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***Toxoplasma gondii*: Effect of infection on expression of 14-3-3 proteins in human epithelial cells**

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Abstract

14-3-3 proteins are expressed in most eukaryotes organisms and play varied and crucial roles in a wide range of regulatory processes. In mammalian cells, seven 14-3-3 isoforms have been identified. However; it is not known what effect infection has on 14-3-3 isoform expression. In this study human colonic carcinoma cell lines were infected with *Toxoplasma gondii* for 24 h and expression of 14-3-3 proteins was determined by RT-PCR. HT-29 cells only expressed 3 out of the 7 isoforms while 5 and all 7 isoforms were found in HCT-116 and Caco-2 cells respectively. Infection had little or no effect in the expression of 14-3-3 γ , ϵ , σ , and ξ ; but in HCT-116 cells induced expression of 14-3-3 η and σ , while 14-3-3 β , η , and ξ were induced in HT-29 cells. If 14-3-3 proteins are involved in cell survival and/or prevention of parasite replication, longer incubation times may be required as no differences in percentage of infection were found among the cell lines at 24 h post-infection.

Keywords

14-3-3 isoforms; RT-PCR; *Toxoplasma*; colonic epithelial cells; colon cancer cells

1. Introduction

14-3-3 Proteins are a family of acidic dimeric proteins expressed in all eukaryotic organisms, including parasites (van Hemert et al., 2001; Siles-Lucas and Gottstein, 2003). 14-3-3 proteins are highly conserved in amino acid sequences from yeast to mammals. Seven isoforms encoded by seven distinct genes are identified in mammals while more than 10 isoforms are identified in plants (Wu et al., 1997), and two isoforms are identified in yeast (Ford et al., 1994; van Heusden et al., 1995). In mammals the function of 14-3-3 proteins is not completely understood but they play varied and crucial roles, including signal transduction, cell cycle control, stress responses, vesicular transport, DNA replication, cytoskeleton organization, prevention of apoptosis, and malignant transformation (Baldin, 2000; Fu et al., 2000; van Hemert et al., 2001; Muslin and Xing, 2000; Tzivion and Avruch, 2002; Tzivion et al., 2006).

Homologs of 14-3-3 proteins have been reported in helminth and protozoan parasites (Siles-Lucas and Gottstein, 2003). However, the number of 14-3-3 isoforms, their function, cellular localization, and expression pattern has been studied in only a few of them. Their crucial role in biological processes such as parasite proliferation and survival has made them attractive targets for vaccination (Zhang et al., 2000; Schechtman et al., 2001; Zhang et al., 2001; Siles-

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Lucas et al., 2003). In *Toxoplasma gondii* 14-3-3 isoforms have been reported in feline enteroepithelial gametocyte stages (Koyama et al., 2001) and in tachyzoites (Assossou et al., 2003). Tachyzoites contain two isoforms, one is mainly cytosolic with a small fraction membrane-associated, while the other is only present in the lipid rafts of detergent-resistant membranes (Assossou et al., 2003).

Expression of 14-3-3 isoforms is generally increased in human cancer cells (Qi et al., 2005; Tzivion et al., 2006) but their regulation by infection by an intracellular parasite is not known. In this study, human colonic carcinomas epithelial cells were infected with the obligated protozoan parasite *Toxoplasma gondii* to determine 14-3-3 isoform regulation by infection.

