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Original article

Infection of *Ixodes* spp. tick cells with different *Anaplasma phagocytophilum* isolates induces the inhibition of apoptotic cell death

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ABSTRACT

Anaplasma phagocytophilum is an intracellular rickettsial pathogen transmitted by *Ixodes* spp. ticks, which causes granulocytic anaplasmosis in humans, horses and dogs and tick-borne fever (TBF) in ruminants. In the United States, human granulocytic anaplasmosis (HGA) is highly prevalent while TBF has not been reported. However, in Europe the situation is the opposite, with high prevalence for TBF in sheep and low prevalence of HGA. The origin of these differences has not been identified and our hypothesis is that different *A. phagocytophilum* isolates impact differently on tick vector capacity through inhibition of apoptosis to establish infection of the tick vector. In this study we used three different isolates of *A. phagocytophilum* of human, canine and ovine origin to infect the *Ixodes ricinus*-derived cell line IRE/CTVM20 and the *Ixodes scapularis*-derived cell line ISE6 in order to characterize the effect of infection on the level of tick cell apoptosis. Inhibition of apoptosis was observed by flow cytometry as early as 24 h post-infection for both tick cell lines and all three isolates of *A. phagocytophilum*, suggesting that pathogen infection inhibits apoptotic pathways to facilitate infection independently of the origin of the *A. phagocytophilum* isolate and tick vector species. However, infection with *A. phagocytophilum* isolates inhibited the intrinsic apoptosis pathway at different levels in *I. scapularis* and *I. ricinus* cells. These results suggested an impact of vector-pathogen co-evolution on the adaptation of *A. phagocytophilum* isolates to grow in tick cells as each isolate grew better in the tick cell line derived from its natural vector species. These results increase our understanding of the mechanisms of *A. phagocytophilum* infection and multiplication and suggest that multiple mechanisms may affect disease prevalence in different geographical regions.

1. Introduction

Anaplasma phagocytophilum is an intracellular Gram-negative bacterium that is transmitted by ticks of the genus *Ixodes* and infects vertebrate host myeloid and non-myeloid cells, mainly neutrophils (Severo et al., 2015). In both invertebrate and vertebrate cells, *A. phagocytophilum* multiplies within a parasitophorous vacuole, thus evading host cell defenses (Severo et al., 2015). Infection with *A. phagocytophilum* has been shown to cause the emerging tick-borne disease human granulocytic anaplasmosis (HGA). This is one of the most prevalent tick-borne diseases in the United States (Goodman, 2005) while, although increasingly detected, it is still rare in Europe (Edouard et al., 2012). *A. phagocytophilum* is also an economically important pathogen of sheep in Europe where it causes tick-borne fever (TBF) (Stuen, 2007; Stuen et al., 2009). This disease has not been reported in the United States but cases of equine and canine granulocytic anaplasmosis caused by *A. phagocytophilum* have been recorded (Severo et al., 2015).

A. phagocytophilum intraspecific genetic variability is associated with different reservoir host and tick vector species (Stuen et al., 2013). In the United States, the main vector is *Ixodes scapularis*, with a prevalence ranging from <1% to 50% (Goltz et al., 2013). In Europe, the main vector is *Ixodes ricinus*, with pathogen-prevalence ranging

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from <1% to 20% although it varies between geographical areas and stages of tick development (Stuen, 2007).

The prevalence of *A. phagocytophilum* infection is very high in domestic and wild animals in Europe with up to 37.5% prevalence in sheep in Norway and 98.9% prevalence in roe deer in Germany (Stuen et al., 2013) whereas few cases of HGA are reported (Heyman et al., 2010). However, cases of HGA are common in many areas of the United States. In fact, seroepidemiological data suggest that many cases of anaplasmosis have not been recognized and, in endemic areas of the United States, 15–36% of the human population could be infected (Dumler et al., 2005). In the United States, mortality has been estimated to be up to 5% and most deaths are associated with opportunistic infections due to immunosuppression or underlying disease (Dumler et al., 2007). In Europe, no death has been reported to be associated with HGA, suggesting that in European regions the main disease produced by *A. phagocytophilum* is TBF in sheep while in the United States HGA predominates. Cross-infection experiments indicate that *A. phagocytophilum* isolates of distinct host origin are not uniformly infectious for heterologous hosts (Jin et al., 2012). Recently, multilocus sequence typing of almost 400 *A. phagocytophilum* strains from humans and animals has shown that strains from humans, dogs, horses, wild boars and hedgehogs from Europe belong to the same clonal complex whereas cattle, sheep, roe deer, vole and shrew strains are distantly related suggesting that they are unlikely to be infectious for humans (Huhn et al., 2014).

