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Adenovirus E1A and E1B-19K Proteins Protect Human Hepatoma Cells from Transforming Growth Factor β 1-induced Apoptosis

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Abstract

Primary and some transformed hepatocytes undergo apoptosis in response to transforming growth factor β 1 (TGF β). We report that infection with species C human adenovirus conferred resistance to TGF β -induced apoptosis in human hepatocellular carcinoma cells (Huh-7). Protection against TGF β -mediated cell death in adenovirus-infected cells correlated with the maintenance of normal nuclear morphology, lack of pro-caspases 8 and 3 processing, maintenance of the mitochondrial membrane potential, and lack of cellular DNA degradation. The TGF β pro-apoptotic signaling pathway was blocked upstream of mitochondria in adenovirus-infected cells. Both the N-terminal sequences of the E1A proteins and the E1B-19K protein were necessary to protect infected cells against TGF β -induced apoptosis.

Keywords

Adenovirus; transforming growth factor beta; apoptosis; E1A; E1B-19K

1. Introduction

Infection of epithelial cells by species C human adenoviruses (Ad), serotypes 5 (Ad5) and 2 (Ad2), ultimately leads to cell lysis and release of progeny virus. The mechanism of cell lysis is poorly understood. In many cell types, infection with certain Ad early gene mutants leads to apoptosis. This apoptosis is caused by expression of the adenovirus E1A proteins, and the mechanism includes p53-dependent and independent pathways (reviewed in (White, 2006; Berk, 2005; Miller et al., 2009)). The p53-dependent apoptosis is countered by the Ad E1B-55K and E4orf6 proteins, which act together as an E3 ubiquitin ligase to cause degradation of p53 (reviewed in (Berk, 2005; Blackford & Grand, 2009)).

E1A-induced intrinsic apoptosis is also counteracted by the Ad E1B-19K protein. E1B-19K is the first viral BCL-2 homologue discovered (Boyd et al., 1994; Chinnadurai, 1983; Takemori et al., 1968); reviewed by (Wold & Chinnadurai, 2000; Cuconati & White, 2002; Berk, 2005). The E1A proteins cause degradation of the anti-apoptotic protein MCL-1, causing release of the pro-apoptotic protein BAK from BAK-MCL-1 complexes (Cuconati et al.,

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2003). The anti-apoptotic function of E1B-19K is in part dependent on the interaction of E1B-19K with BAK, preventing BAK and BAX from co-oligomerizing and forming pores in the outer membrane of mitochondria and thereby allowing the release of pro-apoptotic proteins such as cytochrome c and Smac/DIABLO (White, 2001; Cuconati & White, 2002; Berk, 2005; Cuconati et al., 2002). By preventing the BAK-BAX assembly, E1B-19K acts upstream of mitochondria, and is therefore capable of inhibiting apoptosis induced by a variety of death stimuli that trigger the intrinsic apoptotic pathway. E1B-19K also binds to the pro-apoptotic BH3-only protein BIK/NBK (Boyd et al., 1995; Han et al., 1996). BIK/NBK functions in the apoptotic pathway activated through inhibition of protein synthesis by disrupting BAK-MCL-1 and BAK-BCL-X_L complexes; E1B-19K blocks this apoptotic pathway (Shimazu et al., 2007). Among many BH3-only proteins examined, BIK/NBK was predominantly activated in Ad-infected cells, and the authors concluded that activation of BIK/NBK initiates the apoptotic pathway that occurs when cells are infected by Ad mutants lacking E1B-19K (Subramanian et al., 2007).

Ad also encodes a number of proteins that counteract apoptosis of the infected cell following an extrinsic stimulus. Specifically, Ad-infected cells are protected against apoptosis induced by the classical death signals such as Fas ligand (FasL), Tumor Necrosis Factor α (TNF α), and TNF-Related Apoptosis-Inducing Ligand (TRAIL) (reviewed in (Berk, 2007; Horwitz, 2004; Lichtenstein et al., 2004; McNees & Gooding, 2002; Wold & Horwitz, 2007; Windheim et al., 2004). The protection against extracellular death ligands is mediated by several Ad early proteins including E1B-19K, the E3-RID complex, and the E3-14.7K protein. The E3 RID complex contributes to the protection against FasL, TRAIL, and TNF α at least in part through internalization and degradation of the corresponding receptors, specifically Fas, TNF receptor 1, TRAIL receptor 1, and TRAIL receptor 2. Another E3 protein, E3-14.7K, blocks TNF α , FasL, and TRAIL-induced apoptosis downstream of the corresponding receptors. The 14.7K protein reportedly acts by inhibiting internalization of cell surface TNF receptor 1 (Schneider-Brachert et al., 2006).

Transforming growth factor beta 1 (TGF β) is a multifunctional cytokine that regulates many biological responses including apoptosis, cell proliferation, cell differentiation, cell migration, extracellular matrix synthesis, epithelial-to-mesenchymal transition, angiogenesis, and the immune response (reviewed in (Heldin et al., 2009; Huang & Huang, 2005). There are a number of TGF β receptors, including TGF β receptor 1 (T β RI), TGF β receptor II (T β RII), TGF β receptor III (T β RIII), and TGF β receptor V (T β RV). The canonical TGF β signaling pathway initiates with ligand binding to a T β RI/T β RII complex. T β RI and T β RII are ser/thr kinases. Binding of ligand causes the constitutively active T β RII to phosphorylate T β RI; this is followed by phosphorylation of Smad 2 and 3, which then associate with Smad4 and translocate to the nucleus to induce transcription in cooperation with other transcription factors. Depending on the cell type and context, the TGF β signaling pathway can cross-talk with other pathways, including the phosphatidylinositol 3 kinase (PI3K)/AKT, ras/MAPK, JNK, p38, Rho/ROCK, mTOR, Wnt/ β -catenin, and Jagged/Notch pathways (Guo & Wang, 2009; Xu et al., 2009). TGF β can also signal through the ERK, JNK, p38, Rho-like GTPase, and PIK3/AKT pathways in a manner independent of the Smads (Zhang, 2009; Xu et al., 2009).