2. Material and methods

Three human colorectal carcinomas, HT-29 (ATCC, HTB-38), Caco-2 (ATCC, HTB-37) and HCT-116 (ATCC, CCL-247) were used in these studies. These cells are well established models for studies of intestinal epithelial cell biology. Cells were maintained in DMEM medium supplemented with 10% FBS, 2 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids solution, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine at 37° C in a humidified atmosphere of 5% CO₂. Tachyzoites of the virulent *T. gondii* RH strain were maintained *in vitro* by infection of human foreskin fibroblasts. Parasites were collected and purified using a 3-µm polycarbonate membrane filter (Millipore, Bedford, MA, USA) (Monroy et al., 1999; Aviles and Monroy, 2001). Cells (1×10^5 /ml) were grown at 70% confluence in 6 well plates until exposed to *T. gondii*. Infection of colonic epithelial cells was performed at a 1:2 cells to parasites ratio and incubated for up to 24 h. Cells were collected at 3, 6, 9, 12 and 24 h after infection. A set of wells was used to determine percentage of infection after 24 h. In these wells, cells were grown on 1 cm round tissue culture cover slips. Cover slips were fixed in methanol, stained with DiffQuick (Baxter, Miami, FL, USA), and observed under 100X magnification. At collection times, cells were washed briefly with sterile PBS to remove non-intracellular parasites before processing for RNA collection. Total RNA was isolated from cultured cells using Trizol (Gibco, Rockville, MD, USA) according to the manufacturer's instructions. Reverse transcription was carried out at 42° C for 50 min using 5 µg of each RNA with 0.5 µg/µl Oligo(dT)12–18, 10 mM dNTP mix, 5X RT buffer, 50 mM MgCl₂, 0.1 mM DTT, RNase inhibitor and 50 U of M-MuLV reverse (Fermentas, Hanover, MD, USA). Primers that would specifically amplify each of the 7 human 14-3-3 isoforms according to their cDNAs from GenBank are shown in Table 1. The PCR reaction consisted of 12.5 µl 2X PCR mix (Fermentas, Hanover, MD, USA), 1.5 µl RT product, 1 µl each of forward and reverse primers (10 mM), and water to 25 µl. The reaction was carried out for 30 cycles of 94° C for 40 sec, 55° C for 50 sec and 72° C for 60 sec. Five microlit expression was performed in a FluorChem FC2 (Alpha Innotech Corporation, San Leandro, CA, USA). At each time point pixels for the isoforms were compared to those of β-actin and expressed at percent relative expression.

3. Results

Total RNA was isolated and RT-PCR was carried out using isoform specific primers. These primers have been used for amplification of isoforms in breast tissue samples and their expected amplification fragment sizes have been corroborated by sequencing (Martinez personal communication). There is a different pattern of 14-3-3 isoforms in normal and cancerous tissues (Qi and Martinez, 2003). All isoforms were detected in Caco-2 (Fig. 1C) while 14-3-3γ, ε, σ, and θ were minimally expressed by HT-29 cells (Fig. 1B). HTC-116 cells did not express 14-3-3η, and θ (Fig. 1A). Infection with *T. gondii* in Caco-2 cells primarily decreased the expression of 14-3-3η, θ and σ with this effects taking place after 12 h for 14-3-3θ and 24 h for 14-3-3σ (Fig. 1C). In HT-29 cells infection resulted in induced expression of 14-3-3ξ at 6 h while 14-3-3γ and η were expressed at 12 h after infection (Fig 1B). In HTC-116 cell infection

induced expression of 14-3-3 θ only at 6 h and 14-3-3 η was minimally detected at 3 h after infection (Fig. 1A). This expression was evident when densitometry analysis was performed to obtain relative expression of all isoforms (Fig. 2). Infection had no effect in the expression of 14-3-3 ϵ in all cell lines which was constitutively expressed in control as well as in infected cells throughout the study. When cells were investigated to determine percentage of infection, we found no significant differences ($n = 3$; $p < 0.19$) in the number of infected cells among the three cell lines at 24 h post-infection; although, HCT-116 and Caco-2 cells had lower number of infected cells (Fig. 3).

4. Discussion

We have investigated the effect on *T. gondii* infection on 14-3-3 protein expression in human colonic epithelial cells. All three cell lines used in this study have been well characterized and are useful models to study infection (Dimier and Bout, 1997) but most importantly, as models of cell immune responsiveness and integrity during inflammatory bowel disease. Although it is widely accepted that cancerous cells exhibit enhanced expression of 14-3-3 proteins compared to normal cells, the role of an intracellular pathogen on 14-3-3 is not clear. All three cells used in this study were of human colorectal carcinoma origin and displayed different patterns of 14-3-3 isoform expression. RT-PCR amplified all isoforms in Caco-2 cells while HCT-116 and HT-29 strongly expressed 5 and 3 isoforms respectively.

The role of 14-3-3 isoforms has not been studied in detail but among the seven distinct 14-3-3 genes, 14-3-3 σ has been directly implicated in the etiology of human cancer (Chan et al., 1999; Suzuki et al., 2000). In this type of cancer, 14-3-3 σ expression is lost in 95% of breast carcinomas by methylation-mediated silencing (Umbrecht et al., 2001; Tzivion et al., 2006). In contrast 14-3-3 σ seems to be over expressed in a subset of colorectal cancers and predicts colorectal carcinoma progression (Perathoner et al., 2005). In a different study, it was found that hypermethylation of 14-3-3 σ was an uncommon event and 14-3-3 σ expression was kept even in established colorectal cancer cell lines (Ide et al., 2004). Our data shows that infection with *T. gondii* has no effect on 14-3-3 σ expression as this isoform was expressed throughout the length of this study in all three cell lines with the exception of Caco-2 in which 14-3-3 σ was not expressed at 24 h post-infection. *T. gondii* expresses two 14-3-3 isoforms and sequence similarities predicts one of them to be a homolog of 14-3-3 σ (Koyama et al., 2001). The parasite contribution to the observed 14-3-3 σ expression can be ruled out as no amplification was observed when human primers were used to amplify tachyzoites DNA.

