The Role of Pidd Protein in Adenoviral Induction of Apoptosis

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LOYOLA UNIVERSITY CHICAGO

THE ROLE OF PIDD PROTEIN IN ADENOVIRAL INDUCTION OF APOPTOSIS

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ABSTRACT

The Adenovirus E1A gene sensitizes cells to genetic insults and apoptosis, most notably in response to cytotoxic factors from innate immune cells. The mechanisms by which E1A sensitizes cells to apoptotic stressors have not been fully elucidated, however E1A actively represses NF-κB anti-apoptotic defenses, thereby sensitizing cells to alternative apoptotic cascades. Recent studies alternatively indicate that E1A also actively induces Caspase-2 activation and mitochondrial injury in the presence of certain cytotoxic injuries.

Caspase-2 is cleaved and activated in a large complex consisting of p53-inducible protein with a death domain (PIDD), an adaptor protein RAIDD, and pro-caspase 2. PIDD auto-proteolyzes into several fragments and the C-terminal PIDD-CC fragment is required for activation of the PIDDosome in response to cytotoxic stress. We hypothesized that E1A-induced sensitivity and induction of Caspase-2 activity is dependent on PIDD, and in particular PIDD autoproteolysis to the PIDD-CC fragment. A PIDD knockdown was generated in mouse fibroblast cells that also expressed E1A to determine the contribution of PIDD protein to apoptotic sensitivity. A PIDD processing-mutant, incapable of terminal processing to the PIDD-CC fragment, was also generated to establish whether PIDD auto-cleavage is required for E1A-induced sensitivity. We observed that knockdown of PIDD and inhibition of PIDD processing significantly decreased sensitivity of E1A-expressing cells, indicating that PIDD indeed plays a role in
the effects of E1A on caspase-2 activation and sensitization to cytotoxic stressors. These results may ultimately contribute to improvements on the application of Adenovirus E1A in anti-cancer therapeutics.
CHAPTER I
INTRODUCTION

Adenoviruses are a family of viruses that were discovered in the mid-1950s from independent attempts to isolate the causative agent of the “common cold” and identify the cause of a flu-like epidemic that had infected large groups of army recruits during WWII\(^1\text{-}^3\). Since that time, the antigenic variety and ubiquity of adenoviruses has been revealed in studies identifying a variety of mammalian hosts and high percentages of people possessing antibodies to one or more adenovirus serotypes. For instance, one study found that nearly 100% of adults in Washington, DC had been exposed to adenoviruses by age 34\(^3\text{-}^4\). Most serotypes infect respiratory tissues and cause mild cold symptoms, but many also affect a variety of organs: besides respiratory disease, common maladies associated with adenoviruses are gastroenteritis and conjunctivitis\(^5\text{-}^6\). Symptomatic adenoviral infection may be as low as 15% or as high as 45%, but these estimates depend on the adenovirus serotype and symptoms represented\(^7\text{-}^8\). While adenoviruses have been studied for over half a century, their pathological mechanisms have not been fully defined.

For decades, adenoviruses have had applications within research, as the structure and genome of the virus can be easily manipulated and used as vectors for delivering genetic material to foreign targets. The adenoviral genome has been well characterized and utilizes much of the cellular machinery involved in DNA replication, transcription,
and translation; thus, research on adenoviruses has provided important insights into the molecular biology of the cell (reviewed in 9). Adenoviruses are uniquely suited as genetic vectors for several reasons: They produce high titers and infect a wide variety of cells; the genome can be stably expanded to encode large segments of foreign DNA 10; and integration of the genome into the host chromosome is rare unless it is hybridized with retroviruses, allowing investigators plenty of control for producing either transient or stable gene expression in hosts 11-13. Importantly, certain adenoviral factors, such as the Early Region 1 A (E1A) gene, have properties that have been applied to successful cancer-fighting therapies 14,15. A comprehensive understanding of the adenoviral genetic arsenal can vastly improve both scientific and therapeutic applications of the virus.

**Adenoviral influence on tumor development and apoptosis**

Many adenovirus serotypes are tumorigenic within the right immunological context, such as immunocompromised species or newborn rodents with undeveloped immune systems 16. Early investigations determined that immune competent species, including rodents beyond a few weeks of age, easily defeated Adenovirus-transformed tumors, while models lacking innate and/or adaptive immune function exhibited high tumorigenicity (Figure 1a) 17. A series of studies revealed that the E1A gene specifically reduced tumorigenicity of transformed cell lines and generally increased sensitivity to immune cells and their various cytotoxic factors 18 (Figure 1b). Several mechanistically distinct classes of insults from innate and adaptive immune cells were able to sensitize E1A-expressing cells to cytolytic injuries, including Fas ligand, Tumor Necrosis Factor alpha (TNFalpha), TNF-related Apoptosis Inducing Ligand (TRAIL), and nitric oxide
(NO), among others\textsuperscript{19-24}. The diversity of apoptotic insults resulting in E1A-induced sensitivity raised questions about the potential mechanism(s) by which E1A might be promoting apoptosis in transformed cells.

\textbf{Figure 1. Adenoviral effects on tumor development and apoptosis.} a) Adenovirus infection or transfection with Adenovirus-transformed cells induce tumor formation in immunocompromised rodents, but not in immune competent models. b) Innate killer cells promote death in E1A-expressing cell lines, but not in E1A-negative cells.

