Placenta 35 (2014) 345–350

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Human cytomegalovirus infection induces leukotriene B4 and 5-lipoxygenase expression in human placentae and umbilical vein endothelial cells

M. Benard [a,b,1], K. Straat [c,1], S. Omarsdottir [c], K. Leghmari [a], J. Bertrand [d], C. Davrinche [a], I. Duga-Neulat [e], C. Söderberg-Nauclér [c], A. Rahbar [c,**,2], C. Casper [a,b,c,*,2]

[a] INSERM UMR 1043, Hospital Purpan, Paul Sabatier University, Toulouse 31024, France
[b] Neonatal Unit, Children's Hospital, Toulouse 31059, France
[c] Department of Medicine, Solna Center for Molecular Medicine, Karolinska Institutet, Karolinska University Hospital, SE 171 76 Stockholm, Sweden
[d] INSERM UMR U1048, I2MC, Hospital Purpan, Toulouse 31432, France
[e] Department of Anatomopathology, Hospital Purpan, Toulouse 31059, France

ARTICLE INFO

Article history:
Accepted 26 March 2014

Keywords:
Placenta
Human cytomegalovirus
Histoculture
5-Lipoxygenase
Endothelial cells
LTB4

ABSTRACT

Introduction: Human cytomegalovirus (HCMV) can cause congenital infection with risk of neurological disability. Maternal-fetal transmission is associated with placental inflammation. 5-lipoxygenase (5-LO) is the key enzyme in the biosynthesis of Leukotrienes (LTs), which are proinflammatory mediators. This study investigated the effect of HCMV infection on 5-LO expression and Leukotriene-B4 (LTB4) induction in human placentae and umbilical vein endothelial cells (HUVEC).

Methods: Seven placentae from fetuses with congenital HCMV infection and brain damage and six controls were stained with HCMV-immediate-early-antigen (HCMV-IEA) and 5-LO by immunohistochemistry. 5-hydroxyeicosatetraenoic acid (5-HETE) and LTB4 were measured in culture supernatant from *ex vivo* HCMV-infected placental histocultures by liquid chromatography. *In vitro*, HCMV infected HUVEC cells were analyzed for 5-LO mRNA and protein expression by real time PCR and immunofluorescence staining.

Results: HCMV-IEA was abundant in all HCMV infected placentae but absent in control placentae. 5-LO expression was higher in endothelial and smooth muscle cells of HCMV-infected placentae, compared to control placentae.

HCMV infection induced an up-regulation of LTB4 in *ex vivo* placental explants with higher levels of LTB4 at 72 h compared to controls ($p = 0.002$). *In vitro*, 5-LO transcript and protein expression were significantly induced in HCMV-infected HUVEC, compared to the control cultures ($p = 0.036$).

Conclusion: The presence of HCMV coincided with high 5-LO expression in cells of *in vivo* HCMV infected placentae. HCMV induced up-regulation of 5-LO in both *ex vivo* HCMV-infected placental explants and HUVEC. HCMV induced LT-biosynthesis in congenitally infected placentae may have a role in pathogenesis of congenital HCMV disease.

1. Introduction

Human cytomegalovirus (HCMV) is a common cause of congenital infection with a prevalence of around 1% [1].

Congenital HCMV infection occurs either after a primary HCMV infection, or secondary to HCMV reactivation or reinfection in the mother [2]. Congenital HCMV infection can lead to severe neurological sequel in the offspring, such as sensorineuronal hearing loss and mental disability [3–5].

Abbreviations: 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-LO, 5-lipoxygenase; HCMV, human cytomegalovirus; HUVECs, human umbilical vein endothelial cells; LTs, leukotrienes; LTB4, leukotriene B4; vWF, von Willebrand factor.

* Corresponding author. INSERM UMR 1043, Hospital Purpan, Paul Sabatier University, Toulouse 31024, France. Tel.: +33 534558695; fax: +33 534558684.
** Corresponding author. Department of Medicine, Solna Center for Molecular Medicine, Karolinska Institutet, Karolinska University Hospital, SE 171 76 Stockholm, Sweden. Tel.: +46 851779897; fax +46 831347.
E-mail addresses: afsar.rahbar@ki.se (A. Rahbar), casper.c@chu-toulouse.fr, chca66@yahoo.fr (C. Casper).