Another isoform that has been investigated in colorectal carcinoma is 14-3-3 ϵ (Liou et al., 2007). Nonsteroidal anti-inflammatory drugs (sulindac sulfide) inhibited 14-3-3 ϵ expression in HT-29 cells resulting in increased cell apoptosis. Furthermore, stable expression of 14-3-3 ϵ in HT-29 cells significantly protected them from sulindac sulfide-induced apoptosis. This 14-3-3 isoform was the only one expressed throughout infection in all cell lines. Studies in other cell lines have shown that reduced expression of 14-3-3 ξ in lung cancer cell lines increases their sensitivity to ionizing radiation (Qi and Martinez, 2003). In addition, decreased expression of 14-3-3 β in the rat hepatoma AFB1-K2 cell line resulted in reduced cell proliferation and tumorigenicity in mice (Sugiyama et al., 2003).

Based on the central role observed for 14-3-3 σ , and ϵ in colonic epithelial cell lines it was expected that infection might result in decreased expression of these isoforms, but this was not the case as both isoforms remained expressed throughout infection suggesting that their expression was not affected by *T. gondii*. Furthermore, we found no differences in the number of infected cells despite decreased expression of 14-3-3 isoforms in HT-29 cells. We can conclude that infection resulted in specific induction of some isoforms (14-3-3 η and θ in HCT-116 and 14-3-3 η and ξ in HT-29 cells) but no differences were found percentage of

infection. It is clear that our 24 h post-infection time point was too short to observe parasite-induced cell lysis. Therefore, if 14-3-3 proteins are involved in prevention of parasite replication, this remains to be determined. *T. gondii* infection leads to alteration or inhibition of key cell signaling pathways (Heussler et al., 2001; Butcher et al., 2001; Tato et al., 1993;) and cellular processes (Luder et al., 2003; Payne et al., 2003; Nishikawa et al., 2007) resulting in increased parasite survival. Although, these studies were performed in well characterized human colonic epithelial cells; these cells are derived from colorectal adenocarcinomas, and expression of 14-3-3 proteins in cancer cells is commonly altered (Qi et al., 2005). Therefore, it is reasonable to speculate that infection of intestinal epithelial cells may result in *T. gondii*-induced regulation of 14-3-3 proteins expression leading to increased cell survival and proliferation, and suppression of apoptosis of parasitized cells. Future studies will investigate the significance of *T. gondii* interaction with these key cell regulators in non cancerous cell lines as well as in immune cells.

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References

- Assossou O, Besson F, Rouault JP, Persat F, Brisson C, Duret L, Ferrandiz J, Mayençon M, Peyron F, Picot S. Subcellular localization of 14-3-3 proteins in *Toxoplasma gondii* tachyzoites and evidence for a lipid raft-associated form. *FEMS Microbiology Letters* 2003;224:161–168. [PubMed: 12892878]
- Aviles HO, Monroy FP. Immunomodulatory effects of cold stress on mice infected intraperitoneally with a 50% lethal dose of *Toxoplasma gondii*. *Neuroimmunomodulation* 2001;9:6–12. [PubMed: 11435747]
- Baldin V. 14-3-3 proteins and growth control. *Progress in Cell Cycle Research* 2000;4:49–60. [PubMed: 10740814]
- Butcher BA, Kim L, Johnson PF, Denkers EY. *Toxoplasma gondii* tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NF-kappa B. *Journal of Immunology* 2001;167:2193–2201.
- Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3 σ is required to prevent mitotic catastrophe after DNA damage. *Nature* 1999;401:616–620. [PubMed: 10524633]
- Dimier IH, Bout DT. Inhibition of *Toxoplasma gondii* replication in IFN-gamma-activated human intestinal epithelial cells. *Immunology and Cell Biology* 1997;75:511–514. [PubMed: 9429902]
- Ford JC, al-Khodairy F, Fotou E, Sheldrick KS, Gri.ths DJ, Carr AM. 14-3-3 protein homologs required for the DNA damage checkpoint in fission yeast. *Science* 1994;265:533–535. [PubMed: 8036497]
- Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. *Annual Reviews of Pharmacology and Toxicology* 2000;40:617–647.
- Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B. 14-3-3 σ is a p53-regulated inhibitor of G2/M progression. *Molecular Cell* 1997;1:3–11. [PubMed: 9659898]
- Heussler VT, Kuenzi P, Rottenberg S. Inhibition of apoptosis by intracellular protozoan parasites. *International Journal for Parasitology* 2001;31:1166–1176. [PubMed: 11563357]
- Ide M, Nakajima T, Asao T, Kuwano H. Inactivation of 14-3-3 σ by hypermethylation is a rare event in colorectal cancers and its expression may correlate with cell cycle maintenance at the invasion front. *Cancer Letters* 2004;207:241–249. [PubMed: 15072834]
- Koyama T, Shimada S, Ohsawa T, Omata Y, Xuan X, Inoue N, Mikami T, Saito A. Antigens expressed in feline enteroepithelial-stages parasites of *Toxoplasma gondii*. *Journal of Veterinary Medical Sciences* 2000;62:1089–1092.

