Microbes and Infection 17 (2015) 845–849
www.elsevier.com/locate/micinf

Survival of rat cerebrocortical neurons after rickettsial infection

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Received 8 June 2015; revised 19 September 2015; accepted 22 September 2015
Available online 9 October 2015

Abstract

Neuroinvasive microorganisms are suspected to play an important role in the etiopathogenesis of neurological diseases. However, direct evidence for the pathogenic function is still missing. The main aim of this study was to investigate biochemical and morphological changes that may occur as a result of an *in vitro* infection of rat cerebrocortical neurons by selected members of the genus *Rickettsia*. Our results showed that survival of the neurons is significantly reduced after the infection. Intracellular level of ATP is gradually decreased and inversely correlates with the load of rickettsiae. Immunofluorescence revealed that rickettsiae can enter the neurons and are localized in perinuclear space and also in neuronal processes. Data obtained in this study correspond to the idea of possible involvement of rickettsiae in the etiopathogenesis of various neuropathies.

Keywords: Rickettsiae; Cerebrocortical neurons; Survival; ATP; Immunofluorescence

1. Introduction

The central nervous system (CNS) is one of the targets for many *Rickettsia* species [3,7,12,25]. Rickettsiae primarily infect the microvascular endothelium leading to direct increase in microvascular permeability very early after invasion of the endothelium [32]. It has been demonstrated in mouse model that rickettsiae when injected intravenously, can cross the blood–brain barrier to reach brain parenchyma [22]. Moreover, rickettsial invasion of vascular endothelial cells into the brains was histologically confirmed also in humans [28,29].

Joshi and Kovács demonstrated that cerebellar granule neurons can be efficiently infected by *Rickettsia rickettsii*. Upon infection the neurons underwent apoptotic death, as detected by TUNEL assays [13].

Since rickettsiae may cause a whole spectrum of diseases with clear neurological manifestation, we employed an experimental model of rat embryonal and *in vitro* differentiated cerebrocortical neurons for investigation of direct effect of rickettsiae in the neurons, which may have implications for etiopathogenesis of serious neurological diseases [19]. We have provided first data on intraneuronal detection and neurotoxicity of selected rickettsial species, such as *Rickettsia helvetica*, *Rickettsia akari* and *Rickettsia slovaca* in rat cerebrocortical neurons.

2. Materials and methods

Prior to their purification by isopycnic density gradient centrifugation [2,31], rickettsiae were multiplied in Vero cell line (ATCC® CCL-81™) plated on 20 cell culture flasks with 175 cm^2 growth area. The purity of the rickettsial solution was checked by Gimenez staining [11]. *R. akari* strain MK (Kaplan), *R. slovaca* strain 13-B and *R. helvetica* strain C9P9

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http://dx.doi.org/10.1016/j.micinf.2015.09.024
1286-4579/

were used for infection of differentiated rat neurons cultured *in vitro* for 8 days.

Rickettsiae were quantified by Real-Time qPCR using rickettsiae specific primers and Taqman probe designed in our laboratory, based on the gene sequence coding for 30S ribosomal protein S12 (*rpsL* gene) available in GenBank (NC009881). The sequence of forward and revers primers are as follows: MQ25 5′-CCCGGTGAAAAGCATAGTGT-3′ and MQ26 5′-ATTTCACACCCGGAAGATCA-3′ respectively. The sequence of the probe is as follows: 5-FAM®-TTAG-TAAGAGGCGGTCAGGTG-TAMRA®-3′. Based on the result of qPCR we have calculated amount of genomes used for infection of neurons. For infection of neuronal cultures we have used MOI (multiplicity of infection) 25 and 50 rickettsiae genomes per one neuron.

Cell cultures of rat cerebrocortical neurons were prepared as described previously [9]. Briefly, brains of anaesthetized rats (18-day-old embryos) were removed and cortices were dissected. After enzymatic treatment a cell suspension was prepared and cells were plated onto poly D-lysine-coated 96-well plates at densities of 70,000 and 25,000 cells/cm^2 for determination of cell survival and immunofluorescence, respectively. The neurobasal medium was supplemented with B27 (2%) and Pen/Strep, which were washed out 24 h before experiment. The neurons were maintained in a humidified atmosphere of 5% CO_2 at 37 °C for more than 8 days *in vitro* (DIV) and medium was replaced every 3–4 days.