\textbf{Adenovirus E1A}

Adenoviruses typically infect terminally differentiated cells and E1A is the first gene to be transcribed within the nucleus. The E1A genomic unit codes for two alternative E1A mRNAs, a 12S and a 13S form that have experimentally identical functionality and differ in that the 13S form encodes an additional region of 46 amino acids\textsuperscript{25,26}. E1A proteins across different adenoviral serotypes contain a series of conserved regions: CR1, CR2, CR3 (specific to the 13S form), a PXDLS motif, and a conserved arginine at aa position...
2. All of the confirmed activities of these conserved motifs involve protein-protein interactions, with no specific DNA-binding activity. Each of the conserved regions interacts with different important cellular factors involved in cell cycle and transcriptional control, and while the CR3 region of the 13S form has unique protein interactions within the cell, 12S and 13S are able to carry out equivalent functions to influence cell cycle control\textsuperscript{25}.

The most extensively studied function of E1A is the deregulation of the cell cycle G1/S transition by binding and disrupting the activity of several cell cycle regulators, including key tumor suppressors such as Retinoblastoma protein (pRb). pRb and related proteins interact with and inhibit the E2F family of transcription factors that promote expression of genes required for transition to S phase\textsuperscript{27}. E1A binds pRB and precludes binding and inhibition of E2F, which is then free to influence transcription and cell cycle progression\textsuperscript{28} (Figure 2). This inhibition of key cell cycle regulators is the reason that E1A is historically labeled an oncoprotein, even though it has been shown to sensitize cells to a variety of immune apoptotic factors.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{E1A promotion of cell cycle progression by inhibition of pRB. E1A promotes cell cycle progression by binding and inhibiting pRB, an inhibitor of E2F transcription factor. E2F is then free to induce expression of genes required for DNA synthesis.}
\end{figure}
E1A-induced sensitivity to apoptotic stress by inhibition of NF-κB defenses

Several mechanisms have been proposed for E1A-induced apoptotic sensitivity. For example, it has been established that E1A promotes the accumulation of the anti-tumor factor p53, a stimulator of apoptosis, suggesting p53 as a potential mediator of E1A-induced sensitivity. However, there has been no supporting evidence to indicate that p53 leads to E1A-induced sensitization to apoptosis.

It has been confirmed that the sensitivity to several classes of apoptotic stimuli is due, at least in part, to the influence of E1A on NF-κB defenses against apoptosis. NF-κB defenses have been studied extensively in the context of apoptosis induced by the Tumor Necrosis Factor (TNF) family of cytokines. NF-κB is a transcriptional regulator that leads to the expression of several anti-apoptotic proteins, and has been proven to be the primary mode of defense against TNF. Interestingly, E1A sensitizes certain types of cells to apoptosis under stress by TNF, suggesting a disruption of the NF-κB defenses (Figure 3). It was also demonstrated that E1A is able to inhibit NF-κB-dependent activity in the repression of HIV promoters. Several mechanisms of E1A inhibition of NF-κB defenses have been proposed and have been supported by experimental evidence. For example, E1A has been shown to inhibit IκB-kinase (IKK) activity required for the activation of NF-κB, and also to inhibit the binding of NF-κB subunits to their target promoters. In 2002, Cook et al. presented evidence that the inhibition of NF-κB was related to E1A’s function in repression of Rb proteins. While several potential mechanisms have been implicated, the full spectrum of E1A effects on NF-κB activation has not been resolved.
Figure 3. E1A inhibition of NF-κB anti-apoptotic defenses. E1A promotes apoptosis in response to TNF by inhibiting NF-κB anti-apoptotic defenses.

E1A-induced sensitization to cytotoxic stress by regulation of caspase-2 activation

Due to the extensive evidence supporting E1A repression of NF-κB, a hypothesis developed that this was the central mechanism in all modes of E1A-induced sensitivity. In 2008, Radke et al. conducted a study to determine whether E1A repression of NF-κB defenses could be implicated in E1A-induced sensitivity to several classes of apoptotic injuries. Macrophages are important innate immune cells involved in anti-tumor activities and produce nitric oxide (NO), which efficiently kills E1A-expressing cells. NO is able to activate NF-κB defenses, inspiring the hypothesis that the observed sensitivity in response to E1A could be due to interruption of NF-κB by the viral oncoprotein as previously described. Unexpectedly, the investigators found that E1A-induced sensitivity to macrophage- and chemically-produced NO was not dependent on the inhibition of NF-κB pathways. As NO injures mitochondria and causes a loss of
mitochondrial membrane potential, leading to cell death, the group investigated whether the pathway leading to mitochondrial injury was required for the observed sensitivity. Caspase-2 directly injures the mitochondrial membrane, and NO had previously been confirmed to activate caspase-2. The investigators found that inhibition of caspase-2 reduced sensitivity in E1A-expressing cells, and that activation of caspase-2 was critical to the increased sensitivity of these cells. Thus, the study indicated that E1A-dependent sensitization in response to NO injury is dependent on caspase-2 activation (Figure 4). This suggests a qualitatively different mechanism by which E1A induces sensitivity in cells: in addition to inhibiting anti-apoptotic defenses, E1A may be positively influencing a pro-apoptotic cascade. Further research has focused on determining the mechanism by which E1A might influence caspase-2 activation.

![Diagram](image)

**Figure 4. E1A promotion of the Caspase-2 pro-apoptotic pathway.** E1A promotes apoptosis after NO cytotoxic stress by enhancing a pro-apoptotic cascade via an unknown mechanism. Activation of caspase-2 is required for E1A-induced apoptosis.
In 2002, Lassus et al. determined that activation of Caspase-2 in response to cytotoxic stress such as mitochondrial NO is required for the permeabilization of the mitochondrial membrane\textsuperscript{48}. When mitochondrial membrane integrity is compromised, cytochrome-C and other mitochondrial factors are released, activating further caspases or amplifying the caspase cascade\textsuperscript{49}. In unpublished data leading up to this study, our group found that Caspase-2 activation is required for loss of mitochondrial membrane potential in response to NO and genotoxic stress in E1A-positive cells.