[1] Shared first authorship.
[2] Shared senior authorship.

http://dx.doi.org/10.1016/j.placenta.2014.03.022
0143-4004/

HCMV infection *in utero* is believed to be caused by the direct action of HCMV that reaches fetal circulation through the placenta [5]. Histological examination of placentae from HCMV infected fetuses demonstrate placental villitis – defined by the presence of lymphohistiocytic infiltrates – affecting different proportions of the placental villous tree [6]. In a recent study on histological findings in fetuses with congenital HCMV infection, more severe lesions are observed when HCMV infection of the placenta itself induces impairment of placental function and consequent hypoxic fetal damage [7].

Leukotrienes (LTs) are potent lipid mediators with proinflammatory and immune modulating properties that play a role in the pathogenesis of inflammatory diseases [8]. LTs are divided into two major classes: the bronchoconstrictor agents cysteinyl leukotrienes and the chemoattractant dihydroxy-acid-LT B4 (LTB4) [9]. 5-lipoxygenase (5-LO) is the key enzyme in the biosynthesis of LTs, catalyzing its conversion from arachidonic acid into 5-hydroperoxyeicosatetraenoic acid and 5-hydroxyeicosatetraenoic acid (5-HETE) and converting 5-HETE into LTs [8].

Our group has demonstrated that HCMV can induce production of LTs in HCMV-infected vascular smooth muscle cells, thus contributing to inflammation in the HCMV infected vascular tissue [10]. However, the inflammatory role of LTs in HCMV infected placental tissue has not yet been investigated. The aim of this study was to examine placental tissue sections for expression of HCMV and 5-LO and to further analyze the induction of LTB4 in an *ex vivo* model of placentae. In order to confirm that the induction of 5-LO was due to HCMV infection, 5-LO protein and transcript expression was analyzed in HCMV infected human umbilical vein endothelial cells (HUVEC), *in vitro*.

2. Material and methods

2.1. In vivo HCMV-infected placentae

2.1.1. Placental collection

Second and third trimester placentae of seven HCMV-congenitally infected fetuses with brain damage were obtained at the Paule de Viguier Hospital, Toulouse.

The diagnosis of congenital HCMV infection was based on maternal HCMV-serology and pathological ultrasound examinations of the fetal brain (microcephaly, ventriculomegaly, white matter abnormalities, intracerebral hemorrhages). Growth restriction defined fetuses whose weight was below the 10th percentile for its gestational age.

Pregnancies were terminated by request of the parents and with acceptance of the prenatal diagnosis center of Toulouse. Termination of pregnancy was performed with feticide and thereafter, vaginal delivery. Fetal and placental tissues were obtained after informed parental consent and according to the procedures approved by the Regulations of the French Ministry of Health. Placentae were fixed in 10% buffered formalin and embedded in paraffin. Fetal brain damage was confirmed by anatomopathological examination.

Primary HCMV infection was confirmed by seroconversion in four pregnancies during the first trimester and one during the second trimester. Reactivation occurred during the second trimester in two pregnancies.

Six second and third trimester placentae from pregnancies without congenital HCMV infection were included as controls, in accordance with French ethical guidelines. These control placentae were collected after preterm labors ($n = 3$), pre-eclampsia ($n = 2$) and labor with abnormal fetal heart rate ($n = 1$).

In all pregnancies, TORCH screening showed no seroconversion for toxoplasmosis during pregnancy, immunity against rubella (vaccination) and was negative for hepatitis B, C and HIV. No maternal bacterial infection was diagnosed during pregnancy. Five sections per placenta were examined blinded. All histopathological examinations were done by our pathologist in the Pathology Department, Toulouse, France.

2.1.2. Histopathological examination

Paraffin embedded placental tissues sections (4 μm) were de-waxed using Xylene and rehydrated in an alcohol series. Immunohistochemistry staining was performed on the tissue sections as describe before [10]. The sections were incubated with following antibodies: mouse anti-human HCMV immediate early antigen (IEA) (IgG2aChemicon, United States) and rabbit anti-human 5-LO (Abcam, UK). For detection of vessels and endothelial cells in the vessels within the placenta, antibodies against smooth muscle cells alpha actin (IgG2a, BioGenex, France) and anti-human von Willebrand factor (vWf, IgG1, Dako, Denmark) were used respectively. Poly rabbit antibodies (R&D systems, United States) and secondary antibodies only served as controls.

Visualization was achieved using horseradish peroxidase detection system (BioGenex) with the chromogen diaminobenzidine (Innovex Biosciences, United States). In case of double staining, slides were stained for IEA and 5-LO and visualized by FastRed and DAB, respectively. Finally, all slides were counterstained with hematoxylin (Sigma–Aldrich, United States) and mounted in permanent mounting medium (Dako, Denmark).

