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BAG3 protein regulates caspase-3 activation in HIV-1-infected human primary microglial cells

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Abstract

BAG3, a member of the BAG co-chaperones family, is expressed in several cell types subjected to stressful conditions, such as exposure to high temperature, heavy metals, drugs. Furthermore, it is constitutively expressed in some tumors. Among the biological activities of the protein, there is apoptosis downmodulation; this appears to be exerted through BAG3 interaction with the heat shock protein (Hsp) 70, that influences cell apoptosis at several levels. We recently reported that BAG3 protein was detectable in the cytoplasm of reactive astrocytes in HIV-1-associated encephalopathy biopsies. Here we report that downmodulation of BAG3 protein levels allows caspase-3 activation by HIV-1 infection in human primary microglial cells. This is the first reported evidence of a role for BAG3 in the balance of death versus survival during viral infection.

INTRODUCTION

Cells infected by intracellular pathogens undergo apoptosis, as a consequence of either attack from immune system or activation of intrinsic apoptotic pathways (Xu et al., 2001; Waterhouse et al., 2004). The ability to trigger anti-apoptotic mechanisms constitutes an advantage for some intracellular pathogens, that by this means sustain the prolonged survival of their host cell (Hiscott et al., 2001; Xu et al., 2001; Hilleman, 2004). In HIV-1 infection, cells of the mononuclear phagocytic lineage, including blood monocytes, exert a major role as virus reservoir. Their long-term survival after HIV-1 infection and reduced sensitivity to apoptosis are of central importance in this respect (Herbein et al., 2002; Crowe et al., 2003; Aquaro et al., 2005).

Infection of the nervous system by HIV-1 is commonly associated with a syndrome of cognitive and motor abnormalities called HIV-1-associated dementia (HAD) (Gonzalez-Scarano and Martin-Garcia, 2005; Trujillo et al., 2005). HAD is caused by productive viral infection of brain mononuclear phagocytes (MPs) (perivascular and parenchymal brain macrophages and microglia) and sustained by paracrine neurotoxic responses. Indeed, HIV-1 enters the brain at the early stage of infection and resides primarily in a limited

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number of macrophages/microglia and astrocytes; their long-term survival after HIV-1 infection renders these cells an important reservoir of the virus (Mollace et al., 2001; Lum and Badley, 2003; Chipitsyna et al., 2004; Gonzalez-Scarano and Martin-Garcia, 2005; Speth et al., 2005). Neuronal cell death is induced by macrophage-produced cytokines (including tumor necrosis factor-related apoptosis-inducing ligand: TRAIL), arachidonic acid and its metabolites, nitric oxide, and viral proteins, including Tat, that can affect neighboring uninfected cells (Mollace et al., 2001; Peruzzi et al., 2002; Regulier et al., 2004; Aquaro et al., 2005; Huang et al., 2005; Jones and Power, 2006). The characterization of mechanisms that sustain cell survival in HIV-1-harboring cells (mononuclear leukocytes, microglial cells, astrocytes) and reduce their sensitivity to apoptosis induced by infection, ROS- and RNS-mediated oxidative stress, cytokines of the TNF family and other agents can contribute to our understanding of HIV-1-related pathogenesis and identification of novel target for therapies. Co-chaperone proteins that share the BAG domain are characterized by their interaction with heat shock proteins and other partners (steroid hormone receptors, Raf-1 and others), involved in regulating a number of cellular processes, including proliferation and apoptosis (Takayama et al., 1999; Doong et al., 2000; Takayama and Reed, 2001; Doong et al., 2002). Among BAG family members there is BAG3, also known as CAIR-1 or Bis (Lee et al., 1999; Antoku et al., 2001; Liao et al., 2001; Takayama and Reed, 2001; Briknarova et al., 2002; Doong et al., 2002; Lee et al., 2002a,b; Chroboczek et al., 2003; Doong et al., 2003; Pagliuca et al., 2003; Romano et al., 2003a,b; Bonelli et al., 2004; Chen et al., 2004; Homma et al., 2006; Chiappetta et al., 2007; Rosati et al., 2007a,b). BAG3 forms a complex with Hsp70 (Takayama et al., 1999), a protein that assists polypeptide folding, can mediate altered peptide delivery to proteasome (Young et al., 2003) and is able to modulate apoptosis by interfering with cytochrome c release, apoptosome assembly and other events in the death process (Beere, 2005). Notably, we observed that BAG3 expression can be induced by some stressful agents, such as high temperature or heavy metals (Pagliuca et al., 2003) mainly through the activation of heat shock factor (HSF)-1 (Franceschelli et al., 2008). In this report we investigated whether BAG3 protein levels influenced apoptotic events in microglial cells infected by HIV-1.