Neuron survival was determined by measurement of intracellular ATP using CellTiter-Glo® assay (Promega), which quantifies ATP level in viable cells and toxicity was determined by measurement of adenylate kinase activity in culture medium. The "ATP assay" was designed for quantitative analysis of viability of rat primary cerebrocortical neurons and was used according to manufacturer's protocol. Medium from cell cultures was mixed with adenylate kinase substrate ADP (AbD Serotec) and subsequently the released adenylate kinase (AK) from damaged cells was measured using a Fluoroscan Ascent FL luminometer (MTX Lab Systems, Inc).

A triple immunofluorescent staining technique (IFA) was used to detect and localize rickettsiae in the infected neuronal cells [27]: rabbit anti-*Rickettsia* primary antibody was overlaid by Alexa Fluor®-488 Goat anti-rabbit secondary antibody to visualize rickettsiae. To label neuronal neurites, tau-specific monoclonal antibody DC-25 (Axon-Neurosciences) and Alexa Fluor®-546 anti-mouse polyclonal antibody were applied. Cells were counterstained with DAPI to selectively colour DNA. We have used confocal microscope Zeiss LSM 510 META for co-localization analysis of the fluorescent probes. The resulting signal was analysed by image analysis software from Zeiss.

One experimental group was represented by data from at least six wells of a 96-well plate. The experimental data were repeatedly confirmed and survival was determined after several different time points. Statistical differences were evaluated by one way ANOVA. Differences were considered to be statistically significant when $p < 0.05$.

3. Results

Rat cerebrocortical neurons cultured *in vitro* for eight days (8 DIV) were infected with three different species of the genus *Rickettsia*. We observed considerable phenotypic disparities between neurons after rickettsial infection when compared to rickettsia-free neuronal cultures by a light microscope. Control cells were pictured with a large round soma and well developed branches of neuronal neurites. The neuronal cultures infected with *R. akari*, *R. slovaca* and *R. helvetica* showed the signs of subtotal neuron destruction, i.e. neurite fragmentation, loss of shape and cell fragmentation (Fig. 1).

The presence of rickettsiae inside the neurons was confirmed by indirect immunofluorescence. Co-localization of anti-*Rickettsia* primary antibodies and antibodies specifically labelling intraneuronal microtubule associated protein tau in neurons demonstrated that all three rickettsiae species tested are able to enter into neurons (Fig. 2). We assume that they can propagate intraneuronally forming large aggregates.

To further investigate the effect of rickettsia infection directly on the neurons we measured intracellular concentration of ATP and the level of adenylate kinase (AK) secreted to the culture medium. The neurons were inoculated with three different *Rickettsia* species at an MOI of 50 rickettsiae per cell, full load (L1), and 25 half load (L1/2), for time period of 24 and 48 h. While there is no difference in concentration of AK in the culture medium between infected and non-infected neurons (data not shown), we found a profound differences in the level of intracellular ATP. The rickettsia induced a strong and statistically significant decrease in viability of the neurons, which can be observed as soon as 24 h after the infection (not shown). After 48 h post infection resulted in the loss of 45.1% of ATP in case of *R. helvetica* and 31.5% in case of *R. akari* when compared to ATP level in mock treated control cultures (Fig. 3). Interestingly we observed no compromised viability of neurons when infected with *R. slovaca*. These data indicate significant differences in neurotoxicity of different species of *Rickettsia*, which can be relevant to their potential role in etiopathogenesis of various neuropathies.

4. Discussion

Rickettsioses are a group of arthropod-borne diseases caused by bacteria of the genus *Rickettsia* and *Orientia*, which belong to the *Rickettsiaceae* family. Clinical manifestations of these diseases affecting various organs, including the CNS, have been assigned within the differential diagnoses [1,5].

We have investigated what can be the role of rickettsiae in the brain, specifically whether or not the rickettsiae can enter the neurons, how they can change cell morphology and affect neuron survival. For this purpose we have performed an *in vitro* infection of rat cerebrocortical neurons with three different species of spotted fever group rickettsiae; *R. helvetica, R. akari* and *R. slovaca*, which represent three genetically different clusters of rickettsiae.

Dermacentor marginatus, the major vector of *R. slovaca*, while feeding blood is known to prefer a neck or head area on