Caspase-2 can be activated by a high molecular weight structure called the PIDDosome, or by active caspase-3\textsuperscript{50,51}. The unpublished experiments referenced above also addressed the mode of caspase-2 activation in E1A-expressing cells in response to cytotoxic stress. Activation of Caspase-3 was not required to observe the caspase-2-dependent sensitization of E1A-expressing cells in response to NO or the chemotherapeutic drug etoposide, an agent that causes DNA damage. In sum, the Cook group has determined that Caspase-2 is the apical mitochondria-injuring caspase in response to NO in E1A-expressing cells. The observed effect of E1A-induced apoptotic sensitization via the mitochondria must necessarily involve Caspase-2 activation upstream of mitochondrial injury under conditions of NO stress. Furthermore, the mode of Caspase-2 activation does not involve Caspase-3 upstream of Caspase-2 activation; instead, caspase-2 activation in response to cytotoxic stress must proceed through the PIDDosome.
**PIDD and the PIDDosome**

Efficient activation of caspase-2 is accomplished on the PIDDosome, composed of P53-induced protein with a Death Domain (PIDD), an adaptor protein (RIP)-Associated ICH-ICED-3 homologous protein with a Death Domain (RAIDD), and procaspase-2 (Figure 5). Accumulation of procaspase-2 in the PIDDosome allows for efficient cleavage and activation of caspase-2.

![Diagram of PIDDosome interactions](image)

**Figure 5. Interactions of proteins in the PIDDosome.** Schematic demonstrating the interactions of PIDD, RAIDD, and pro-caspase-2 in the PIDDosome. PIDD and RAIDD interact via Death Domains (DD), while RAIDD and pro-caspase-2 interact via CAspase Recruitment Domains (CARD).

PIDD is a dynamic protein, able to promote opposing pathways depending on its level of post-translational processing and subsequent protein associations. The prominent PIDD isoform in humans (isoform 1) is expressed as a 910 aa protein with seven Leucine Rich Repeats (LRRs) near the N terminus, two ZU-5 domains, and a C-terminal Death Domain. At least two other PIDD isoforms are expressed in different classes of cells and may have different effects on cell survival. Isoform 1 has been the subject of most studies involving PIDD. The 100 kDa full-length protein is able to autoproteolyze to 48 kDa PIDD-N and 51 kDa PIDD-C fragments at position S446. The PIDD-C fragment
further cleaves at S588 to produce a 37 kDa PIDD-CC fragment (Figure 6). The PIDD-CC fragment is the functional component of the PIDDosome; within the PIDDosome, the PIDD-CC death domain interacts with the death domain of RAIDD, and RAIDD’s CASpase Recruitment Domain (CARD) interacts with the CARD of pro-caspase-2 \(^{53}\) (Figure 5).

While the PIDDosome provides a platform for cleavage of caspase-2, an alternative complex formed by PIDD involves Receptor Interacting Protein 1 (RIP-1) and the NF-κB complex factor NEMO \(^{56}\). NEMO on this complex is subjected to sumoylation, an essential step in the activation of NF-κB anti-apoptotic defenses. In order to interact with RIP-1, PIDD must be processed to the PIDD-C form, as opposed to PIDD-CC (Figure 6).

Thus, PIDD seems to have a dual role in cell survival and apoptotic pathways depending on the degree of processing: PIDD-C promotes NF-κB defenses, while PIDD-CC as part of the PIDDosome promotes caspase-dependent apoptotic pathways (Figure 6).
Figure 6. Schematic of PIDD auto-proteolysis to PIDD-C and PIDD-CC fragments. Cleavage at S446 results in a PIDD-C fragment that promotes NF-κB anti-apoptotic defenses. Further cleavage at S588 results in a PIDD-CC fragment, which facilitates pro-apoptotic caspase-2 activity.

**Hypothesis and Experimental Approach**

It is due to this dual role that we seek to understand the contribution of PIDD to E1A-induced sensitivity in response to cytotoxic stress. PIDD seems to be a switch between pro- and anti-apoptotic pathways, so it is tempting to consider how E1A might influencing PIDD processing to turn the switch towards apoptotic sensitivity, while simultaneously inhibiting anti-apoptotic defenses. In this study, we hypothesized that E1A-induced sensitivity to cytotoxic stress is dependent on PIDD expression. We also propose that E1A-induced sensitivity depends on PIDD processing to the terminal PIDD-CC fragment.
To determine the contribution of PIDD expression to E1A-induced sensitivity, we generated knockdowns of PIDD in E1A-expressing NIH-3T3 cells and assayed sensitivity to chemically-induced NO and the drug Etoposide, a chemotherapeutic agent that leads to DNA damage and subsequent apoptosis. To understand the role of PIDD-processing to the terminal -CC fragment in E1A-induced sensitivity, a PIDD-S588A mutant that is incapable of cleaving to PIDD-CC was generated and also assayed for sensitivity to NO and etoposide. Sensitivity assays included MTS metabolic activity assay, which gave a measure of cell survival after treatment with the cytotoxic stressors. We also measured the change in mitochondrial membrane potential (MMP) of treated and untreated cells by DilC₁(5) staining and global apoptotic character by Annexin V staining. If PIDD expression and PIDD processing is required for E1A-induced sensitivity to cytotoxic stress, PIDD knockdowns and PIDD-processing mutants should demonstrate reduced sensitivity by these various assays.
CHAPTER II
MATERIALS AND METHODS

Generation of PIDD-knockdowns and PIDD-S588A mutants

NIH-3T3 mouse fibroblasts and all derivatives expressing Ad5 12S E1A were grown in DMEM with 5% bovine calf serum\(^{20,40}\). PIDD-knockdown E1A+ cells were generated from 12S E1A+ cells by infection with lentiviral particles targeting mouse PIDD (siRNA target sequence AGCTTTAAACTTGACTCGA) or a standard non-targeting control (Dharmacon). The lentiviral constructs also contained a puromycin resistance marker and encoded for GFP. Single colonies were selected from 7.5 \(\mu\)g/ml puromycin media and screened for GFP expression by flow cytometry, expression of E1A by western blot using M73 monoclonal antibody (Santa Cruz Biotechnology), and suppressed expression of PIDD (Figure 8).

