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SHORT COMMUNICATION

Inhibition of Fibroblast Apoptosis by *Borrelia afzelii*, *Coxiella burnetii* and *Bartonella henselae*

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Abstract

Apoptosis is a genetically controlled mechanism of cell death involved in the regulation of tissue homeostasis. The aim of this study was to investigate the influence of *Borrelia afzelii*, *Coxiella burnetii*, and *Bartonella henselae* bacteria on apoptosis measured as the level of caspase 3 activity in human fibroblast cells HEL-299. Our findings show that *C. burnetii* bacteria may inhibit the process of apoptosis in the host cells for a long time. This can permit intracellular survival in the host and mediatingthe development of chronic disease.

Key words: *Borrelia afzelii*, *Coxiella burnetii*, *Bartonella henselae*, fibroblasts, apoptosis

Apoptosis is a genetically controlled mechanism of cell death involved in the regulation of tissue homeostasis. It has been found that some viral, bacterial and parasitic pathogens affect the viability of the host cell, inhibiting or promoting apoptosis (Carmen *et al*., 2006; Clifton *et al*., 1998; Fischer *et al*., 2004; Radulovic *et al*., 2002). In this process, activation of mediators called caspases plays a key role in the destruction phase of cell apoptosis. At least 13 different caspases have been identified, which belong to three different subfamilies, depending on their substrate specificity.

Caspase 3 is a cytosolic protein found in cells as an inactive 32 kDa proenzyme, and it is activated by various death signals into 20 kDa (p20) and 11 kDa (p11) active subunits. Both subunits contribute to substrate binding and catalysis. This protein cleaves and activates caspases 6, 7, and 9; moreover the protein itself is processed by caspases 8, 9, and 10 (Gołąb 2009).

The aim of our studies was to investigate the influence of *Borrelia afzelii*, *Coxiella burnetii*, and *Bartonella henselae* bacteria on apoptosis measured as the level of caspase 3 activity in human fibroblast cells.

A suspension of *B. henselae* bacterial cells (ATCC 49882) used for evaluations was obtained by growing on chocolate agar containing 5% defibrinated sheep blood in a humid atmosphere with 5% CO_2 at 35°C and harvested after 5 days when bacterial growth was sufficient. A final inoculum of 10^6 cfu/spot was used for inoculation in HEL-299 (Dörbecker *et al*., 2006).

Borrelia afzelii strain VS 461 (ATCC 51567) was grown at 35°C in BSK-H Medium Complete (Sigma-Aldrich, USA) to a cell density of 10^7/ml (Pollack *et al.,* 1993).

C. burnetii (strain Henzerling) was cultured in HEL-299 (ATCC-CCL-137) human fibroblast cells in shell-vials containing 5 ml of Eagle's Minimum Essential Medium (EMEM) medium with Earle's BSS, 2 mM L-glutamine and supplemented with 5% fetal bovine serum for 14 days. Cells were infected with the supernatant containing *C. burnetii* to a fresh monolayer and incubated in 5% CO_2 atmosphere at 35°C (Raoult *et al.,* 1990).

HEL-299 – human fibroblasts cells (ATCC-CCL-137) were cultured in shell-vials (Bibby Sterilin, Staffordshire, United Kingdom) containing 2 ml of EMEM with Earle's BSS, 1 mM sodium pyruvate, 2 mM L-glutamine (ATCC, Manassas, Canada) and supplemented with 5% fetal bovine serum. After two days the cells were infected with 100 µl *C. burnetii*, *B. henselae* and *B. afzelii* cultures. As a control, uninfected HEL299 cells were tested. Both infected and uninfected cells were incubated in 5% CO_2 atmosphere at 35°C.

The course of human fibroblast apoptosis was evaluated by determination of caspase-3 activity at the same time in infected cell cultures after six hours post infection, on 7^{th}, 14^{th} 21^{st} and 28^{th} day of infection. All tests were run in triplicate.

The presence of enzyme activity in cells lysates was determined with a Human Caspase-3 Instant ELISA

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(Bender MedSystems, Austria) according to the manufacturer's protocol. Infected and uninfected cells adhering to cover slips in shell-vials were washed twice with PBS. Cells were incubated 60 minutes at room temperature with gentle shaking in lysing buffer (Lysis buffer, Bender MedSystems, Austria), then centrifuged at 1000 g for 15 minutes. Supernatants were stored at –80°C and assayed at the same time. Absorbance of each sample was measured in duplicate on spectrophotometer at wavelength 450 nm. Concentration was calculated from a standard curve, created by plotting the mean absorbance for each standard concentration.