TGF β acts as a pro-apoptotic agent for a limited number of human cell types (Ramesh et al., 2009; Ramjaun et al., 2007; Schuster & Krieglstein, 2002). TGF β -mediated apoptosis seems to be dependent on caspase activation and new protein synthesis; the exact mechanism of death induction by TGF β is not known and appears to vary with the cell type (Schuster & Krieglstein, 2002; Heldin et al., 2009). The BH3-only proteins BIM and BMF in particular have been implicated in TGF β -induced apoptosis in a number of cell types including hepatocytes (Ramesh et al., 2009; Ramjaun et al., 2007; Romano et al., 2008).

The PI3K pathway, which is activated by a variety of growth factors including epidermal growth factor (EGF), provides a strong pro-survival signal and is deregulated in many types of human malignancies (Zhang, 2009). The PI3K pathway counteracts TGF β -mediated apoptosis (Schuster & Kriegstein, 2002; Song et al., 2006). EGF, by activating PI3K, provides a survival effect against TGF β -induced cell death in fetal hepatocytes (Fabregat et al., 2000). Interleukin-6 as well as insulin act primarily through the PI3K pathway to block TGF β -induced apoptosis in human hepatoma cells (Chen et al., 1998; Chen et al., 1999). The inhibition of the death signal occurs downstream of Smad transcription but upstream of caspase 3 activation.

Pro-caspase 8 processing occurs in many cell types sensitive to TGF β -induced apoptosis. Furthermore, inhibition of caspase-8 by a specific inhibitor blocks the TGF β -mediated death signal (Inman & Allday, 2000; Shima et al., 1999). Caspase 8 activation occurs upon ligation of death receptors, such as Fas or TNF receptor I; efficient caspase 8 activation is dependent on the formation of the death inducing signaling complex (DISC) at the cell membrane (Wilson et al., 2009). Interestingly, TGF β -mediated apoptosis and pro-caspase 8 processing that occurs upstream of caspase-3 activation are not dependent on Fas/FasL interactions, TNF α , or TRAIL (Inman & Allday, 2000; Shima et al., 1999).

Human and rat hepatocytes are very sensitive to TGF β -induced apoptosis (Fan et al., 2002; Fan et al., 2004a; Wang et al., 2006; Coyle et al., 2003; Ramjaun et al., 2007; Shima et al., 1999). TGF β is important in normal liver homeostasis *in vivo*, as overexpression of TGF β leads to hepatocyte cell death and liver fibrosis in transgenic mice (Ueberham et al., 2003). Not surprisingly, many hepatocellular carcinomas have become resistant to TGF β -induced apoptosis (reviewed in (Fabregat, 2009)). On the contrary, Huh-7, a human hepatocellular carcinoma cell line, has retained the ability to undergo apoptosis in response to TGF β . Huh-7 cells treated with TGF β display diagnostic features of apoptosis, including processing of pro-caspases 8, 9, and 3 and fragmentation of cellular DNA (Huang & Chou, 1998; Shima et al., 1999; Fan et al., 2002; Fan et al., 2004a). Interestingly, Huh-7 cells lack expression of BCL-2 (Huang & Chou, 1998). Treatment of Huh-7 cells with TGF β results in decreased levels of BCL-X_L, and reintroduction of BCL-2 by stable overexpression renders this cell line resistant to TGF β -induced cell death (Huang & Chou, 1998; Shima et al., 1999). In addition, treatment with EGF completely inhibits TGF β -induced apoptosis in Huh-7 cells (Shima et al., 1999).

Infection with Ad5 or Ad2 inhibits the TGF β signaling pathway in infected cells (Tarakanova & Wold, 2003). This inhibition is a function of the Ad E1A proteins, and includes downregulation of T β RII (Tarakanova & Wold, 2003) and interference with the transcriptional activity of Smad-containing complexes (Datto et al., 1997; Nishihara et al., 1999; Tarakanova & Wold, 2003). In this report we show that Ad5-infected Huh-7 cells are resistant to TGF β -induced apoptosis. Induction of cell death by TGF β is blocked upstream of mitochondria in infected cells, and expression of both E1A and E1B-19K is required to confer full protection against TGF β -triggered cell death.

2. Materials and Methods

2.1. Cell lines

The human Huh-7 hepatocellular carcinoma cell line was obtained from ATCC. Cells were grown in DMEM/F12 medium supplemented with 10% FCS (HyClone, Logan, UT).

2.2. Adenovirus mutants and infections

rec700 is a recombinant virus derived from Ad2 and Ad5 with an E1A region from Ad5 (Wold et al., 1986). Most Ads used in this study were described previously (Tarakanova & Wold, 2003). *dl250* is an Ad2 mutant that lacks the gene for the E1B-19K protein (Subramanian et

al., 1984a). *dl110* and *dl111* lack the gene for E1B-55K and E1B-19K, respectively, and are derived from *dl309* (Babiss et al., 1984). *dl309* is an Ad5 mutant that lacks the genes for the E3 RID α , RID β , and 14.7K proteins. For infections, virus was diluted in 0.5 ml of serum free DMEM/F12 medium, added to cells, and incubated for 1 h at 37 C, 5% CO₂.

2.3. Treatment of infected cells

Mock- and Ad-infected cells were maintained in the presence of freshly supplied 20 μ M AraC throughout the infection. Treatment agents were diluted in DMEM/F12 medium containing 3% FCS. LY294002 was used at a final concentration of 15 μ M, and TGF β was used at 10 ng/ml, as indicated. Treatment started at 1 h postinfection (p.i.) and continued for indicated time period.