Human fibrosarcoma HT1080 cells (H4) and E1A-expressing derivatives (P2) were maintained in 10% Fetal Bovine Serum in RPMI. E1A+ cells were transfected with one of five pLKO.1 vectors encoding shRNA sequences targeting human PIDD (CTTTGTCTTCTACTCGCACCT; GACTGTTCTGACCTCAGATT; GACCTCAGATTTGGACAGCTT; CTGCTTTGTTCTTACTCGA; CCTCGATCTCTCTCAATCT; Thermo Scientific). Single colonies were selected in 2.5 \(\mu\)g/ml puromycin and screened for suppressed expression of PIDD by western blot using anti-PIDD monoclonal antibody (AL233, Alexis; Figure 10).
PIDD S588A mutants were generated in 12SA E1A-expressing cells using a plasmid encoding PIDD-S588A flag-tagged at the C-terminal end, which had been obtained from Jurg Tschopp. The PIDD gene was cloned into vector pcDNA3.1 (+) Hygromycin (Invitrogen) by HindIII/NotI digestion and ligation, and the resulting construct was electroporated into the E1A+ line. S588A mutant cells were selected in media containing 200 µg/ml hygromycin, then screened for expression of E1A and Flag-tagged PIDD-S588A by western blot (Sigma anti-Flag M2 antibody; Figure 1).

**Cell death assays**

Cell lines were treated with increasing doses of DETA-NONOate (Cayman Chemical) or etoposide (Sigma) overnight (18 hours) in 96 well plates and subjected to a colorimetric cell viability assay (CellTiter 96 Non-Radioactive Cell Proliferation assay from Promega). Absorbance values were read on a plate reader at 570 nm as per instructions and cell death was expressed as a ratio of treated/untreated absorbance values for each cell line.

**Flow cytometry**

To assess mitochondrial membrane potential, cells were treated with DETA-NONOate or etoposide as described, then stained with 25 nM MitoProbe Dil1C(5) as per kit instructions (Life Technologies). To observe global apoptotic phenotype, cells were treated with DETA-NONOate or etoposide and stained with both Dil1C(5) and PE-Annexin V (Life Technologies) as per instructions. Data were acquired on a BD Accuri C6 Flow Cytometer and processed and analyzed using FlowJo X 10.0.7r2 (Tree Star Inc.). Forward scatter versus side scatter plots were used to gate around non-debris.
values and the same gate was applied to all corresponding data to generate histograms of DilC\(_1\)(5) staining. All assigned quadrants in the DilC\(_1\)(5)/Annexin V multiplex experiments remained consistent, permitting a comparison of cell populations across cell lines.

**Statistical Analysis**

Student’s \(t\)-test was used for statistical analysis, using Systat SigmaPlot 12.3 software. \(n \geq 3\) for all statistically analyzed data, and data are expressed as mean ± SEM.
CHAPTER III

RESULTS

Generation and selection of PIDD-knockdown and PIDD-mutant E1A-positive cells

PIDD knockdown cell lines

To determine whether PIDD is required for E1A-induced sensitization to apoptosis, I generated PIDD knockdowns of mouse fibroblast NIH-3T3 E1A-expressing mutants. The E1A positive parent line had been used previously by the Cook lab for studies investigating the effect of E1A on NF-κB and apoptotic pathways\(^4^0\); therefore, I generated the knockdowns in this background. I introduced into the E1A positive cell line lentiviral vectors that encoded shRNAs specific for PIDD as well as puromycin resistance and GFP, such that expression of the shRNAs correlated with GFP expression in the transduced lines. Cell lines resistant to puromycin were initially screened for GFP expression by flow cytometry. Two separate PIDD knockdown lines (iPIDD-1 and iPIDD-2) displayed GFP expression over the 3T3/E1A-negative and E1A-positive parent lines (Figure 7). In parallel, I introduced a lentiviral vector that encoded a control scrambled shRNA sequence; this transduced E1A+ line also expressed GFP, indicating successful expression of the construct and shRNA (Figure 7).
Figure 7. GFP expression in PIDD knockdown lines. Cell lines were screened for GFP expression by flow cytometry to indicate successful expression of PIDD shRNAs.

To verify that levels of PIDD protein were reduced in the knockdown cell lines, I evaluated PIDD protein levels by western blot (Figure 8). The parental E1A-negative 3T3 cell line and the E1A-positive cell line (lanes 1 and 2, respectively) exhibited equivalent expression of full-length and PIDD-C processed fragments. The E1A-positive, PIDD-knockdown cell lines (iPIDD-1 and iPIDD-2) showed reduced PIDD expression for each fragment (lanes 3 and 4). The E1A-positive line expressing the scrambled shRNA had no alteration of PIDD levels (lane 5). iPIDD-1 exhibited a more extensive knockdown of PIDD than did iPIDD-2. Despite several repeated attempts to create knockdowns by infection with the lentiviral constructs, we could not produce or confirm
a complete PIDD knockdown in either of these lines, and performed further experiments on these partial knockdowns.

