognate protein hsc70, the protein disulfide isomerase, gangliosides, and the tight junction protein JAM-A to enter the cell, although the only protein that has been found so far to be required for cell infection of all tested strains is hsc70 (Calderon et al., 2012; López and Arias, 2004; Martínez et al., 2013; Torres-Flores et al., 2015). Rotavirus internalization is mediated by endocytosis; the endocytic route employed is dependent on the rotavirus strain, and is determined by the VP4 protein (Díaz-Salinas et al., 2013; Wolf et al., 2011). Rhesus rotavirus (RRV) enters cells by a clathrin- and caveolin-independent pathway, while all other strains evaluated employ a clathrin-dependent endocytosis. (Díaz-Salinas et al., 2013; Gutiérrez et al., 2010; Sánchez-SanMartín et al., 2004; Wolf et al., 2011). The intracellular traffic of different rotavirus strains may also differ; thus, the simian rotavirus strains RRV and SA11 seem to reach the cytosol from maturing endosomes, while others, such as the bovine rotavirus UK, the swine virus TFR-41 and the human rotavirus strain Wa, traffic to late endosomes before reaching the cytosol (Arias et al., 2015; Díaz-Salinas et al., 2014; Silva-Ayala et al., 2013; Wolf et al., 2012). During the process of, or shortly after, reaching the cytoplasm, the triple-layered particle (TLP) loses the external protein layer and is converted to the transcriptionally active double-layered particle (DLP) (Lawton et al., 1997). The viral RNA transcripts direct the synthesis of six structural (VP1 to VP4, VP6, and VP7) and six nonstructural (NSP1 to NSP6) proteins.

The phosphorylation of viral and cellular proteins can have a major impact on viral replication. In fact, the genome of several viruses code for protein kinases (for review see Keating and Skriker, 2011). The rotavirus nonstructural protein NSP5 is a phosphoprotein that has been suggested has kinase activity (Afrikanova et al., 1996; Blackhall et al., 1997). In addition, several changes in protein phosphorylation are induced during the replication of rotavirus, including the increase in phosphorylated forms of several kinases, such as JNK, p38, Akt, GSK3, mTor, p70S6K, CAMK1, and AMPK (Bagchi et al., 2010; Bhowmick et al., 2012; Bhowmick et al., 2014; Crawford et al., 2012; Dutta et al., 2009; He et al., 2012; Holloway and Coulson, 2006), as well as of proteins that have no kinase activity, such as eIF2-alpha (Montero et al., 2008), 4EBP1 and p65 (Dutta

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et al., 2009), retinoblastoma (Bhowmick et al., 2014), and IRF3 and STAT1 (Feng et al., 2009). Additional kinases that are activated during rotavirus replication, but for which there is no evidence of phosphorylation, are CaMKK-β and PKR (Crawford et al., 2012; Rojas et al., 2010)

In this study we evaluated the effect of inhibiting protein tyrosine kinases (PTK) on the infectivity and the production of viral progeny of rotavirus, and found that genistein inhibits virus entry.

2. Materials and methods

2.1. Cells and viruses

The epithelial cell line MA104 was grown as described (Silva-Ayala et al., 2013). The rhesus rotavirus strain RRV (G3P[3]) and the human rotavirus strains Wa (G1P[8]) and DS-1 (G1P[4]) were obtained from H. B. Greenberg (Stanford University, Stanford, CA), bovine rotavirus UK (G6P[5]) was donated by D. R. Snodgrass (Moredun Research Institute, Edinburgh, United Kingdom), Simian rotavirus strain SA11 (G3P[2]) was obtained from M. Estes (Baylor College of Medicine, Houston TX), bovine rotavirus strain RF (G6P[1]) was obtained from J. Cohen (CNRS-INRA, Gif-sur-Yvette Cedex, France), porcine rotavirus strain TFR-41 (G5P[7]) was obtained from I. Holmes (University of Melbourne, Victoria, Australia), the neuraminidase-resistant rotavirus strain nar3, and the porcine rotavirus strain YM (G11P[7]) were previously described (Méndez et al., 1993; Ruiz et al., 1988).

2.2. Antibodies and inhibitors

The rabbit polyclonal sera to rotavirus structural proteins, and to vimentin have been described previously (Ayala-Breton et al., 2009), monoclonal antibody (mAb) 159 against VP7 was kindly provided by H. B. Greenberg (Stanford University, Stanford, CA). The goat anti-rabbit coupled to Alexa-488, and Alexa-568-coupled phalloidin were obtained from Molecular Probes (Eugene, OR). All PTK inhibitors tested were obtained from Merck Biosciences (Darmstadt, Germany).