2.2. Ex vivo HCMV-infected placental explants

2.2.1. Placental collection

Three HCMV-negative placentae were obtained by elective caesarean section after uncomplicated pregnancies at the Paule de Viguier Hospital, Toulouse, France. The HCMV status of all placentae was verified by immunochemistry [11].

2.2.2. Placental histocultures

Placental tissues were cultured *ex vivo*, and were infected with HCMV, as described previously [11]. The placentae were processed within 2 h and chorionic villi were isolated and infected with HCMV. Briefly, five days before *ex vivo* HCMV infection of placenta, MRC5 cells (human fetal lung fibroblast, gift from the Laboratory of Virology, Pr Izopet, Toulouse, France, BioMérieux) were seeded in 6-well plates (BD Biosciences, CA, USA) and were infected with HCMV strain VHL/E (gift from Sinzger C., University of Tübingen, Germany, originally from James Waldmann (Columbus, Ohio)) at a multiplicity of infection (MOI) of 0.75–3. Placentae were incubated with supernatant from HCMV-infected MRC5 at 37 °C in a 5% CO_2 incubator overnight. After incubation, placental villi were rinsed with PBS and placed on collagen sponge gels into the 6-well plates seeded with infected or uninfected MRC5 cells. There was no direct contact between the MRC5 cells and the placental blocks. The placental villi fragments were cultured at the liquid air interface and supernatant was collected at 24, 48 and 72 h. The supernatants were aliquoted and frozen at −80 °C for later use.

2.2.3. Chromatography and mass spectrometry

Production of LTB4 and 5-HETE were examined in supernatant of uninfected and *ex vivo* HCMV infected placentae.

2.2.3.1. Chemicals. Chemicals were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Methanol (MeOH) (HPLC gradient Grade), methyl formate (MeFor) (Reagent Grade), acetonitrile (ACN) (HPLC gradient Grade) and formic acid (FA) were obtained from Sigma–Aldrich.

2.2.3.2. Stock solution and standards. Internal standard mixture (IS): Deuterium-labeled compounds were mixed at final concentration of 400 ng/mL in MeOH and used as internal standard (IS).

All compounds were mixed together in MeOH to a first calibration solution of 2000 ng/mL. Then, a series of dilutions were prepared in MeOH (500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL, 15.6 ng/mL, 7.8 ng/mL, 3.9 ng/mL). IS was added to each, at a final concentration of 200 ng/mL.

2.2.3.3. Liquid chromatography/tandem mass spectrometry measurements. LC-MS/MS analysis was performed on UHPLC system (Agilent LC1290 Infinity) coupled to Agilent 6460 triple quadrupole MS (Agilent Technologies) equipped with electro-spray ionization interface. Separation was done at 40 °C on a Zorbax SB-C18 column (2.1 mm–50 mm–1.8 μm) (Agilent Technologies). The compositions of mobile phase A and B were respectively water, ACN and FA (75/25/0.1) and ACN, FA (100/0.1). Compounds were separated with a linear gradient from 0 to 85% B in 8.5 min and then to 100% B at 9 min. Isocratic elution continued for 1 min at 100% B then100% A was reached at 10.2 min and maintained to 11 min. The flow rate was 0.35 mL/min. The autosampler was set at 5 °C and the injection volume was 5 μL.Source conditions were as follows: negative ESI mode; source temperature = 325 °C, nebulizer gas (nitrogen) flow rate = 10 L/min, sheath gas (nitrogen) flow rate = 12 L/min, sheath gas temperature = 400 °C and spray voltage = −3500 V. Data were acquired in MRM mode.

For each compound the best conditions of separation: retention time in minute (RT), and of quantification were defined: specific Q1/Q3 transition (T) fragmentor (F) and collision energy (CE). 5-HETE (RT: 6.31; T: 319/115, F: 110; CE: 4), 5-HETE-d8 (RT: 6.27; T: 327/155, F: 100; CE: 2).

Peak detection, integration and quantitative analysis were performed using Mass Hunter Quantitative analysis software (Agilent Technologies).

2.3. In vitro HCMV-infected HUVEC

2.3.1. HUVEC preparation

Since mostly HCMV infected endothelial cells express 5-LO in placentae tissue sections, we examined the levels of 5-LO transcript and protein expression in HCMV infected HUVEC *in vitro*. HUVEC were used because isolation of endothelial cells from the microvasculature of the placenta was very difficult. HUVEC (Cascade Biologics, Portland, USA) were maintained in M200 medium, including supplement, at 37 °C, 5% CO_2. The cells were infected with an endotheliotropic stain of HCMV (TB40E) at a MOI of 0, 0.5 for 3 days. mRNA levels of 5-LO were determined by