Figure 8. Immunoblot of mouse PIDD-knockdown cell lines. Anti-PIDD immunoblots of PIDD-knockdowns (iPIDD-1 and iPIDD-2) were compared to 3T3 E1A-negative and E1A-positive parent lines, as well as an E1A+ line transfected with a construct expressing a scrambled shNA. Whole cell lysates were run on SDS-PAGE gels and blotted with mouse anti-PIDD antibody to determine positions of PIDD protein. Anti-actin blotting confirmed equal applications of lysates, and anti-E1A confirmed expression of E1A across the E1A+, PIDD knockdown, and scrambled shNA lines.

In order to correlate apoptotic sensitivity to PIDD expression, transduced cell lines that were resistant to puromycin and expressed GFP were screened for reduced sensitivity to NO over several concentrations of the NO generator DETA-NONOate. Figure 9 represents a selection of cell lines that were tested for sensitivity to NO by MTS metabolic assay. Cell lines 2D and 2E consistently showed lower cell death than the E1A+/PIDD-competent parent line (red line labeled 12SA), and comparable cell death as
the 3T3 E1A-negative line (blue line labeled 3T3). Cell lines 2D and 2E were labeled iPIDD-1 and iPIDD-2 respectively, and chosen for further analysis to determine if the decreased sensitivity to NO is related to PIDD function. Cell lines 1E and 2B had comparable NO cell death to the E1A+ parent line and were not chosen for further analysis.

Figure 9. Selection of PIDD knockdown lines with reduced sensitivity to NO treatment. Puromycin-resistant and GFP-positive lines were treated with several concentrations of DETA-NONOate for 18 hours overnight. Cell death was assessed by MTS assay and expressed as a percent of cell death of untreated cells.
We also attempted to generate PIDD knockdowns of an E1A+ derivative of the H4 human fibroblast cell line. I transfected E1A+ cells with one of several vectors designed to target several distinct regions of transcript for the human PIDD protein, then assessed the presence of PIDD by immunoblot with anti-PIDD antibody. Unfortunately, I did not observe a knockdown effect on any protein bands predicted to be the various fragments of PIDD (Figure 10, lanes 1-7). Additionally, the exact identity of the PIDD fragments was unclear, with several bands approaching but not exactly matching the predicted sizes. Full-length PIDD is 100 kD; we observed two bands between 100 and 150 kD (position A on Figure 10). PIDD-C is 51 kD, however the immunoblot shows a strong band at around 60 kD and several lighter bands between 50 and 60 kD (position B). Finally, the PIDD-CC fragment should be 37 kD, but the blot showed no band at this position. Rather, the lowest band at position C was positioned well above 37 kD and is our best approximation of PIDD-CC. The complicated banding profiles of PIDD in this blot (and others) may represent at least three isoforms that are known to be expressed in human cell lines.55 Regardless, we were unsuccessful in knocking down any of the predicted PIDD fragments in the transfected lines: the banding patterns clearly matched those of the H4 and E1A+ parent lines, indicating that all PIDD fragments were equally expressed and processed in all transfected lines.
**Figure 10. Immunoblot of human PIDD-knockdown lines.** Whole cell lysates were run on SDS-PAGE gels and blotted with human anti-PIDD antibody to determine positions of PIDD protein. A represents potential full-length PIDD fragments. B represents potential PIDD-C fragments. C represents a potential PIDD-CC fragment.

**PIDD mutant cell lines**

We hypothesized that PIDD processing to the PIDD-CC fragment is required for E1A-induced sensitivity to cytotoxic stress. To test this hypothesis and determine the effect of PIDD processing on E1A-induced sensitivity, a C-terminal flag-tagged PIDD processing-mutant (S588A) was expressed in the E1A-positive mouse fibroblast cell line. This mutant construct has been described and used in several studies, and is unable to auto-cleave to the PIDD-CC fragment required for PIDDosome activation of Caspase-2\(^{53}\). The overexpressed mutant PIDD only cleaves to PIDD-C and results in a dominant-negative mutation, accumulating to prevent the formation of the PIDDosome with the necessary endogenous PIDD-CC fragments. On western blots, we observed the flag-tagged full length and PIDD-C proteins (Figure 11); we did not observe the PIDD-CC band (data not shown). These data confirm that the cell line successfully overexpressed the flag-tagged PIDD processing mutant.
Figure 11. Immunoblot of mouse PIDD-S588A mutant cell lines. Whole cell lysates were run on SDS-PAGE gels and blotted with anti-flag to determine positions of flag-tagged PIDD. Anti-actin blotting confirmed equal applications of lysates, and anti-E1A confirmed expression of E1A in E1A+ parent line and the S588A mutant. * = non-specific antibody interactions.

Cytotoxic sensitivity of PIDD-knockdown and PIDD-processing mutant lines

To determine whether PIDD was required for the sensitization of E1A-expressing cells, we compared cell death resulting from NO- and Etoposide-mediated cytotoxic stress of E1A-positive/PIDD-knockdown cells and their PIDD-competent parent lines. Cell death was determined by MTS metabolic assay and represented a measure of sensitivity to the chemical or drug treatment. After treatment with NO, cell death of the knockdown lines was about half of that observed for the E1A+/PIDD+ parent line (Figure 12). The non-
targeting shRNA expression line (labeled scRNA) did not have any unintended effects on cell sensitivity. These results indicate that PIDD expression is required for E1A-induced sensitivity to NO injury.