Apoptosis is a regulated process of cell death that maintains tissue homeostasis. Control of cell death by the host can be used to manage microbial spread and enhance the induction of immunity. Different disease states, including bacterial infection, can lead to inappropriate apoptosis regulation. The manipulation of host cell death pathways is exploited by many viral and microbial pathogens as part of their life cycle. *A. phagocytophilum* is able to inhibit host cell apoptosis pathways as a mechanism to improve its survival and multiplication inside the host cell (Rikihisa, 2010). In vertebrates, the pathogen has the ability to delay host cell apoptosis by activation of an anti-apoptotic cascade since this is crucial for its intracellular survival and reproduction in the neutrophils whose life cycle is normally short (Carlyon and Fikrig, 2003; Galindo et al., 2008). Different studies have shown that *A. phagocytophilum* infection upregulates the expression of anti-apoptotic *blc-2* genes, blocks cell surface fatty acid synthase (FAS) clustering during spontaneous neutrophil apoptosis and inhibits cleavage of pro-Caspase 8 and Caspase 8 activation as well as secretion *Anaplasma* translocated substrate-1 (Ats-1) preventing mitochondrial response to apoptotic signals (Niu et al., 2010; Rikihisa, 2010). Inhibition of apoptosis has also been observed in the *I. scapularis* cell line ISE6 in which *A. phagocytophilum* infection downregulates mitochondrial *porin* inducing mitochondrial dysfunction and thereby inhibition of the intrinsic apoptosis pathway to subvert host cell defenses and increase infection (Ayllón et al., 2013). Functional characterization using RNA interference (RNAi) demonstrated that *porin* knockdown significantly increases colonization of *I. scapularis* ticks by *A. phagocytophilum* but does not affect tick feeding, thus showing how bacterial inhibition of *porin* expression increases tick vectorial capacity for this pathogen (authors' unpublished results). These results demonstrate how *A. phagocytophilum* has evolved to use similar strategies to establish infection in both vertebrate and invertebrate hosts (Ayllón et al., 2013).

A. phagocytophilum strains isolated from a variety of host species are able to grow in both *I. scapularis* (Munderloh et al., 1999) and *I. ricinus* (Dyachenko et al., 2013) tick cell lines, suggesting that the pathogen employs similar infection and multiplication mechanisms in both tick species. Tick-*A. phagocytophilum* interactions are not as well characterized as those between the pathogen and vertebrate hosts. Understanding the mechanisms of pathogen infection and multiplication is essential to answer basic biological questions and to design new strategies for pathogen control. Despite genetic variability between *A. phagocytophilum* strains from different hosts (Huhn et al., 2014), the origin of the differences in disease prevalence between Europe and the United States has not been identified. Our hypothesis is that different *A. phagocytophilum* strains impact differently on tick vector capacity through apoptosis inhibition to establish infection of the tick vector. If proven correct, it would indicate that pathogen strains causing HGA are well adapted to *I. scapularis* but not *I. ricinus*, while strains causing TBF are better adapted to *I. ricinus*. To address this hypothesis, the aim of this study was to characterize cell apoptosis during early *in vitro* infection of tick cell lines derived from two different *Ixodes* species with three different isolates of *A. phagocytophilum* of human, canine and ovine origin.

2. Materials and methods

2.1. *Tick cell cultures and* A. phagocytophilum *isolates*

The *I. ricinus* embryo-derived cell lines IRE/CTVM19 and IRE/CTVM20 (Bell-Sakyi et al., 2007) were maintained in, respectively, complete L-15 and L-15/L-15B media (Bell-Sakyi, 2004). Medium was changed once a week for both cell lines. The *I. scapularis* embryo-derived cell line ISE6 (provided by U.G. Munderloh, University of Minnesota, USA) was maintained in L-15B300 medium as described previously (Munderloh et al., 1999). The tick cell line IDE8, derived from *I. scapularis* embryos (Munderloh et al., 1994) was maintained at 32 °C in L-15B medium in sealed containers as previously described (Munderloh and Kurtti, 1989). Infected IDE8 cultures were propagated in a modified L-15B medium further supplemented with 0.1% $NaHCO_3$ and 10 mM HEPES. The pH of the medium was adjusted to 7.5. This modified medium is referred to as *Anaplasma* culture medium (ACM). IRE/CTVM20 and ISE6 cells were inoculated with three different isolates of *A. phagocytophilum*: the human isolate NY18 (Asanovich et al., 1997), a dog isolate from Germany (L610) and an sheep isolate from Norway obtained in this study as described below. Infected cultures were propagated at 34 °C in 25 cm^2 plastic culture flasks in 5 ml of the medium.

2.2. In vitro *isolation of* A. phagocytophilum *from canine blood in IDE8 cells*

An EDTA blood sample from a German dog was obtained from the Diagnostic Laboratory of the Institute for Comparative Tropical Medicine and Parasitology, University of Munich (Germany). Five hundred μl of EDTA blood were diluted into 10 ml of physiological PBS, and centrifuged for 5 min at 515 × *g*. The supernatant was removed and the blood cell pellet was resuspended in 9 ml of sterile, double distilled water for approximately 30 s to lyse most of the erythrocytes by hypotonic shock. Physiological tonicity was restored by the addition of 1 ml of 10-fold-concentrated Hanks' balanced salt solution. The lysate was centrifuged for 5 min at 290 × *g*. The supernatant was discarded and the pellet resuspended in ACM and inoculated into an IDE8 culture. The cultures were incubated at 34 °C. Three ml of medium were changed twice weekly. The strain isolated here is referred to as strain L610.

2.3. *Isolation of* A. phagocytophilum *from sheep blood in IRE/CTVM19 cells*

Blood samples were collected from Norwegian White Sheep experimentally inoculated with sheep *A. phagocytophilum* variant2 strain from Norway (Stuen et al., 2009). The presence of *A. phagocytophilum* DNA in the blood samples was confirmed by