MATERIALS AND METHODS

Cells and HIV-1 infection

Primary human fetal microglial cells were prepared from 8- to 12-week-old human fetal brain tissue (purchased from Advanced Bioscience Resources Inc., Alameda, CA) by a modified procedure based on the methods by Cole and Vellis (1997). Human fetal microglial cells plated at 70% of confluency were infected with JR-FL of HIV (obtained from the AIDS Research and Reference Reagent Program, division of AIDS, NIAID, NIH). Briefly: 50 ng of p24 containing virus stock were added to every 1×10^6 cells. Cells were incubated with virus stock in a small volume of serum free media for 2 h at 37°C, the cells were then washed twice with PBS and a new fresh media containing 2% of FBS was added. Cells were harvested at indicated days and HIV-1 infection was assessed by Western blot for HIV-1 Tat.

Western blot and antibodies

Cells were harvested and whole cell lysates were obtained in TNN buffer (50 mM Tris PH 7.5, 150 mM NaCl, 0.5% NP40) supplemented with a protease inhibitor cocktail (Sigma–Aldrich Corp., St. Louis, MO), with five cycles of freeze and thawing. Protein amount was determined by Bradford assay and 30 µg of total protein were loaded in a SDS–PAGE and blotted on a nitrocellulose membrane. Total cell lysates were examined for the amount of BAG3 protein (monoclonal antibody AC-1: Alexis Biochemicals, San Diego, CA), anti- α -tubulin (Sigma), pAKT and cleaved caspase-3 (polyclonal antibody purchased from Cell Signaling Inc., Beverly, MA). Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary antibody (GE HealthCare, Pittsburgh, PA) and enhanced chemiluminescence reagents (SuperSignal West Dura Extended Duration Substrate, Pierce, Rockford, IL) following standard protocols.

Adenoviral vectors

BAG3siRNA-Ad construct was made using the BD Adeno-X Expression Systems 2 PT3674-1 (Pr36024) and BD knockout RNAi Systems PT3739 (PR42756) (BD Biosciences-Clontech, Palo Alto, CA). We inserted a dsDNA oligonucleotide against a specific bag3 mRNA (5'-AAG GUU CAG ACC AUC UUG GAA-3) in a RNAi-ready pSIREN-DNR vector, designed to express a small hairpin RNA (shRNA) driven by the human Pol III-dependent U6 promoter. After ligation, this vector was used to transfer the shRNA expression cassette to the Adenoviral Acceptor Vector pLP-Adeno-X-PRLS viral DNA (BD Adeno-X Expression Systems 2), containing E1/ E3 Ad5 genome, by Cre-loxP mediated recombination. An AdNull empty adenoviral vector was used as control. Infectious adenoviruses were propagated in the HEK-293 cell line and CsCl gradient purified; functional plaque-forming unit (pfu) titers were determined by limiting dilution plaque titration on HEK-293 cells according to standard techniques. Microglial cells were transduced by thawing the titrated virus stocks at 37°C, mixing the appropriate volume of virus (10 PFU for single cell) in serum free medium (Optimem: GIBCO SRL, Life Technologies, Milano, Italy) and adding the mixture to the target cells. After 1 h incubation, regular medium was added to the cells.