Data were compared with the Mann-Whitney's statistical test, and *p-value* less than 0.05 (level of significance) was considered statistically significant. Calculations were performed using the statistical package R Development Core Team, 2011 (Vienna, Austria).

Caspase 3 activity in HEL299 cell line infected with *B. afzelii, B. henselae* and *C. burnetii* was compared. During 28 days a slight increase from 0.43 to 0.48 ng/ml was detected in uninfected cells.

In cells infected with *B. afzelii* strain, the initial level of caspase 3 activity was 0.43 ng/ml. It showed a steady increase from 0.41 ng/ml, 0.44 ng/ml, 0.55 ng/ml to 0.56 ng/ml on 7th, 14th, 21st and 28th days after infection, respectively.

In cell cultures infected with *B. henselae* strain, a decrease of caspase-3 activity was observed, from 0.45 ng/ml on the first day of infection to 0.34 ng/ml and 0.36 ng/ml after 7 and 14 days, followed by an increase to 0.48 ng/ml and 0.49 ng/ml after 21 and 28 days of incubation.

In cell culture inoculated with *C. burnetii* a decrease in the level of the enzyme activity from 0.45 ng/ml on the 1st day to the level of 0.35 ng/ml on 7th day and 0.31 ng/ml on 14th day was observed. After 21 and 28 days the level stabilized at 0.34 ng/ml and 0.35 ng/ml (Table I).

Comparing caspase 3 activity levels on the 1st and 28th day, a 17% increase in *B. afzelii* infected HEL299 cultures ($p = 0.1$) and 2% increase in cell cultures infected with *B. henselae* ($p = 0.4$) was observed, compared to uninfected HEL-299.

In cell culture infected with *Coxiella burnetii* a 27% decrease in caspase 3 activity was detected ($p = 0.1$).

The p-values greater than 0.05 and equal or less than 0.10 may be treated as the border of statistical significance due to the small number of tests.

In the present studies, the process of apoptosis on the basis of caspase 3 activity in human fibroblasts *in vitro* was monitored. In *C. burnetii* infected HEL 299 caspase activity decreased after 7 days and was 22% less after 28 days than the initial level on the first day of infection. Inhibition was observed throughout the incubation period. At the same time, caspase 3 activity increased during four weeks of incubation in uninfected cell culture and in cells infected with *B. azelii* and *B. henselae* (Fig. 1).

Several reports have described interactions between *B. burgdorferi* bacteria and various host cells. It has been shown that the spirochetes can enter mammalian immune cells and other cells as well as tick tissue. This process allows the pathogen to survive in host tissues, to infect them and to escape the host defense (Hu and Klempner, 1997; Klempner *et al.,* 1993; Linder *et al.,* 2001; Peters and Benach, 1997; Sigal 1997; Szczepanski *et al.,* 1990; Thomas and Comstock 1989). Our study reveals that *B. afzelii* bacteria have the ability to inhibit apoptosis only for a short period of time compared to *C. burnetii*. Electron microscopic studies have revealed the consecutive steps of the *B. burgdorferi* life cycle *in vitro*. The spirochetes penetrate into fibroblasts.

Table I
Levels of caspase 3 activity (ng/ml) in cultures HEL 299 infected with *Bartonella henselae*, *Borrelia afzelii*, *Coxiella burnetii*

Day	Caspase 3 activity measured in ng/ml with standard deviation in cultures HEL 299 infected with			Caspase 3 activity measured in ng/ml in uninfected HEL 299 culture
	Bartonella henselae	*Borrelia afzelii*	*Coxiella burnetii*	
1	0.45 ± 0.04	0.39 ± 0.04	0.45 ± 0.04	0.43 ± 0.047
7	0.34 ± 0.04 (↓ 17%)*	0.43 ± 0.07 (↑ 5%)*	0.35 ± 0.03 (↓ 15%)*	0.41 ± 0.04
14	0.36 ± 0.06* (↓ 18%)	0.44 ± 0.03 (↑ 2%)*	0.31± 0.03 (↓ 28%)*	0.43 ± 0.04
21	0.48 ± 0.04 (↑ 4%)*	0.55 ± 0.04 (↑ 20%)*	0.34 ± 0.02 (↓ 26%)*	0.46 ± 0.04
28	0.49 ± 0.03 (↑ 2%)*	0.56 ± 0.04 (↑ 17%)*	0.35 ± 0.03 (↓ 27%)*	0.48 ± 0.04