2.4. Antibodies and Reagents

Laboratory chemicals used in this study were purchased from Sigma (St. Louis, MO). Recombinant human TGF β 1 was purchased from R&D Systems (Minneapolis, MN); it was reconstituted and stored per manufacturer's instructions. LY294002 was purchased from Calbiochem (La Jolla, CA) as a 10 mM solution in DMSO. DAPI was purchased from Sigma and used at a final concentration of 1 μ g/ml. Rabbit anti-caspase 3 antibody was purchased from Stressgen (Cat. # AAP-113; Victoria, BC Canada) and used at a 1:3000 dilution for western analysis. Mouse anti-caspase 8 was purchased from Alexis Biochemicals (clone 12F5; San Diego, CA) and used at a 1:1000 dilution. For western analysis, rabbit anti-E1B-19K antibody was used at 1:500, mouse anti-E1A antibody was used at 1:140 (Tollefson et al., 1998).

2.5. Western analysis

Western analysis was performed essentially as described previously (Tarakanova & Wold, 2003). Equal protein concentrations of 50 (caspase-3, caspase-8) or 20 (E1B-19K and E1A) μ g were loaded per lane.

2.6. Nuclear staining

Huh-7 cells were seeded onto sterile glass coverslips placed in 35 mm tissue culture dishes. After overnight attachment, cells were infected and treated as described. Following treatment, cells were washed three times with phosphate buffered saline (PBS) and fixed for 10 min at room temperature in a 3.7% paraformaldehyde solution in PBS. The cells were subsequently permeabilized and stained for 6 min in DAPI containing methanol solution chilled to -20 C. Excess DAPI was removed with a cold methanol wash and cells were rehydrated with three PBS washes. Coverslips were mounted onto glass slides and examined with a fluorescent microscope.

2.7. DNA fragmentation analysis

Huh-7 cells were seeded in 100 mm tissue culture dishes. Following overnight attachment cells were mock- or Ad-infected and treated as described. At the end of treatment, floating cells from the supernatant were combined with adherent cells dislodged with a cell scraper and collected by centrifugation. The cell pellet was subsequently resuspended in 0.5 ml PBS; 55 μ l of 10X lysis buffer (5% Triton X-100, 50 mM Tris-Cl, pH 8.0, 0.2 M EDTA) were added per sample followed by an incubation on ice for 20 min. Cellular debris was pelleted down by centrifugation at 12,000 g for 25 min at 4 C. The supernatant was extracted with an equivalent volume of 1:1 mixture of phenol:chloroform. The nucleic acid from the aqueous phase was precipitated at 4 C overnight in two volumes of cold 95% ethanol and one-tenth volume of 3 M sodium acetate. Following nucleic acid precipitation, samples were centrifuged at 12,000g for 30 min at 4 C. Nucleic acid pellets were resuspended in 30 μ l of deionized water and

incubated with 20 units of RNase ONE (Promega, Madison, WI) for 30 min at 37 C. The entire reaction was subjected to gel electrophoresis (1.8% agarose) and the DNA was visualized with ethidium bromide staining.

2.8. Mitochondria membrane potential analysis

Huh-7 cells were infected and treated as described. During the last 30 min of treatment Rh123 (Molecular Probes, Eugene, OR) was added to the treatment medium at a final concentration of 0.1 μ g/ml. Cells were trypsinized and fluorescence levels were immediately measured by flow cytometry using the FACS Calibur analyzer (Becton Dickinson, San Jose, CA). Cell Quest Pro software was used to analyze the data.

3. Results

3.1. Inhibition of PI3K sensitizes Huh-7 cells to TGF β -induced apoptosis

Unlike most human transformed cells, Huh-7 cells are sensitive to the pro-apoptotic activity of TGF β (Huang & Chou, 1998; Shima et al., 1999). In our hands, treatment of Huh-7 cells with 10 ng/ml of human recombinant TGF β produced initial morphological signs of apoptosis by the third day of treatment; by day four most of the cells have undergone apoptosis (data not shown). PI3K mediates resistance to TGF β -induced apoptosis in several cell lines (Fabregat et al., 2000; Chen et al., 1998; Zhang, 2009). When Huh-7 cells were incubated with TGF β in the presence of LY294002, a specific PI3K inhibitor, dead cells appeared between 20 and 24 h post treatment (Fig. 1A, middle). No dead cells were seen in the presence of the PI3K inhibitor alone (Fig. 1A, left). Therefore, inhibition of the PI3K pathway sensitized Huh-7 cells to cell death induced by TGF β . Data presented below indicate that the cell death is due to apoptosis.

3.2. The pro-apoptotic function of TGF β is attenuated in cells infected with wild type adenovirus

In order to examine the sensitivity of Ad-infected cells to TGF β -induced apoptosis, Huh-7 cells infected with wild-type Ad *rec700* (Wold et al., 1986) at 50 plaque forming units (PFU)/cell were incubated with TGF β in the presence of the PI3K inhibitor. The PI3K inhibitor was used in this experiment because it accelerated TGF β -induced cell death and therefore made it possible to differentiate between TGF β -induced cell death and cell death that might result from prolonged expression of early Ad proteins. Infection was maintained in the early phase by the inclusion of AraC, a DNA synthesis inhibitor. No cell death was seen in treated infected cells at 24 h p.i., whereas dying cells were present in treated mock-infected cells at the same time (Fig. 1A, compare the right and middle micrographs).

In a separate experiment, DAPI staining was used to assess the nuclear morphology of mock-infected or *rec700*-infected cells treated with a combination of the PI3K inhibitor and TGF β . The nuclei of mock-infected cells maintained in the presence of the PI3K inhibitor alone (Fig. 1B, panel a) had homogenous nuclear staining typical of interphase chromatin. Mock-infected cells that were treated with a combination of the inhibitor and the cytokine displayed typical apoptotic morphology including condensed nuclei and scattered nuclei debris that were located slightly above the plane of the adherent cells (Fig. 1B, panel b). However, *rec700*-infected cells treated with a combination of the PI3K inhibitor and TGF β were indistinguishable from mock-infected cells treated with PI3K inhibitor only (Fig. 1B, compare panels a and c).