**Figure 12.** Cell death in PIDD-knockdowns and E1A+ cells after NO treatment. Cells were exposed to 250 μM NO generator DETA-NONOate for 18 hours overnight. Cell death was assessed by MTS assay and expressed as a percent of cell death of untreated cells. n=3, mean±SEM.

The PIDD knockdown lines also had lower cell death when compared to the PIDD-competent line as a result of etoposide treatment (Figure 13). However, cell death was significantly higher for both knockdown lines when compared to the 3T3 parent line.
It is possible that the partial knockdown of PIDD accounts for the intermediate phenotype between the E1A+ and E1A-negative parent lines in response to etoposide. Alternatively, the intermediate phenotype may be due to the fact that etoposide has a distinct DNA-damaging mechanism compared to NO. Regardless, we can conclude that PIDD expression is required for the complete sensitization effect of E1A for both NO and etoposide treatment.

Figure 13. Cell death in PIDD-knockdowns and E1A+ cells after etoposide treatment. Cells were exposed to 10 µM Etoposide for 18 hours overnight. Cell death was assessed by MTS assay and expressed as a percent of cell death of untreated cells. n=3, mean±SEM.
To determine whether E1A-induced sensitivity is due to PIDD processing to the PIDD-CC fragment, we also compared the cell death sensitivity of the PIDD S588A mutant to the parent lines. We found that the E1A+/PIDD-S588A mutant exhibits significantly less cell death than the E1A+/PIDD-competent line (Figure 14), indicating that the inability to process to the PIDD-CC form makes the mutant cell line less sensitive to NO challenge than the E1A line with endogenous PIDD. Thus, PIDD processing to the −CC fragment is also required for E1A-induced sensitivity to NO.

The PIDD-processing mutant was also significantly less sensitive to the effects of etoposide poisoning (Figure 15). The mutant exhibited an intermediate phenotype between the 3T3 and E1A+ cell lines in response to etoposide. As with the PIDD knockdowns, this could be due to the difference in cytotoxic damage caused by NO and etoposide. Regardless, the data indicate that PIDD processing to the −CC fragment is required for the full sensitization effect of E1A due to cytotoxic stress.
Figure 14. Cell death in PIDD-S588A mutant and E1A+ cells after NO treatment. Cells were treated with 250 µM NO generator DETA-NONOate for 18 hours overnight. Cell death was assessed by MTS assay and expressed as a percent of cell death of untreated cells. n=3, mean±SEM.

Figure 15. Cell death in PIDD-S588A mutant and E1A+ cells after etoposide treatment. Cells were exposed to 10 µM Etoposide for 18 hours overnight. Cell death was assessed by MTS assay and expressed as a percent of cell death of untreated cells. n=3, mean±SEM.
Mitochondrial Membrane Potential of PIDD knockdown and mutant lines

In unpublished data, our group has confirmed that E1A influence on caspase-2 activation in response to cytotoxic injury is upstream of mitochondrial injury and loss of membrane potential. To determine if this effect on MMP is dependent on PIDD, these new cell lines were assessed for loss of MMP in response to cytotoxic stress by staining with DilC1(5), a dye that accumulates within healthy mitochondria and is lost as the mitochondrial membrane is injured.

In response to NO treatment, E1A-positive cells lose more MMP than their E1A-negative parent line (Figure 16; gray histograms represent untreated cells, empty histograms indicate NO-treated cells). The 3T3 parent line even seems to increase in MMP, possibly as a result of cell survival mechanisms intended to save the cell. Markedly different from the response of the E1A+ line is the E1A+/PIDD-knockdown response, which does not show a change of MMP in response to NO. Thus, the decrease in PIDD expression in these cells seems to decrease E1A-induced mitochondrial injury. The E1A+/PIDD-processing mutant loses MMP in response to treatment, but to a lesser extent than can be observed for the E1A+ cells. The inability to process to the PIDD-CC fragment seems to decrease the effect of E1A on cell mitochondrial sensitivity. Figure 17 represents three repeated experiments and shows the average MMP of NO-treated cells as a percentage of untreated cells. The E1A+ cell line has significantly lower MMP after treatment than either the E1A+/PIDD-kd line or the E1A+/PIDD-mutant line. Thus, PIDD expression and processing are necessary for E1A-induced loss of MMP as a result of NO cytotoxicity.
Figure 16. Loss of MMP in PIDD-knockdown and PIDD-mutant lines after NO treatment. Cell lines were treated with 250 µM of DETA-NONOate for 18 hours and stained with DilC<sub>1</sub>(5), then analyzed by flow cytometry to determine the degree of mitochondrial injury as a result of cytotoxicity. Filled histograms represent untreated cells and empty histograms are the NO-treated cells.
Figure 17. **Quantification of MMP loss for PIDD-knockdown and PIDD-mutant lines.** Three DilC1(5) staining experiments from figure 7 were averaged for each cell line. Loss of MMP of treated cells was expressed as a percent of MMP of untreated cells. n=3, mean±SEM.

![Graph showing MMP loss for different cell lines](image)

E1A+ cells show reduced MMP as a response to etoposide, compared to the E1A-negative parent lines. Both the E1A+/PIDD-kd and E1A+/PIDD-mutant lines exhibit either no loss or reduced loss of MMP compared to the E1A+ line after etoposide treatment. Thus, the E1A-induced mitochondrial sensitivity after either NO or etoposide cytotoxic stress seems to be due to PIDD expression. It is not clear from these data that PIDD processing is required for E1A-induced sensitivity to cytotoxic stress.
Figure 18. Loss of MMP in PIDD-knockdown and PIDD-mutant lines after etoposide treatment. Cell lines were treated with 10 µM of etoposide for 18 hours and stained with DilC$_1$(5), then analyzed by flow cytometry to determine the degree of mitochondrial injury as a result of cytotoxicity. Filled histograms represent untreated cells and empty histograms are the etoposide-treated cells.