2.3. Infectivity assays.

MA104 cells in 96-well plates were pre-incubated with the indicated concentration of drugs for 1 h, cells were then infected with the indicated viruses at an MOI of 0.02 in the presence of the drugs for 1 h, at 37 °C. Unbound virus and drugs were washed-off and the remaining extracellular virus was removed by a brief incubation with EGTA as described (Ayala-Breton et al., 2009). Cells were incubated for 14 h, fixed and infected cells were detected by an immunoperoxidase foci detection assay as described previously (Pando et al., 2002). Where indicated, virus was added to cells at 4 °C in the presence or absence of 100 μM genistein, washed and incubated for an additional hour in the presence or absence of genistein at 37 °C. The drug was then washed three times, the remaining extracellular virus was neutralized with mAb 159, and the infection was left to proceed for 14 h at 37 °C, time at which the cells were fixed and immunostained.

2.4. DLP lipofection

MA104 grown in 96-well plates were incubated with a lipofectamine-DLP mix, as described previously (López et al., 2006). The DLP-lipofectamine mix was left on the cells for 1 h at 37 °C, washed twice with MEM, and the cultures were incubated for 2 h in presence or absence of 100 μM genistein. Cells were washed twice and incubated for 12 h at 37 °C in MEM. The cell monolayers were fixed and immunostained.

2.5. Binding assays

The binding assay was carried out as described with minor modifications (Silva-Ayala et al., 2013), MA104 cells grown in 48-well plates and incubated for 60 min at 37 °C in blocking solution (1% of bovine serum albumin (BSA) in PBS); during this period 100 μM genistein was included, or cells were maintained in blocking solution. Cells were then washed and incubated with a suspension of purified SA11 TLPs in blocking solution, for 1 h at 4 °C in the presence or absence of genistein. Cells were then frozen and thawed twice, and the virus present in the cell lysates was detected by an ELISA (Zárate et al., 2004). An ELISA using known amounts of purified TLPs was performed in parallel to quantify the virus.

2.6. Immunofluorescence

MA104 cells grown on glass coverslips were washed twice and incubated with MEM without serum for 30 min at 37 °C, next the cells were incubated with 0.5% BSA-PBS for 1 h at 4 °C, and purified SA11 TLPs, at a MOI of 50, were added and incubated for 1 h at 4 °C. Unbound virus was removed by washing, and cells were fixed or incubated with MEM or MEM with 100 μM genistein for 1 h at 37 °C, and then fixed with freshly prepared 2.5% formaldehyde for 20 min at room temperature.. Viral particles were detected by staining with a polyclonal anti-rotavirus serum and with an anti-rabbit Alexa488-labeled as secondary antibody as described (Silva-Ayala et al., 2013); cells were also stained with Alexa568-labeled phalloidin. Coverslips were mounted on glass slides and observed as described (Gutiérrez et al., 2010).

2.7. Preparation of radioactively labeled viral particles

MA104 cells grow in 75 cm^2 flasks were infected at an MOI of 3. Three hours post infection (hpi) MEM was replaced by MEM without methionine and cysteine and supplemented with 50 μCi/ml of Easy Tag EXPRESS/^{35}S labeling mix (Dupont, NEN). Twelve hpi, media was removed and cells were collected in TNC buffer (10 mM Tris–HCl [pH 7.5], 140 mM NaCl, 10 mM $CaCl_2$), freeze-thawed twice, and the viral particles were CsCl purified as described previously (López et al., 2005).

2.8. Virus detachment assay

MA104 cells grown in 48-well plates were washed twice and incubated with MEM without serum for 30 min at 37 °C. After this time MEM was removed, and 500 μl of blocking solution (1% of bovine serum albumin (BSA) in PBS) was added for 1 h at room temperature. Cell were washed with an ice-cold washing solution (0.5% BSA–PBS) and incubated with ^{35}S-labeled SA11 TLPs, at an MOI of 50 in blocking solution, for 1 h at 4 °C. The cells were then washed three times with washing solution, or with 10 mM EGTA and then lysed with Laemmli sample buffer. Cells were transferred to 37 °C with or without of 100 μM genistein for 1 h, then the media was collected and cells were washed or not with EGTA as described. Finally the cells were lysed with Laemmli sample buffer. Each sample was divided in two, one portion was analyzed by SDS-PAGE and autoradiography, and other portion was solubilized in Ecolite (ICN) and used to measure the radioactivity by liquid scintillation counting.

2.9. Data analysis

Statistic analysis and IC_{50} calculations were performed using Prism 4.0 for Macintosh (GraphPad Software, Inc.) by a paired, two-tailed t-test, and non-linear regression as indicated.