Pro-caspase 3 processing and caspase activation have been described in TGF β -treated Huh-7 cells undergoing apoptosis (Shima et al., 1999; Huang & Chou, 1998). Pro-caspase 3 processing was undetectable in mock-infected untreated cells or in cells treated with either TGF β or PI3K inhibitor alone for 23 h (Fig. 1C, lanes a, b, c). However, pro-caspase 3 processing was evident in mock-infected cells that were treated with a combination of TGF β

and PI3K inhibitor for the same period (Fig. 1C, lane d). Consistent with the absence of apoptotic cells (Figs. 2A, B), pro-caspase 3 processing was attenuated in *rec700*-infected cells treated with TGF β in the presence of the sensitizing agent (Fig. 1C, compare lanes d and g). Therefore, Ad infection conferred resistance to TGF β -mediated apoptosis in Huh-7 cells.

3.3. The lack of TGF β -induced pro-caspase 3 processing in adenovirus-infected cells maps to the E1A and E1B-19K proteins

Resistance to TGF β -induced apoptosis was maintained in infected cells when the infection was limited to the early phase by blocking DNA synthesis (AraC, Fig. 1); therefore, the observed phenotype is a function of an early Ad protein(s). The E1A proteins inhibit TGF β signaling both independently and in the context of infected cells (Datto et al., 1997; Nishihara et al., 1999; Tarakanova & Wold, 2003). As discussed earlier, several proteins encoded by the E3 region of Ad (E3-14.7K and the RID complex) protect infected cells against exogenous death-inducing ligands such as TNF α , FasL, and TRAIL. Also, the E1B region of Ad encodes two anti-apoptotic proteins; one of them, E1B-19K, is a BCL-2 homologue with a protective function against multiple pro-apoptotic stimuli. Potentially, Ad proteins encoded by the E1A, E1B, or E3 regions could contribute to the observed protection against TGF β -induced apoptosis in infected Huh-7 cells.

In order to map the Ad gene(s) responsible for the resistant phenotype, Huh-7 cells were infected with Ad mutants lacking the entire early region or select early genes, and pro-caspase 3 processing was examined in cells treated with TGF β and PI3K inhibitor for the duration of infection. No pro-caspase 3 processing was observed with mutants lacking either the entire E3 region (*dl7001*) (Fig. 2A, lane g), most of the E3 region with the exception of the gene for the E3-12.5K protein (*dl327*) (lane i), or all of the E4 region (*dl808*) (lane k). Therefore, the E3 and E4 proteins are not required to prevent TGF β -induced processing of pro-caspase 3 in Ad-infected cells.

Interestingly, infection of Huh-7 cells with an Ad mutant lacking the N-terminal sequences of E1A (E1A.2-36) failed to protect against pro-caspase 3 processing in infected cells (Fig. 2B, lane g). Deletion of the N-terminal sequences of E1A has been shown to eliminate inhibition of TGF β signaling in infected cells (Tarakanova & Wold, 2003). Whereas minimal pro-caspase 3 processing was observed in cells infected with wild type Ad (*rec700*, Fig. 2B, lane e) in this experiment, pro-caspase 3 processing was similar to that observed in mock infection in cells infected with the E1A.2-36 virus mutant (Fig. 2B, compare lanes c and g). Pro-caspase 3 processing correlated with the apoptotic morphology observed in cells infected with the E1A.2-36 mutant upon TGF β treatment (data not shown). Infection with Ad mutants lacking amino acids 81-120 of the E1A protein did not result in a loss of protection against TGF β -induced pro-caspase 3 processing (data not shown). E1A levels were similar for all mutants and conditions examined (Fig. 2C). In addition, levels of E1B-19K were similar in cells infected with *rec700* or E1A.2-36 (Fig. 2D), indicating that the loss of protection against TGF β -induced apoptosis is not due to the inability of the N-terminal mutant to transactivate expression of other Ad genes with anti-apoptotic functions.

In addition, enhanced pro-caspase 3 processing was observed in TGF β -treated cells that were infected with two Ad mutants lacking E1B-19K (*dl111* and *dl250*; Fig. 2B lanes k and m). When Huh-7 cells were infected with a mutant lacking the second protein encoded by the E1B region, E1B-55K (*dl110*), minimal pro-caspase 3 processing occurred (similar to that observed in cells infected with *rec700*; Fig. 2B, compare lanes e and i), indicating that the loss of resistance to TGF β -induced apoptosis is specific for the lack of E1B-19K expression.

Therefore, E1A proteins, specifically the N-terminal sequences of E1A as well as the E1B-19K protein, contribute to the attenuation of TGF β -induced pro-caspase 3 processing in Ad-infected cells.

3.4. Both the N-terminal sequences of E1A and the E1B-19K protein are required to protect adenovirus-infected cells against TGF β -induced nuclear disruption

As shown in Fig. 1B, treatment of mock-infected cells but not Ad-infected cells with TGF β in the presence of the PI3K inhibitor resulted in nuclear condensation and disintegration, features typical of apoptosis. The effect of TGF β treatment on nuclear morphology was examined in cells infected with the mutants lacking either the E1B-19K protein or the N-terminal portion of the E1A proteins.