- Koyama T, Ohsawa T, Shimada S, Omata Y, Xuan X, Inoue N, Maeda R, Mikami T, Saito A. 14-3-3 protein homologue is expressed in feline enteroepithelial-stages of *Toxoplasma gondii*. *Veterinary Parasitology* 2001;96:65–74. [PubMed: 11182236]
- Liou JY, Ghelani D, Yeh S, Wu KK. Nonsteroidal Anti-inflammatory Drugs Induce Colorectal Cancer Cell Apoptosis by Suppressing 14-3-3 ϵ . *Cancer Research* 2007;67:3185–3191. [PubMed: 17409426]
- Luder CG, Aligner M, Lang C, Bleicher N, Gross U. Reduced expression of the inducible nitric oxide synthase after infection with *Toxoplasma gondii* facilitates parasite replication in activated murine macrophages. *International Journal for Parasitology* 2003;33:833–844. [PubMed: 12865083]
- Monroy FP, Banerjee SK, Duong T, Aviles HO. Cold stress-induced modulation of inflammatory responses and intracerebral cytokine mRNA expression in acute murine toxoplasmosis. *Journal of Parasitology* 1999;85:878–886. [PubMed: 10577724]
- Muslin AJ, Xing H. 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal* 2000;12:703–709. [PubMed: 11152955]
- Nishikawa Y, Kawase O, Vilemeyer O, Suzuki H, Joiner KA, Xuan X, Nagasawa H. *Toxoplasma gondii* infection induces apoptosis in noninfected macrophages: role of nitric oxide and other soluble factors. *Parasite Immunology* 2007;29:375–385. [PubMed: 17576367]
- Payne TM, Molestina RE, Sinai AP. Inhibition of caspase activation and a requirement for NF-kappaB function in the *Toxoplasma gondii*-mediated blockade of host apoptosis. *Journal of Cell Science* 2003;116:4345–4358. [PubMed: 12966169]
- Perathoner A, Pirkebner D, Brandacher G, Spizzo G, Stadlmann S, Obrist P, Margreiter R, Amberger A. 14-3-3 σ expression is an independent prognostic parameter for poor survival in colorectal carcinoma patients. *Clinical Cancer Research* 2005;11:3274–3279. [PubMed: 15867223]
- Qi W, Martinez JD. Reduction of 14-3-3 proteins correlates with increased sensitivity to killing of human lung cancer cells by ionizing radiation. *Radiation Research* 2003;160:217–223. [PubMed: 12859233]
- Schechtman D, Tarrab-Hazdai R, Arnon R. The 14-3-3 protein as a vaccine candidate against schistosomiasis. *Parasite Immunology* 2001;23:213–217. [PubMed: 11298298]
- Siles-Lucas MM, Gottstein B. The 14-3-3 protein: a key molecule in parasites as in other organisms. *Trends in Parasitology* 2003;19:575–581. [PubMed: 14642768]
- Sugiyama A, Miyagi Y, Komiya Y, Kurabe N, Kitanaka C, Kato N, Nagashima Y, Kuchino Y, Tashiro F. Forced expression of antisense 14-3-3 β RNA suppresses tumor cell growth in vitro and in vivo. *Carcinogenesis* 2003;24:1549–1559. [PubMed: 12896901]
- Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, Imai K. Inactivation of the 14-3-3 σ Gene Is Associated with 5' CpG Island Hypermethylation in Human Cancers. *Cancer Research* 2000;60:4353–4357. [PubMed: 10969776]
- Tato CM, Villarino A, Caamano JH, Boothby M, Hunter CA. Inhibition of NF-kappa B activity in T and NK cells results in defective effector cell expansion and production of IFN-gamma required for resistance to *Toxoplasma gondii*. *Journal of Immunology* 2003;170:3139–3146.
- Tzivion G, Avruch J. 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. *Journal of Biological Chemistry* 2002;277:3061–3064. [PubMed: 11709560]
- Tzivion G, Gupta VS, Kaplun L, Balan V. 14-3-3 proteins as potential oncogenes. *Seminars in Cancer Biology* 2006;16:203–213. [PubMed: 16725345]
- Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S. Hypermethylation of 14-3-3 σ (stratifin) is an early event in breast cancer. *Oncogene* 2001;20:3348–3353. [PubMed: 11423985]
- van Hemert MJ, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signaling and apoptosis. *Bioessays* 2001;23:936–946. [PubMed: 11598960]
- van Heusden GP, Gri.ths DJ, Ford JC, Chin AWTF, Schrader PA, Carr AM, Steensma HY. The 14-3-3 proteins encoded by the BMH1 and BMH2 genes are essential in the yeast *Saccharomyces cerevisiae* and can be replaced by a plant homologue. *European Journal of Biochemistry* 1995;229:45–53. [PubMed: 7744048]
- Wu K, Rooney MF, Ferl RJ. The Arabidopsis 14-3-3 multigene family. *Plant Physiology* 1997;114:1421–1431. [PubMed: 9276953]
- Zhang Y, Bickle QD, Taylor MG. Cloning of *Schistosoma japonicum* 14-3-3 epsilon (Sj14-3-3 ϵ), a new member of the 14-3-3 family of proteins from schistosomes. *International Journal for Parasitology* 2000;30:991–994. [PubMed: 10980288]