To further characterize the sensitivity of cell lines to cytotoxic stress, cells were double stained with DilC$_1$(5) and Annexin-V, an indicator of global cell death. Multiplex staining in two repetitions of this experiment clearly showed the progression of the cells towards a lower MMP in combination with a reduction in global cell injury. In figures
19a and 19b, cells in Quadrant 1 (Q1) have low Annexin-V and high DilC₅(5) counts, indicating healthy cells with high MMP. Those cells in Q3 have lost mitochondrial staining and gained Annexin-V staining, indicating apoptosis.

In Figure 19a, each of the cell lines has roughly equivalent values across all four quadrants in the untreated graphs, with very few cells indicating apoptosis in Q3. Upon the addition of NO, the 3T3 parent line does not transition towards an apoptotic phenotype, while roughly 40% of the E1A+ cells become apoptotic. The E1A+/PIDD-knockdown line (E1A-iPIDD) also transitions to apoptosis at about 17%, but clearly less than the E1A+ cells, indicating less apoptotic sensitivity to cytotoxic stress as a result of a lack of PIDD. We may have observed even starker differences between the PIDD-kd and E1A+ lines if the knockdown effect had been more complete.

The plot for the E1A+/PIDD-processing mutant (E1A-mtPIDD) in figure 19a shows less of a transition to apoptosis after NO treatment than that of the E1A+ line, however the proportions are very close (30% versus 39% apoptotic cells). Thus, the mutant-processing PIDD does not seem to prevent the cells from experiencing some transition to apoptosis as obviously as can be observed by the PIDD knockdown.

The difficulty with this multiplex staining assay is demonstrated in figure 19b, in which the protocol remained the same but the results showed increased progression to the apoptotic quadrant for the E1A+, iPIDD, and mtPIDD lines after NO treatment. For example, the Q3 population of the iPIDD line is only 17% in figure 19a, but 27% in 19b. The stability of the cells may be affected by the successive staining with DilC₅(5) followed by Annexin V, which call for different buffer solutions and extended staining
periods; thus, the assay is fastidious and inconsistent. However, while the lines were less stable in 19b than in 19a, both experiments indicate that the iPIDD line is less sensitive to NO treatment compared to the E1A+ line, suggesting that PIDD expression is necessary for E1A-induced sensitivity to apoptosis in response to NO treatment. The results for the mutant line in these experiments do not clearly indicate that PIDD processing is required for E1A-induced sensitivity to apoptosis.
Figure 19a. Multiplex MMP and apoptosis staining of PIDD-knockdown and PIDD-mutant cell lines. Cells were treated with DETA-NONOate at 250 µM for 18 hours, then stained with DilC₁(5) and PE-Annexin V. Cells were analyzed by flow cytometry to visualize relative MMP and global cell apoptotic character. Quadrant 1 (Q1) represents high MMP and low phosphatidylserine staining of healthy cells, while Quadrant 3 (Q3) represents low MMP and high PS staining of cells undergoing apoptosis. Figures 19a and 19b (below) represent two repetitions of this experiment.
Figure 19b. Repeat of multiplex staining of knockdown and mutant lines. Methods for this experiment were identical as described for figure 19a.
CHAPTER IV
DISCUSSION

The experiments presented in this thesis have attempted to determine if there is a requirement for PIDD protein and terminal PIDD processing in E1A-induced sensitivity to certain cytotoxic stressors. Previous research indicated that E1A-induced sensitivity to NO was caspase-2 dependent, and unpublished data out of the Cook lab indicate that the caspase-2 activation is upstream of mitochondrial injury and likely proceeds via the PIDDosome. Using two E1A+ cell lines with a partial knockdown of PIDD protein, I determined that PIDD deficiency had verifiable effects on the sensitivity of E1A-expressing cells. PIDD-knockdown cells exhibited less cell death in response to cytotoxic stressors than E1A+/PIDD+ cells, as measured by a metabolic activity assay (Figures 12 and 13). PIDD-KD cells also lost less mitochondrial membrane potential, and exhibited less of a global apoptotic phenotype as a result of NO and etoposide treatment (Figures 16-19). Thus, PIDD expression is required for E1A-induced sensitivity to cytotoxic stresses. Unfortunately, I was only able to create partial knockdowns of PIDD protein in these lines, which limits the conclusions that can be drawn from these studies in regards to the exclusive requirement of PIDD in E1A-induced apoptotic sensitivity; that is, I cannot rule out that other PIDD-independent mechanisms may be involved in producing the complete sensitization effect.
PIDD-knockdown lines were consistently less sensitive than the E1A+/PIDD+ lines; however, in some cases, the knockdowns did not display as much resistance to cytotoxic stressors as the E1A-negative parent line 3T3: both knockdowns were significantly more sensitive to Etoposide than the 3T3 line (Figure 13), and iPIDD-2 was also more sensitive to NO than the 3T3 line (Figure 12). There are several possibilities for why our knockdowns do not completely eliminate the E1A-induced sensitivity in response to cytotoxic stresses: 1) The incomplete knockdown of PIDD in our lines means that some of the protein is able to promote caspase-2 activation and subsequent apoptosis. 2) As mentioned above, it is possible that E1A regulates some alternative pathway that leads to caspase-2 activation and subsequent mitochondrial injury. 3) The intermediate sensitivity of the knockdown lines compared to the E1A+ and E1A– lines is obvious when etoposide is the stressor. As etoposide and NO have different mechanisms of DNA damage, E1A may not be working through PIDD activity and caspase-2 activation to induce sensitivity in response to etoposide; another mechanism may be involved instead. Beyond these possibilities, these experiments confirm that the presence of PIDD protein is required for the complete sensitization that cells experience by E1A expression. It seems important to repeat these experiments with complete PIDD knockdowns or in a cell line with deleted PIDD gene, to determine whether sensitivity can be reduced to levels approximating the E1A-negative parent lines.