As shown in Fig. 3, treatment of Huh-7 cells with a combination of TGF β and PI3K inhibitor resulted in nuclear condensation and apoptotic bodies in mock-infected cells but not in cells infected with *rec700* (compare panels b and c). Cells infected with a mutant harboring an E1A deletion outside the N-terminus (E1A.81-120) were protected against TGF β -induced apoptosis (Fig. 3, panel d). However, TGF β treatment of cells infected with Ad mutants lacking either the N-terminal portion of E1A (E1A.2-36; Fig. 3, panel f) or the E1B-19K protein (*dl250*; Fig. 3, panel h) resulted in apoptotic nuclei similar to those observed in mock-infected TGF β -treated cells. Apoptotic nuclei were not present when E1A.2-36- or *dl250*-infected cells were treated with the PI3K inhibitor alone (Fig. 3, panels e and g), indicating that the observed effect was specific to TGF β .

Therefore, the lack of protection against TGF β -mediated pro-caspase 3 processing in cells infected with mutants lacking either the N-terminal E1A sequences or E1B-19K correlated with the inability of these mutants to protect infected cells against TGF β -dependent nuclear disruption typical of apoptosis.

3.5. Genomic DNA cleavage is concurrent with pro-caspase 3 processing in cells sensitive to TGF β -mediated apoptosis

TGF β induces cellular DNA fragmentation in Huh-7 cells (Shima et al., 1999). Therefore, cellular DNA fragmentation was examined in PI3K inhibitor-treated Huh-7 cells infected with either wild-type Ad (*rec700*) or two early gene mutants (E1A.2-36 and *dl250*) susceptible to TGF β -induced pro-caspase 3 processing (see Fig. 2B). DNA ladder formation was apparent in mock-infected cells treated with a combination of TGF β and PI3K inhibitor (Fig. 4, lane b), but not with the PI3K inhibitor alone (Fig. 4, lane a). DNA ladder formation was minimal in cells treated with the combination of the two agents and infected with *rec700* (Fig. 4, lane d). DNA laddering was observed in cells infected with either E1A.2-36 or *dl250* and subsequently treated with a combination of the cytokine and the inhibitor (Fig. 4, lanes f and h). Therefore, processing and presumably activation of caspase 3 correlates with the appearance of the apoptotic DNA ladder; expression of both E1A with intact N-terminal sequences and E1B-19K are necessary for full protection against TGF β -induced cellular DNA fragmentation.

3.6. Both inhibition of TGF β signaling by E1A and expression of E1B-19K are necessary to maintain mitochondria membrane potential upon TGF β treatment of infected cells

The N-terminal sequences of E1A are important for its ability to interfere with TGF β signaling in infected cells. Inhibition of TGF β signaling by E1A is likely to involve at least two mechanisms: clearance of T β R β RII (Tarakanova & Wold, 2003) and interference with the transcriptional activity of Smad-containing complexes (Datto et al., 1997; Nishihara et al., 1999). The E1B-19K protein does not play a role in inhibiting TGF β signaling in Ad-infected cells (Tarakanova & Wold, 2003). E1B-19K functions upstream of mitochondria to inhibit apoptosis induced by agents such as FasL and TNF α . TGF β -induced apoptosis has been shown

to proceed through the mitochondrial pathway with BCL-2 family members exerting a protective function (Huang & Chou, 1998). In order to better characterize the apoptotic pathways in cells infected with wild type Ad or deficient mutants, the status of mitochondria was examined.

Huh-7 cells were mock infected or infected with Ad mutants and treated with a combination of PI3K inhibitor and TGF β or PI3K kinase inhibitor alone for the duration of infection. During the last 30 min of treatment, cells were incubated with Rh123, a fluorescent dye that is selectively retained in mitochondria with intact membrane potential, and cells were analyzed by flow cytometry. At the conclusion of the experiment, approximately one-third of mock-infected, TGF β -treated cells showed a significant decrease in mitochondrial retention of Rh123, whereas control cells treated with the PI3K inhibitor alone were minimally affected (Fig. 5, compare A and B). The mitochondria membrane potential was not affected by TGF β treatment of cells infected with either wild-type Ad (*rec700*) (Fig. 5C, D) or an E1A mutant with a deletion outside the N-terminal region (E1A.81-120) (Fig. 5G, H).

Infection with the Ad mutant lacking the N-terminal E1A sequences (E1A.2-36) resulted in a loss of mitochondria membrane potential in infected TGF β -treated cells (Fig. 5F). However, there were fewer E1A.2-36-infected cells with changed mitochondria permeability than mock-infected cells (20% vs. 32%), indicating that other Ad proteins, most likely E1B-19K, offered some protection. An increase in cell population with disrupted mitochondrial potential was also evident in *dl250*-infected cells treated with TGF β (Fig. 5J). Similar to the E1A.2-36 infection, the population of TGF β -treated cells exhibiting a loss of mitochondria membrane potential was smaller upon infection with *dl250* as compared to the mock-infected cells (26% vs. 32%). Upon treatment with the PI3K inhibitor alone, there were more *dl250*-infected cells that had decreased mitochondrial function as compared to *rec700*-infected cells (13% vs. 8%; compare Fig. 5I and 5C). This is most likely a reflection of a cytotoxic phenotype of Ad mutants lacking E1B-19K protein (Subramanian et al., 1984b).

These results indicate that TGF β -induced apoptosis is blocked upstream of mitochondria in cells infected with wild type Ad. In addition, the data suggest that the inhibition of the TGF β signaling pathway provided by E1A proteins and maintenance of mitochondrial integrity by E1B-19K are both necessary for the resistance to TGF β pro-apoptotic functions.

3.7. Both the N-terminal sequences of E1A and the E1B-19K protein are required to block TGF β -induced pro-caspase 8 processing in adenovirus-infected cells

Upon interaction with the corresponding receptors, extracellular death ligands such as FasL and TNF α induce DISC formation at the plasma membrane that results in pro-caspase 8 processing and activation. Pro-caspase 8 cleavage occurs in cells susceptible to TGF β -induced apoptosis, however, the classical pro-caspase 8 activation pathway does not seem to be involved (Inman & Allday, 2000; Shima et al., 1999). TGF β -mediated pro-caspase 8 processing was examined in Huh-7 cells infected with wild-type Ad or deficient mutants.