Zhang Y, Taylor MG, Johansen MV, Bickle QD. Vaccination of mice with a cocktail DNA vaccine induces a Th1-type immune response and partial protection against *Schistosoma japonicum* infection. 2001;20:724–730.

Figure 1.

PCR amplification of 14-3-3 isoforms in HCT-116 (A), HT-29 (B) and Caco-2 (C) cells. Control (Con) and infected cells with *T. gondii* RH tachyzoites were collected at 3, 6, 9, 12 and 24 h after infection. Experiments were repeated three times with similar results, and a representative set of results is presented.

Figure 2.

Semi-quantitative determination of 14-3-3 isoforms displayed in Figure 1. Gels were scanned and the intensity of each band determined using the Alpha Innotech FluorChem FC2. Pixels for each isoforms were compared to those of β -actin for each time point and expressed at percent relative expression.

Figure 3.

Percentage of infected human epithelial cells at 24 h after infection. Cell grown on cover slips were stained and counted under 100X magnification. A minimum of 100 cells were counted on three different cover slips.

Table 1

List of primers used for amplification of all seven 14-3-3 isoforms.

Accession Number	14-3-3 Isoform	Forward Primers	Reverse Primers	Expected Size (bp)
X57346	β	5'-CATTCGGCTGTGGATAGAGA-3'	5'-CATCTGCTGCTTCTTCATTC-3'	269
AM024334	γ	5'-CAGTGTCTCTCTCTCTCC-3'	5'-CTGGCACACAGCCTCCAACTCTTCT-3'	291
NM_0067613	ε	5'-GAGCGATACGACGAAATGGTG-3'	5'-CCTTGGACTCGCCAGGTGTAG-3'	263
X80536	η	5'-CTAGCGAGCCAGCGGTGGA-3'	5'-CTGTCTCTCCAGCTCTTCTCAATCTCTCC-3'	287
AF029082	σ	5'-AGAGACACAGAGTCCGGCATTTGG-3'	5'-TCCACCTTCTCCCGGTACTCAGGC-3'	290
NM_006826	θ	5'-CGTGAAGCTCTCGAGGCTCTCT-3'	5'-AATTCCAGCACCGTGGTGCAGATG-3'	344
U28964	ξ	5'-GCCGTGAGCAGCGAGATCC-3'	5'-AGCATGGATGACAAAATGGTC-3'	842
AK223055		5'-GTGGGGCGCCCGAGGCACCA-3'	5'-CTCCTTAATGTACGCGACGATTTC-3'	516

* Sequences obtained from Qi and Martinez (2003)