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E1B and E4 oncoproteins of adenovirus antagonize the effect of apoptosis inducing factor

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ABSTRACT

Adenovirus inundates the productively infected cell with linear, double-stranded DNA and an abundance of single-stranded DNA. The cellular response to this stimulus is antagonized by the adenoviral E1B and E4 early genes. A mutant group C adenovirus that fails to express the *E1B-55K* and *E4ORF3* genes is unable to suppress the DNA-damage response. Cells infected with this double-mutant virus display significant morphological heterogeneity at late times of infection and frequently contain fragmented nuclei. Nuclear fragmentation was due to the translocation of apoptosis inducing factor (AIF) from the mitochondria into the nucleus. The release of AIF was dependent on active poly(ADP-ribose) polymerase-1 (PARP-1), which appeared to be activated by viral DNA replication. Nuclear fragmentation did not occur in AIF-deficient cells or in cells treated with a PARP-1 inhibitor. The E1B-55K or E4ORF3 proteins independently prevented nuclear fragmentation subsequent to PARP-1 activation, possibly by altering the intracellular distribution of PAR-modified proteins.

Introduction

Entry of adenovirus into a host cell and the replication of the linear, double-stranded DNA viral genome inundates the cell with what may be sensed as double-stranded DNA breaks, regions of single-stranded DNA, and stalled replication forks (Karen et al., 2009; Nichols et al., 2009; Shepard and Ornelles, 2004). Adenovirus dampens the host DNA-damage response (DDR) by the action of viral proteins encoded in the E1B and E4 early genes (Turnell and Grand, 2012; Weitzman et al., 2004). One mechanism of signaling DNA damage proceeds through the phosphorylation of latent cellular proteins (Karran, 2000). Among the first responders to DNA damage is the sensor protein complex Mre11/Rad50/Nbs1 (MRN) (Williams et al., 2010). Once localized to sites of DNA damage, the MRN complex recruits apical kinases related to the phosphoinositol-3′-kinase, including the DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated protein (ATM), and ATM- and Rad3-related protein (ATR) (Durocher and Jackson, 2001; Nam and Cortez, 2011). ATM is most closely associated with the double-stranded DDR through checkpoint kinase 2 (Chk2), ATR is associated with the single-stranded DDR though checkpoint kinase 1, and DNA-PK is associated with the repair of double-stranded breaks through non-homologous end-joining (NHEJ) (Karran, 2000; Turnell and Grand, 2012). Once active, these apical kinases activate distal kinases, scaffolding proteins such as H2AX, and effector proteins such as p53 that direct DNA repair (Khanna and Jackson, 2001), cell cycle arrest (Zhou and Elledge, 2000), or cell death (Biss and Xiao, 2012). Adenovirus inhibits multiple steps of the DDR pathway. The MRN complex is inhibited by the adenoviral E1B-55K/E4ORF6 protein complex that targets Mre11 for degradation and by the adenovirus serotype 5-specific E4ORF3-mediated sequestration of MRN protein members into nuclear tracks and cytoplasmic aggresomes (Araujo et al., 2005; Stracker et al., 2002,2005). Inactivation of the MRN complex blocks signaling through the kinases ATM (Carson et al., 2003; Mathew and Bridge, 2008) and ATR (Carson et al., 2009). The ATR arm of the DDR is targeted during adenovirus serotype 12 infections by E4ORF6-mediated degradation of TOPBP1 independently of E1B-55K (Blackford et al., 2010) and by the E1B-55 K-associated protein 5 (E1B-AP5) and its ability to promote phosphorylation of ATR substrates. The NHEJ arm of the DDR is targeted by E4ORF6 in complex with E1B-55K through the degradation of DNA ligase IV (Baker et al., 2007) and independently of E1B-55K by disrupting the association of XRCC4 with DNA ligase IV, thus precluding binding to DNA (Jayaram et al., 2008a, 2008b). E4ORF6

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also inhibits protein phosphatase 2A (PP2A) leading to the sustained phosphorylation of DNA-PK and H2AX with hyperactivation of the DDR in response to ionizing radiation (Hart et al., 2005, 2007). The master regulator of cell survival, p53, is inhibited at the transcriptional level by the E1B-55K protein alone (Hartl et al., 2008; Martin and Berk, 1998; Yew and Berk, 1992), through degradation by the E1B-55K/E4ORF6 complex (Querido et al., 1997, 2001), and through the inhibitory methylation of p53-target promoters by the E4ORF3 protein (Soria et al., 2010). Cells infected with an E4-deleted adenovirus show evidence of a strong DDR, including activation of non-homologous end-joining resulting in concatenation of the viral genome (Weiden and Ginsberg, 1994). Genome concatenation is believed to contribute to the inhibition of late protein synthesis in some E4-mutant adenoviral infections (Jayaram and Bridge, 2005). Defects in viral DNA replication in the absence of E4 adenoviral proteins are due to the Nbs1-dependent, Rad50-stabilized binding of Mre11 to viral DNA (Mathew and Bridge, 2007, 2008). Cells infected with a virus bearing deletions in the *E1B-55K* and *E4ORF3* genes also show robust DNA damage signaling and typically die as quickly as cells infected with single-mutant viruses (Shepard and Ornelles, 2004).