Inhibition of PI3K sensitizes Huh-7 cells to TGF β -induced apoptosis (Fig. 1). Pro-caspase 8 processing was detected as early as 18 h post treatment in mock-infected cells that were treated with a combination of the PI3K inhibitor and TGF β (Fig. 6A, lane f). No pro-caspase 8 cleavage was observed in mock-infected cells treated with the PI3K inhibitor alone for 24 h (Fig. 6A, lane b), or in mock-infected cells treated with TGF β without the inhibitor for either 24 or 48 h (Fig. 6A, lanes d, e). Whereas the amount of cleaved pro-caspase 8 increased in a time-dependent manner in mock-infected cells treated with a combination of agents (Fig. 6A, lanes f-i), no pro-caspase 8 processing was detected in *rec700*-infected Huh-7 cells (Fig. 6A, lanes j-m).

Pro-caspase 8 processing and activation occurs upstream of pro-caspase 3 in the TGF β -induced apoptotic pathway. E1A proteins could interfere with TGF β apoptosis by acting upon T β RII while the E1B-19K protein could prevent TGF β proapoptotic signaling immediately upstream of mitochondria. Therefore, we hypothesized that a potential difference in pro-caspase 8 processing could exist in cells infected with two Ad mutants expressing either the N-terminal-deleted E1A or lacking expression of E1B-19K. Huh-7 cells were infected with wild-type Ad or deficient mutants and pro-caspase 8 cleavage was examined at 19 h p.i.. Similar to mock-infected cells (Fig. 6B, lane c), TGF β treatment induced pro-caspase 8 processing in cells infected with either the mutant Ad lacking the N-terminal sequences of E1A (E1A.2-36; Fig. 6B, lane g) or the E1B-19K mutant (*dl250*; Fig. 6B, lane i). Cells infected with wild-type Ad (*rec700*) showed minimal pro-caspase 8 cleavage at the time examined (Fig. 6B, lane e). The corresponding levels of E1A proteins in infected cells are shown in Fig. 6C.

These data indicate that TGF β -induced pro-caspase 8 processing in mock-infected cells is accelerated by inhibition of the PI3K pathway; TGF β -triggered pro-caspase 8 cleavage is dramatically reduced upon infection with wild type Ad, and both the E1A and E1B-19K proteins are required for this reduction.

4. Discussion

Our results show that the pro-apoptotic function of TGF β is strongly attenuated in Huh-7 cells infected with wild type Ad. Ad proteins encoded by the E3 region play a crucial role in protection against death-inducing cytokines, such as FasL, TNF α , and TRAIL. Our study shows that protection of Ad-infected cells against TGF β does not require the E3 proteins.

The N-terminal sequences of Ad E1A proteins are responsible for the inhibition of TGF β signaling in infected and E1A-transformed cells (Tarakanova & Wold, 2003). The inhibition of signaling in infected cells could be mediated by several mechanisms, including downregulation of T β RII, displacement of p300 from Smad-containing nuclear complexes (Datto et al., 1997; Nishihara et al., 1999), or possibly disruption of signaling from other TGF β receptors. The E1A N-terminus mediates interaction with at least 15 different cellular proteins (Berk, 2005; Turnell & Mymryk, 2006; Pelka et al., 2008; Bruton et al., 2007) including factors that directly regulate gene expression such as p300/CBP, p400, TRRAP, pCAF, AP-2, YY1, CtIP, thyroid hormone receptor, myogenin, and TBP. In addition, the E1A N-terminus binds the Ran GTPase, the receptor for protein kinase C (RACK1), the RII α subunit of protein kinase A, and the Nek9 kinase. Interestingly, the binding of STAT1 by the N-terminal sequences of E1A is important for the inhibition of interferon signaling (Look et al., 1998). Also, the N-terminal portion of E1A interacts with the S4 and S8 components of the 19S regulatory components of the proteasome; this interaction may regulate proteasomal activity (Turnell et al., 2000). The results of the present study indicate that the presence of an intact N-terminal portion of E1A is also important for the resistance of infected cells to TGF β -induced apoptosis. E1A proteins have been known for their ability to induce p53-dependent or independent apoptosis, to sensitize cells to various death-inducing stimuli such as TNF α and irradiation, to cause stabilization of apoptosis-promoting p53, and to cause degradation of the anti-apoptotic BCL-2 family member MCL-1 (Cuconati et al., 2003; Berk, 2005; White, 2006; Wold & Chinnadurai, 2000). Infection with adenovirus mutant incapable of expressing 13S isoform of E1A relieved TGF β -mediated inhibition of proliferation and apoptosis of Madin-Darby canine kidney (MDCK) cell line (Quinlan, 1993); interestingly, E1B viral genes were dispensable for this phenotype. To our knowledge, our report is the first to show that E1A protects against cell death in the context of an Ad-infected human cell.

We also found that the E1B-19K protein contributes to the resistance of Ad-infected cells to TGF β -induced apoptosis. E1B-19K is a viral BCL-2 functional homologue that protects

infected cells against both intrinsic and extrinsic apoptotic stimuli that involve the mitochondrial pathway, such as TNF α , FasL, and DNA damage (Cuconati & White, 2002; White, 2006; Wold & Chinnadurai, 2000; Berk, 2005). This report is the first to show the involvement of E1B-19K in the inhibition of TGF β -induced apoptosis.