Analysis of hypodiploid (apoptotic) nuclei

Primary microglial cells were transduced with an adenoviral vector capable of expressing siRNA against bag3 gene (BAG3siRNA-Ad). As a control another set was transduced with empty adenoviral vector (AdNull). Forty-eight hours later cells were either mock infected or infected with the JRFL strain of HIV. Four days after HIV infection cells were collected, permeabilized and stained with propidium iodide and quantification of apoptotic cells was done by following standard guidelines recommended by Guava Technology and by using a Guava Citometer.

RESULTS AND DISCUSSION

To investigate the influence of BAG3 protein levels on apoptotic events in microglial cells harboring HIV-1, we infected human primary fetal microglial cells with the virus. BAG3 expression was increased in HIV-1-infected compared with uninfected cells (Fig. 1A).

Those findings are in accord with reported results in reactive astrocytes from HIV-1-associated encephalopathy biopsies (Rosati et al., 2007a).

We reduced BAG3 expression by using an adenovirus carrying a BAG3-specific small interfering (si) RNA (BAG3siRNA-Ad); a void adenoviral vector (AdNull) was used as control. Forty-eight hours following adenoviral infections, microglial cells were infected with HIV (JR-FL) and harvested after 4 days. In BAG3siRNA-Ad-infected cultures, and not in AdNull-infected control cells, we observed a decrease in BAG3 protein levels (Fig. 1A). We then analyzed the percentage of hypodiploid (apoptotic) nuclei in those cells. We found that in the BAG3siRNA-Ad treated microglia infected by HIV-1, apoptosis was enhanced by more than 250% ($P < 0.0001$) in respect to AdNull treated microglia cells (Fig. 1B). Although the knockdown of BAG3 was only partial (Fig. 1A), the effect on apoptosis is clearly evident (Fig. 1B). This observation is in agreement with analogous findings in other cell systems (Rosati et al., 2007b) and strengthens the role of BAG3 in maintaining cell survival. To verify the activation of the apoptotic pathways, we then analyzed cell extracts for the presence of the activated form (p17/p12) of caspase-3, a pivotal actor of cell apoptosis. In uninfected cells or in cells infected with HIV-1 alone, we did not detect significant levels of activated caspase-3. On the other hand, when HIV-1-infected cells were treated with BAG3siRNA-Ad, activated caspase-3 was clearly evident, 3 and 6 days after HIV-1 infection (Fig. 1C); it was not detectable in control (Mock) or AdNull-treated cells. Therefore, it appeared that HIV-1 infection was able to activate caspase-3, but only in cells where BAG3 protein levels were downmodulated. Those results indicated that a pro-apoptotic impulse was produced by HIV-1 infection, but counteracted by BAG3 protein activity, in microglial cells. Among elements with a pro-survival role in HIV-1-infected cells, there is the protein kinase Akt (Yang et al., 2006; Zhao et al., 2007). BAG3 protein reportedly interferes with the Hsp70-mediated delivery of Akt kinase to proteasome sustaining its intracellular levels (Doong et al., 2003). We found that phospho (P)-Akt levels, raised in HIV-1-infected cells, were downmodulated by effect of BAG3siRNA-Ad (Fig. 1D). The influence of BAG3 on the level of Akt kinase activity is likely to be responsible, at least in part, for the apoptosis-modulatory effect of this co-chaperone.