The phosphorylation-mediated DNA damage signaling to p53 has been studied extensively in the context of a viral infection (reviewed in Turnell and Grand, 2012); however, signaling also proceeds through the activation of poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 activation occurs in response to DNA-damage and promotes the addition of poly(ADP-ribose) (PAR) to PARP-1 itself, the ribosylation of cellular proteins such as histones, and the accumulation of free PAR chains (Halldorsson et al., 1978; Wang et al., 2009a). PARP-1 automodification and the localization of PAR at sites of DNA damage recruit DNA signaling and repair proteins and leads to PAR-modification of these proteins (Haince et al., 2007; Li and Yu, 2013; Sousa et al., 2012; Wang et al., 2012). Not all forms of DNA damage activate PARP-1 to an equivalent extent. In neuronal cells, cell death associated with PARP-1 activation tends to result from excitotoxic signals or in response to the DNA-alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (Andrabi et al., 2006; Yu et al., 2002, 2003). PARP-1 activation is also required for apoptosis inducing factor (AIF) to translocate to the nucleus (Yu et al., 2002).

Translocation of AIF into the nucleus can lead to profound changes to the integrity of the cell nucleus and the survival of the cell. AIF provokes a caspase-independent form of cell death accompanied by PARP-1-dependent nuclear fragmentation (Susin et al., 1999; Yu et al., 2002). The mechanism by which active PARP-1 signals for death and facilitates AIF release from the mitochondria remains unclear. Studies have demonstrated that free PAR chains are sufficient to induce caspase-independent cell death (Andrabi et al., 2006). Still other studies indicate that AIF itself must be PAR ribosylated in order to be released from the mitochondria and to translocate to the nucleus (Wang et al., 2002). Translocation of AIF to the nucleus was found in irradiated cells radiosensitized by the E4ORF6 protein of adenovirus and AIF was found to be required for this radiosensitization (Hart et al., 2007). Perhaps this interaction of AIF with an adenoviral protein suggests the existence of a relationship between adenovirus and caspase-independent death signaling, much in the same way that adenovirus inhibits caspase-dependent apoptosis (Burgert et al., 2002; Degenhardt et al., 2000).

We report here that adenovirus-infected cells that fail to express the E1B-55K and E4ORF3 proteins show striking heterogeneity in cellular morphology and nuclear integrity. The emergence of this heterogeneity unmasks the importance of these two adenoviral proteins in orchestrating the apparent uniformity seen in a typical adenoviral infection. Viral genome replication was found to activate PARP-1 and, when signaling was unhindered, showed similarities to excitotoxic cell death. Either the E1B-55K or E4ORF3 proteins were sufficient to prevent translocation of AIF from the mitochondria to the nucleus and to prevent nuclear fragmentation. The mechanism by which the E1B-55K and E4ORF3 proteins prevent AIF translocation remains to be determined, although evidence is presented to suggest that these proteins alter the distribution of PAR-modified proteins, thus preventing nuclear fragmentation.

Results

Striking morphological heterogeneity among cells infected with the E1B-55 K/E4ORF3 double-mutant virus

Adherent HeLa cells in a subconfluent monolayer exhibit the characteristic morphology of epithelial cells where each cell adopts an irregular polygonal shape (Fig. 1A, mock). After 72 h, cells infected with the E1B-55K single-mutant virus (Fig. 1A, ΔE1B-55K) were loosely attached to the substrate and appeared uniformly round. The loosely-attached rounded cells showed a median diameter of 11 μm (Fig. 1B, ΔE1B-55K). Cells infected with either the wild-type or the E4ORF3 single-mutant viruses appeared similar to E1B-55K single-mutant virus-infected cells (data not shown) and the detached rounded cells were of similar size to the E1B-55K single-mutant virus-infected cells (Fig. 1B). By contrast, cells infected by the E1B-55K/E4ORF3 double-mutant virus included detached and rounded cells as well as adherent, polygonal cells (Fig. 1A, ΔE1B-55K/ΔE4ORF3). These attached cells were infected because over 99% of similarly infected cells stained for an early adenovirus protein (data not shown). The diameter of the detached and rounded double-mutant virus-infected cells varied considerably with a median diameter of 16 μm and an interquartile range nearly twice that of the wild-type and single-mutant virus-infected cells (Fig. 1B). These cells also appeared significantly larger than wild-type, E4ORF3- and E1B-55K-mutant virus-infected cells ($p=0.01$, 0.001 and 0.0002, respectively). The heterogeneous morphology of double-mutant virus-infected cells was confirmed by flow cytometry. Forward scatter reflects the volume of the cell while side scatter reflects properties such as the presence of cytoplasmic granules, nuclear shape, and membrane roughness. Although there was no appreciable difference in cell volume, side scatter increased for all infected cells and the heterogeneity in this measurement was especially pronounced in cells infected with the double-mutant virus (Fig. 1C).

The striking variability in cell shape determined by phase contrast microscopy and flow cytometry was evident in the morphology of the plasma membrane visualized by scanning electron microscopy. Mock-infected cells were flat, polygonal and displayed numerous microvilli (Fig. 1D, mock). Wild-type virus-infected cells, which were uniformly round in appearance, displayed numerous small blebs and ruffles. Very few microvilli were evident in these cells (Fig. 1D, wild-type). By contrast, the representative image of E1B-55K/E4ORF3 double-mutant virus-infected cells illustrates the heterogeneity in cell shape and the variability in membrane morphology (Fig. 1D, ΔE1B-55K/ΔE4ORF3). Some cells appeared smooth with few membrane blebs and appeared similar to wild-type virus-infected cells. Additional cells contained small blebs that were larger in extent and protruded further from the membrane than in wild-type virus-infected cells. Some cells appeared to retain long thin cytoplasmic processes attached to the substrate. Still other cells appeared to have fragmented and possibly damaged membranes showing a granular texture (Fig. 1D, ΔE1B-55K/ΔE4ORF3). It should be noted that although all infected cells displayed a substantial cytopathic effect, over 90% of the cells infected with the single-mutant viruses and over 75% of the