In the course of Ad infection, E1B-19K counteracts E1A-triggered BAX-dependent apoptosis (Lomonosova et al., 2002). Protection against apoptosis is accomplished at least in part through the interaction of E1B-19K with BAX and BAK, two BH1-BH3 proteins that comprise the core apoptotic machinery (Sundararajan et al., 2001; Cuconati et al., 2002; White, 2006; Lomonosova et al., 2005). Also, E1B-19K binds to and inhibits the proapoptotic BH3-only protein BIK/NBK and thereby inhibits apoptosis in response to inhibition of protein synthesis (Shimazu et al., 2007). Unlike its cellular functional homologue, E1B-19K does not interact with BAD and BID, BH3-only proapoptotic proteins that function upstream of BAK and BAX (Cuconati & White, 2002). Further, E1B-19K lacks the transmembrane domain which may differentially affect its localization and functions in the cell (Cuconati & White, 2002). E1B-19K also forms a complex with p53 in mitochondria where it may inhibit p53-mediated apoptosis (Lomonosova et al., 2005).

The molecular details of the TGF β -mediated apoptotic pathway in hepatocellular carcinoma cells are beginning to be understood (Fabregat, 2009). Normal hepatocytes are susceptible to growth inhibition by TGF β and TGF β -induced apoptosis, but some hepatocellular carcinoma cells are resistant to TGF β -induced apoptosis. Mechanisms of resistance include downregulation of TGF β receptors, disruption of TGF β signaling, expression of certain microRNAs, and upregulation of survival pathways. However, in Huh-7 cells, an hepatocellular carcinoma cell line, the intrinsic pathway of cell death proceeding through the mitochondria is triggered by TGF β (Huang & Chou, 1998). Not surprisingly, stable transfection of Huh-7 cells with BCL-2 conferred resistance to TGF β -induced apoptosis as compared to control cells that lacked BCL-2 expression (Huang & Chou, 1998).

According to our data, the pro-apoptotic pathway of TGF β is blocked upstream of mitochondria in infected cells. It is noteworthy that wild-type levels of expression of E1B-19K in cells infected with the Ad mutant lacking the N-terminal sequences of E1A were insufficient to fully protect infected cells against TGF β -induced apoptosis and disruption of the mitochondria membrane potential. This could be explained by the relative levels of E1B-19K expression in infected cells as compared to the levels observed in the transfected cells (Huang & Chou, 1998). Alternatively, E1B-19K may lack certain functions of cellular BCL-2 that allow the latter to be sufficient in blocking TGF β -induced apoptosis. Finally, it is possible that under the conditions of Ad infection, a cooperation between E1A and E1B-19K is required to confer full resistance against TGF β -mediated cell death.

TGF β -induced pro-caspase 8 processing was significantly reduced in cells infected with wild type Ad. Both E1A and E1B-19K proteins were required to prevent TGF β -induced cleavage of pro-caspase 8 in infected cells, suggesting that both proteins cooperate to block events upstream of TGF β -triggered mitochondria disruption and pro-caspase 8 processing in Ad-infected cells.

Regarding the mechanisms by which the E1A and E1B-19K proteins inhibit TGF β -induced apoptosis, one obvious possibility is that they interfere with the canonical T β RI/T β RII/Smad signaling pathway, acting on the T β RI/T β RII complex, on Smad signaling, and on further downstream events. As discussed, the E1A N-terminus is involved in downregulating T β RII in Ad-infected cells (Tarakanova & Wold, 2003). However, it is quite possible that E1A and E1B-19K may act upon additional steps in the signaling pathway, or act instead on other mechanisms in order to block TGF β -induced apoptosis in infected cells. In fact, a recent study

concluded that TGF β signaling in Huh-7 cells is altered because T β RII levels are very low and because these cells lack β 2SP, a protein that associates with Smad3 and presents it to the T β RI/T β RII complex and that is required for nuclear transduction of Smad3 (Lin et al., 2009). Also, other TGF β signaling pathways could also play a role. It is known that TGF β can signal through T β RIII (reviewed in (Margulis et al., 2008) or T β RV (reviewed in (Huang & Huang, 2005). There are also type IV and VI TGF β receptors, as well as a number of membrane-associated proteins that bind TGF β , and these could be involved in TGF β -induced apoptosis in Huh-7 cells.

With respect to T β RIII, enforced expression of this receptor in a clear cell renal carcinoma cell line led to apoptosis (Margulis et al., 2008). This T β RIII-induced apoptosis required the cytoplasmic domain of T β RIII, it occurred in cells lacking T β RII, and also in cells where transcription-induction through the T β RI/T β RII/Smad pathway did not occur. The apoptosis appeared to be mediated through the p38 mitogen-activated protein kinase (MAPK) pathway (Margulis et al., 2008). In another study, enforced expression of T β RIII in myoblasts or fibroblasts induced transcription from TGF β target promoters in a manner independent of the canonical TGF β signaling pathway but involving the p38 MAPK pathway (Santander & Brandan, 2006). In studies by still another group, T β RIII was found to regulate epithelial and cancer cell migration (Mythreye & Blobe, 2009), and to mediate growth inhibition of primary human epithelial cells and rat L6 myoblasts through both p38 MAPK and T β RI/Smad3 pathways (You et al., 2007).

