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Dual-color bioluminescent assay using infected HepG2 cells sheds new light on *Chlamydia pneumoniae* and human cytomegalovirus effects on human cholesterol 7α-hydroxylase (*CYP7A1*) transcription

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ABSTRACT

Chlamydia pneumoniae and human cytomegalovirus (HCMV) are intracellular pathogens able to infect hepatocytes, causing an increase in serum triglycerides and cholesterol levels due to the production of inflammatory cytokines. We investigated whether these pathogens could interfere with cholesterol metabolism by affecting activity of hepatic cholesterol 7α-hydroxylase (*CYP7A1*) promoter. *CYP7A1* is the rate-limiting enzyme responsible for conversion of cholesterol to bile acids, which represents the main route of cholesterol catabolism. A straightforward dual-reporter bioluminescent assay was developed to simultaneously monitor *CYP7A1* transcriptional regulation and cell viability in infected human hepatoblastoma HepG2 cells. *C. pneumoniae* and HCMV infection significantly decreased *CYP7A1* promoter activity in a dose-dependent manner, with maximal inhibitions of 33 ± 10% and 32 ± 4%, respectively, at a multiplicity of infection of 1. To support in vitro experiments, serum cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides and glucose levels were also measured in Balb/c mice infected with *C. pneumoniae*. Serum cholesterol and triglycerides also increased in infected mice compared with controls. Although further investigation is required, this work presents the first experimental evidence that *C. pneumoniae* and HCMV inhibit *CYP7A1* gene transcription in the cultured human hepatoblastoma cell line.

It is well recognized that during inflammation and infections, serum triglycerides and cholesterol levels increase [1] as a consequence of alterations in gene expression caused by pro-inflammatory cytokines. Hypercholesterolemia is caused both by activation of the positive acute response gene HMG-CoA reductase, coding the rate-limiting enzyme in de novo cholesterol synthesis, and by a decrease in cholesterol 7α-hydroxylase (*CYP7A1*) gene transcription, in response to cytokines [2,3]. The latter is the rate-limiting enzyme responsible for cholesterol catabolism [3]. Changes in lipid metabolism have been receiving much attention since the pioneering work of Fabricant and coworkers, who suggested the link between infection/inflammatory diseases and atherosclerosis [4–8].

Several studies have focused on the atherogenic role of *Chlamydia pneumoniae* and human cytomegalovirus (HCMV)[1] [9–12] by direct or indirect interaction with endothelial cells [13,14]. *C. pneumoniae* and HCMV have been shown to infect liver cells [15–17], and more recently *C. pneumoniae* has been demonstrated to perturbate cholesterol homeostasis in TPH-1 macrophages via JNK–PPARγ-dependent signal transduction pathways [18].

Moreover, an increasing body of evidence based on epidemiological studies suggests an association between *C. pneumoniae* infections and serum lipid abnormalities in healthy obese female subjects [19].

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[1] *Abbreviations used:* HCMV, human cytomegalovirus; BL, bioluminescent; HDL, high-density lipoprotein; EB, elementary body; EMEM, Eagle's minimum essential medium; HSV, herpes simplex virus; MOI, multiplicity of infection; PpyWT, *P. pyralis* luciferase wild type; PpyRE-TS, thermostable red-emitting luciferase of *P. pyralis*; SV40, Simian virus 40; *IBABP*, ileal bile acid-binding protein; RLU, relative light units; PBS, phosphate-buffered saline; FXR, farnesoid X receptor.

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Transcriptional in vitro assays represent a suitable tool to preliminarily investigate whether these pathogens could directly interfere with the transcriptional regulation of *CYP7A1*.

Although they have limitations, bioluminescent (BL) cell-based assays are sensitive, cost-effective, and easy to perform. As we reported previously [20], an internal viability control can be introduced to overcome one of their main pitfalls (i.e., scarce robustness and high variability). Indeed, by exploiting spectral resolution of red- and green-emitting firefly luciferases requiring the same substrate, it is possible to develop cell-based assays with improved reproducibility.

This approach has been employed to develop a multiplexed cell-based assay that provides highly valuable and reliable functional information, although no applications to mammalian cell lines infected with bacterial or viral pathogens have been reported yet.

In the current study, we have investigated the ability of *C. pneumoniae* and HCMV to inhibit human *CYP7A1* promoter in the human hepatoblastoma HepG2 cell line. We developed a dual-reporter BL transcriptional assay to simultaneously monitor *CYP7A1* transcriptional regulation and cell viability after *C. pneumoniae* or HCMV infection. To the best of our knowledge, this is the first multicolor BL assay using infected mammalian cells.

To support results obtained with the BL cell-based assay, serum cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and glucose levels were evaluated in *C. pneumoniae* infected Balb/c mice.