* percentage of increase (↑) or decrease (↓) of caspase 3 activity levels calculated as a ratio of the levels in infected to uninfected HEL299 cells

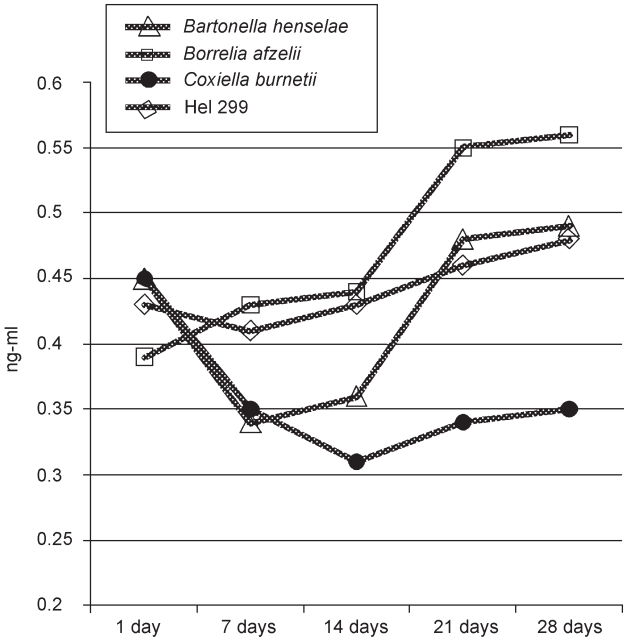

Fig. 1. Caspase 3 activity in uninfected HEL-299 cells and in cells infected with *B. henselae*, *B. afzelii* and *C. burnetii*

They have been observed in the fibroblasts and after 48 hours they are released to the extracellular space. This indicates that they stay in a cell only a short time (Chmielewski and Tylewska-Wierzbanowska, 2010). Thus, their ability to inhibit apoptosis is limited.

Bacteria of the genus *Bartonella*, in the human body attach to epithelial cells and in the process of phagocytosis, they penetrate into the cells and multiply inside. They have an ability to form large aggregates. This structure creates perfect conditions for bacterial replication, protecting them from the host immune defense and degrading enzymes, present in lysosomes. Next, these organisms are released into the cytoplasm, in which produce and secrete factors stimulatinge cell proliferation, activation of pro-inflammatory factors and inhibiting apoptosis. After 4 days of infection, bacteria are released to the blood stream and penetrate into the erythrocytes and multiply intracellularly (Guz and Goroszkiewicz, 2009; Kordick *et al.,* 1999). Ability to inhibit apoptosis in fibroblast culture *in vitro*, was observed especially between 7 to 14 days of infection.

C. burnetii after successfully evading host defense mechanisms is able to inhibit apoptosis to survive and to multiply inside the cells. Inhibition of apoptosis has been observed among intracellular pathogens with characteristic slow multiplication to establish a productive infection. (Carmen *et al.*, 2006; Clifton *et al.*, 1998; Fischer *et al.,* 2004). *C. burnetii* infection affects the expression of multiple apoptosis-related genes and resulting in increased synthesis of the antiapoptotic proteins such as A1/Bfl-1 and c-IAP2, prosurvival kinases Akt and Erk1/2 (extracellular signal-regulated kinases 1 and 2). *C. burnetii* infection of THP-1 human macrophage-like cells caused increased levels of phosphorylated c-Jun, Hsp27, Jun N-terminal protein kinase, and p38 protein. This pathogen can interfere with the intrinsic cell death pathway during infection by producing proteins that either directly or indirectly prevent release of cytochrome c from mitochondria. To summarize, these results indicate the importance of *C. burnetii* modulation of host signaling in successful intracellular parasitism and maintenance of host cell viability (Voth *et al.,* 2007; Voth and Heinzen, 2009; Lührmann and Roy 2007).

C. burnetii is the one of the obligate intracellular pathogens that can infect mammalian monocytes and macrophages *in vivo* and can grow in Vero, fibroblast and macrophagelike cells *in vitro*. Our findings show that *C. burnetii* bacteria may inhibit the process of apoptosis in the host cells for a long time. It can be the crucial pathogenic mechanism which permits the pathogen to survive intracellularly in the host and to mediate the development of chronic disease.

C. burnetii has to inhibit host cell death to provide a stable, intracellular niche for the course of the pathogen's infectious cycle. In cultures *C. burnetii*-infected cells can be maintained for weeks.

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