We also examined whether a dependence on PIDD expression meant that the mechanism for E1A-induced sensitivity required PIDD-processing to the terminal PIDD-CC fragment and, thus, activation of the PIDDosome towards caspase-2 activation. The
E1A+/PIDD-S588A processing mutants were less sensitive to cytotoxic stress than the E1A+/PIDD+ cell line. Cell death as measured by metabolic activity was significantly reduced in the PIIDD-S588A line as compared to the E1A+ line for both NO and etoposide treatment, although it was also significantly greater than the E1A-negative 3T3 parent line in the case of etoposide (Figures 14 and 15). Quantification of MMP loss in response to NO indicated that the mutant did have significantly more retention of MMP in response to NO treatment (Figure 17). That the PIIDD processing mutant had intermediate phenotype between the E1A+ and E1A-negative lines could point to at least two different possibilities: 1) PIIDD is acting independently of PIIDD processing and the PIIDDosome to activate caspase-2 or induce mitochondrial injury via the NF-κB pathway, or 2) that the level of exogenous expression of the PIIDD S588A mutant is not enough to overcome the effect of endogenous wild-type PIIDD and PIIDD-CC in formation of functional PIIDDosomes. Further studies may be performed with either stronger PIIDD S588A mutant expression or by inhibiting the cleavage of endogenous PIIDD to the PIIDD-CC form. Also, inhibition of PIIDDosome formation with knockdowns or mutants of the RAIDD adaptor protein could reveal the importance of the functional PIIDDosome on E1A-induced sensitivity.

For this study we also attempted to generate PIIDD knockdowns of human fibroblast cell lines: a HT1080 parent line (H4), and its derivative expressing E1A. Anti-human PIIDD antibodies seem to have several non-specific interactions, making it difficult to identify the full-length PIIDD and processed forms. Alternatively, it is possible that several isoforms of PIIDD may be present in this fibroblast cell line, and that
the interactions reflect the processing of diverse PIDD isoforms. Regardless, we could not observe knockdowns of any band predicted to be the PIDD full-length or processed fragments in western blots. Previous studies have confirmed the existence of at least 3 PIDD isoforms expressed differentially across many human cell lines\(^5\). As opposed to our mouse cell lines—where there is not yet evidence for more than one PIDD isoform—these human isoforms may function in opposing fashions and complicate the interpretation of studies examining PIDD function. Studies attempting to determine the effect of PIDD on E1A-induced sensitivity in humans may require consideration of the activity of a whole suite of PIDD proteins, as opposed to a seemingly simpler homologous system in mice. Such studies may involve the overexpression of individual PIDD isoforms in E1A-expressing cells to determine their effect on E1A-induced sensitivity.

Immediate experiments following this study should confirm and correlate the processing of Caspase-2 in PIDD-knockdown or mutant cells to E1A-induced sensitivity. Western blots of Caspase-2 processing could confirm activation of the caspase in PIDD-knockdown cells in response to NO and etoposide-induced stress. Flow cytometry experiments with staining for active caspase-2 in combination with indicators of MMP will provide visual evidence of whether cells that lose MMP also exhibit caspase-2 activation, and whether that condition is dependent on PIDD expression or processing.

Further studies should attempt to elucidate potential mechanisms of E1A-induced caspase-2 activation and cytotoxic sensitivity. We have provided evidence that PIDD expression is required for E1A-induced sensitivity to cytotoxic stress: how might E1A be
directly or indirectly influencing PIDD or the PIDDosome? One possibility is that E1A drives efficient autoproteolysis of PIDD to the PIDD-CC form, leading to formation of the PIDDosome and caspase-2 activation. It will be important to determine whether E1A interacts with potential factors involved in PIDD processing. PIDD is auto-proteolyzed to the PIDD-C and subsequently PIDD-CC form, but the process at either cleavage site may be constitutively repressed by unknown factors. E1A may be interacting with and inhibiting factors involved in repression of PIDD-CC cleavage, or indirectly driving processes that inhibit such cleavage repressors. This may have a compound effect, as efficient cleavage to the PIDD-CC site not only contributes to PIDDosome formation and caspase-2 activation, but also depletes the pool of PIDD-C fragments required for NF-κB activation, as described in the introduction. To understand the potential interactions and pathways by which E1A is involved in PIDD processing, we will need to clarify the endogenous mechanisms involved in regulation of PIDD.

Ultimately these studies will lead to greater understanding of the many mechanisms utilized by the E1A adenoviral protein to sensitize cells to a variety of apoptotic insults. Hopefully, this knowledge will contribute to improvement of the arsenal of E1A-based cancer therapeutics by directing more targeted and efficient activity of the viral protein on endogenous PIDD.
REFERENCE LIST


VITA

Iris Figueroa is a native of Chicago, Illinois and graduated from the University of Chicago with a Bachelor of Arts in Biological Sciences in 2007. She has worked in the laboratories of Dr. Stuart Johnson and Dr. Dale Gerding at Edward Hines, Jr. VA Hospital since 2008, identifying strains and researching epidemics of Clostridium difficile outbreaks from North America and Europe. As a graduate student in Dr. James Cook’s laboratory at Loyola University Chicago, Iris studied the influence of Adenovirus proteins on mechanisms of programmed cell death.