Materials and methods

Microorganisms and cultures

C. pneumoniae strain FB/96 was isolated from a patient with pneumonia at the Sant'Orsola Polyclinic in Bologna, Italy [21]. *C. pneumoniae* was grown in LLC-MK2 cells, and elementary bodies (EBs) were purified by sucrose gradient centrifugation. A clinical isolate of HCMV, obtained from a urine sample of a newborn with congenital infection, was used. The virus was propagated in human embryo fibroblasts and grown in Eagle's minimum essential medium (EMEM). A clinical isolate of herpes simplex virus (HSV) was obtained from a male patient with skin eruption.

Growth of microorganisms in HepG2 cells

Human hepatocarcinoma HepG2 cells were grown in EMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 10 mg/L gentamicin, and 1% (v/v) L-glutamine. Cultures of HepG2 cells grown on cover slips in 24-well plates (10^5 cells/well) were infected with sucrose-purified chlamydiae at a multiplicity of infection (MOI) of 1. At 24, 48, 72, and 96 h postinfection, cells were fixed with methanol and stained by indirect immunofluorescence using genus-specific monoclonal antibody (Meridian Diagnostics, Cincinnati, OH, USA). Similarly, the growth of HCMV and HSV was evaluated in HepG2 cultures by indirect immunofluorescence using isothiocyanate-conjugated specific monoclonal antibody against HCMV or HSV (Argene, Segrate, Italy).

Reporter plasmids

The plasmid *pCYP7A1*–PpyWT containing the portion of the human *CYP7A1* promoter −1887/+24 and *Photinus pyralis* luciferase wild type (PpyWT) as reporter gene was a gift from John Y.L. Chiang (Northeastern Ohio Universities). The thermostable red-emitting luciferase of *P. pyralis* (PpyRE-TS) was excised from the plasmid pGEX–PpyRE-TS, kindly provided by Bruce Branchini (Connecticut College), and inserted into pGL3 control vector with Simian virus 40 (SV40) promoter (Promega, Madison, WI, USA) by blunt ligation; the plasmid was named pGL3–PpyRE-TS and verified by restriction digestions and DNA sequencing [22]. The human ileal bile acid-binding protein (*IBABP*)–Luc reporter plasmid was kindly provided by S. Spampinato (University of Bologna, Italy).

Correlation between PpyRE-TS luciferase BL and viable cell number

Approximately 1.5×10^5 HepG2 cells per well were transiently transfected with 0.5 μg of pGL3–PpyRE-TS in a 24-well culture plate. Cells were incubated for 48 h with various concentrations of G418 (range = 0–800 μg/ml). Viable cells were then counted by trypan blue exclusion. Correlation between BL emission, expressed as relative light units (RLU), and viable cell count was evaluated using the BL measurement procedure described in the following paragraph.

Dual-reporter BL assays: Bioassay procedure

A half-day (12 h) after the infection of HepG2 by *C. pneumoniae,* HCMV, and HSV, the cells were washed with phosphate-buffered saline (PBS) and then transiently cotransfected with the plasmids p*CYP7A1*–PpyWT and pGL3–PpyRE-TS or p*IBABP*–Luc and pGL3–PpyRE-TS using Exgen500 (MBI Fermentas, Vilnius, Lithuania). Three days (72 h) after cotransfection, cells were washed in PBS and lysed with 200 μl of 1% Triton X-100 for 5 min at 25 °C. After centrifugation (5 min at 1000*g*), a 100-μl aliquot of supernatant was transferred to a white 96-well microtiter plate and analyzed for the presence of PpyWT and PpyRE-TS by the addition of 100 μl luciferase assay system (Promega). A Varioskan Flash spectral scanning multimode reader (Thermo Scientific, Waltham, MA, USA) equipped with bandpass filters was used for BL measurements [23].

Statistical analysis

Assays were performed in triplicate, and each experiment was repeated at least four times. The Student's *t* test was used for statistical analysis. The *P* values less than 0.05 were considered statistically significant. Data are presented as means ± standard deviations.

Animal studies

Experiments were conducted in conformity with the Public Health Service Policy on Human Care and use of Laboratory Animals and approved by the ethical committee of the University of Bologna. A total of 20 male BALB/c mice (8 weeks old, 22–26 g body weight) purchased from Charles River Laboratories (Calco, Milan, Italy) were used [16]. Animals were housed 2 weeks before the experiments and fed the usual commercial diet and water ad libitum. They were kept at constant light/dark cycling throughout the study. Of the sample, 10 animals were infected intraperitoneally by injection of purified *C. pneumoniae* EB suspension (titre: 5×10^5 inclusion forming units, IFUs)Page: 8. As negative controls, 10 animals were challenged with sucrose/phosphate/glutamate buffer. One week (7 days) after infection, animals were anesthetized with zolazepam/tiletamin (20 mg/kg body weight (Virbac Laboratories, France) and sacrificed by cardiac puncture.

Serum lipid analysis

Total cholesterol, HDL cholesterol, and triglycerides concentrations were measured using a Dimension RxL Max system (Siemens Healthcare Diagnostics, Newark, DE, USA).