The above reported observations indicate that BAG3 protein induction counteracts apoptotic events induced by HIV-1 in human primary microglial cells. It is well known that HIV-1 infection causes cell stress, and particularly oxidative stress, through more than one mechanism (Perl and Banki, 2000; Mollace et al., 2001; Gil et al., 2003; Pocernich et al., 2005). A few reports showed an increase in Hsp70 and/or Hsp27 levels in lymphocytes (Agnew et al., 2003) or blood (Espigares et al., 2006) from HIV-1-infected patients or in HIV-1 infected cells (Wainberg et al., 1997; Pocernich et al., 2005). *bag3* gene is induced by stressful agents, mainly through the activation of heat shock factor (HSF)-1 (Franceschelli et al., 2008). Therefore, the increase in BAG3 expression is likely part of the cell's response to virus infection and appears to represent a homeostatic mechanism for cell defence. The findings reported here provide a sound basis for investigating the role of BAG3 in maintaining an HIV-1 reservoir in infected cells which do not undergo apoptosis.

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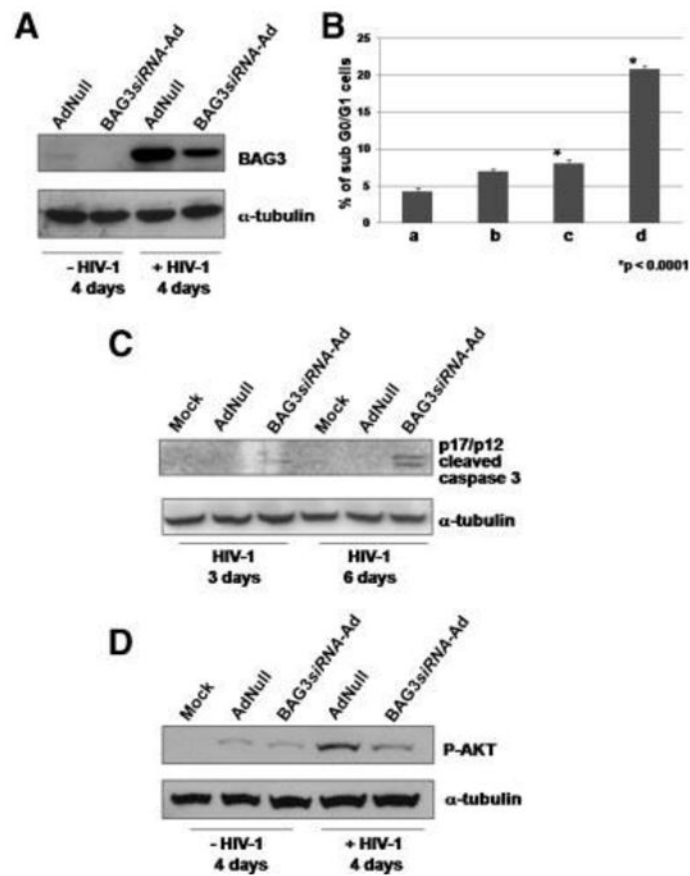


Figure 1.

Primary human fetal microglial cultures were prepared from 8- to 12-week-old human fetal brain tissue. Adenoviral constructs carrying BAG3-specific siRNA (BAG3siRNA-Ad) or AdNull were made using the BDAdeno-X Expression Systems 2 and BDknockout RNAi Systems. Microglial cells were plated at 70% confluency and transductions were performed using 10 PFU for target cell. After 48 h, cells were infected with HIV-1 JRFL obtained from the AIDS Research and Reference Reagent Program, division of AIDS, NIAID, NIH. Whole cell lysates were obtained and analyzed by Western blot as described in Materials and Methods Section with the following antibodies: (A) anti-BAG3 monoclonal antibody (mAb) AC-1 (Alexis Corp., San Diego, CA); anti- α -tubulin mAb (Sigma); (C) anti-cleaved caspase 3 (Asp175) and (D) anti-P-AKT antibodies (Cell Signaling, Danvers, MA). B: Sub-G0/G1 cell population was measured after 4 days of HIV-1 infection in the following samples: (a) mock cells; (b) mock cells + HIV-1; (c) AdNull infected cells + HIV-1; (d) BAG3siRNA-Ad infected cells + HIV-1.