Regarding T β RV, this TGF β receptor is co-expressed together with T β RI, T β RII, and T β RIII in all non-cancerous cell lines tested (reviewed in (Huang & Huang, 2005). T β RV is also the receptor for insulin-like growth factor binding protein-3 (IGFBP-3), and it mediates cell growth inhibition by IGFBP-3 (Leal et al., 1997). Both TGF β and IGFBP-3 act through T β RV to inhibit epithelial cell proliferation (Leal et al., 1997; Wu et al., 2000; Huang et al., 2004; Huang & Huang, 2005). In mutant Mv1Lu mink lung epithelial cells that lack functional T β RII, the T β RV-mediated growth inhibiting signal is operational (Liu et al., 1997)

Based on published descriptions of TGF β -induced apoptosis in Huh-7 cells (Huang & Chou, 1998; Shima et al., 1999) and our report we propose the following simplified working model for the inhibition of the TGF β pro-apoptotic pathway in Ad-infected cells (Fig. 7). Ligation of the TGF β receptors by the cytokine leads to assembly of transcription-competent complexes. In the case of the T β RI/T β RII complex, signal transduction occurs via the Smad 2/3/4 pathway and other cellular cofactors that control transcription of apoptosis-regulating genes. With the other TGF β receptors, the signaling mechanisms are poorly understood including those leading to apoptosis. The mechanism of TGF β -induced apoptosis differs among cell types (Heldin et al., 2009). In Huh-7 cells, p53 and pRB are reported to mediate TGF β -induced apoptosis, with p53 acting upstream of pRB (Fan et al., 2002; Fan et al., 2004b). Subsequent steps lead to processing and activation of pro-caspase 8. The caspase 8 activity leads to the disruption of the mitochondrial membrane followed by the activation of pro-caspases 3 and 9. The activity of caspases 3 and 9 further amplifies pro-caspase 8 processing and the apoptotic cascade that ultimately results in cell death.

We suggest that upon infection with-wild type Ad, E1A, acting through its N-terminal domain, mediates a gradual decrease in the levels of T β RII that does not become evident until later in infection (Tarakanova and Wold, 2003) and interferes with the transcriptional activity of Smad-containing complexes (Fig. 7). The E1A N-terminal domain could also possibly interfere with signaling through the other TGF β receptors. The E1B-19K protein inhibits the apoptotic signaling just upstream of mitochondria by binding BAK, and possibly also by binding BIK/NBK (Fig. 7). When E1A expressed in Ad-infected cells lacks the N-terminal sequences, the unrestricted TGF β signal transduction throughout the infectious cycle eventually overcomes

the protection offered by E1B-19K and premature cell death ensues. On the other hand, in the absence of E1B-19K, TGF β signaling is eventually inhibited by E1A in infected cells, however, the initial burst of pro-apoptotic signal transduction prior to the establishment of adequate E1A levels and significant downregulation of TGF β receptor II or a low level of TGF β signaling that may persist in infected cells is not countered by E1B-19K. Without mitochondrial protection, the caspase-8 activity is amplified leading to the demise of the infected cell.

In summary, our study provides evidence that TGF β -induced apoptosis is inhibited by Ad infection. The intact N-terminal sequences of the E1A proteins and E1B-19K are important for the resistance to TGF β -triggered cell death in Ad-infected Huh-7 cells. Our results are consistent with microarray analysis of Ad2-infected primary human lung fibroblasts which indicated that the TGF β signaling pathway is downregulated (Zhao et al., 2007). Suppression of TGF β -mediated cell death expands an existing arsenal of tools human Ads use to keep the infected host cell alive.

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Figure 1. Adenovirus-infected cells are resistant to TGF β -induced apoptosis

Huh-7 cells were mock-infected or infected with *rec700* at 50 PFU/cell in the presence of 20 μ g/ml of AraC. **A.** Cells were treated with either 15 μ M LY294002 or 10 ng/ml TGF β , or a combination of two agents at 1-24 h p. i.. Representative phase contrast images are shown. Arrows point to the loci of apoptotic cells. **B.** In a separate experiment, Huh-7 cells were seeded on coverslips, treated as described in A, and stained with DAPI at 22 h p.i. (21 h post treatment). Representative fluorescent micrographs are shown. **C.** Huh-7 cells were treated as described in A. Cell lysates normalized for protein concentration were examined for pro-caspase 3 processing by Western analysis. The arrow indicates the processed forms of caspase 3. **D.** The

general schematic of infection and treatments used for experiments in this study is shown unless otherwise specified.

Figure 2. E1A and E1B-19K proteins contribute to the lack of TGF β -induced caspase 3 processing in infected cells

Huh-7 cells were mock infected or infected with indicated viruses at 35 PFU/cell and treated as described in Fig.1. Cell lysates were normalized against protein concentration and probed with anti-caspase 3 (**A**, **B**), anti-E1A (**C**), or anti-E1B 19K (**D**) antibodies.

Figure 3. TGF β -induced apoptotic nuclear morphology is dependent on expression of both the N-terminal sequences of E1A and E1B-19K

Huh-7 cells were mock-infected or infected with adenovirus mutants and treated as in Fig. 1B. Representative fluorescent micrographs are shown.

Figure 4. Cleavage of cellular DNA correlates with the caspase 3 processing in cells sensitive to TGFβ-induced apoptosis

Huh-7 cells treated with PI3 kinase inhibitor were mock infected or infected with indicated viruses and treated or not treated with TGFβ as in Fig. 1. Cellular DNA was extracted and DNA fragmentation was analyzed by agarose gel electrophoresis. Molecular weight markers are in the rightmost and leftmost lanes.

Figure 5. Both E1A and E1B-19K proteins contribute to the maintenance of mitochondria potential in infected TGFβ-treated cells

Huh-7 cells were infected and treated as described in Fig. 1. The treatment was extended to 30 h post infection; cells were incubated with Rh123 for the last 30 min of treatment. Live cells were trypsinized and immediately analyzed by flow cytometry.

Figure 6. Both E1A and E1B-19K proteins are required to block TGFβ-induced procaspase 8 processing in adenovirus-infected cells

Huh-7 cells were infected and treated as described in Fig. 2. **A.** At indicated times post infection/treatment or at 24 h p.i. (lanes a-c) cell lysates were collected and analyzed for caspase 8 processing by western analysis. The arrow indicates the cleaved forms of caspase 8. **B.C.** At 19 h p.i. cell lysates were analyzed for pro-caspase 8 cleavage (**B**) or E1A levels (**C**).

Figure 7. Possible model for inhibition of TGFβ-induced apoptosis by E1A and E1B-19K in adenovirus-infected cells