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ADENOVIRUS E1A GENE-INDUCED TUMOR REJECTION THROUGH CELLULAR SENSITIZATION TO IMMUNE AND NONIMMUNE APOPTOTIC INJURIES

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1. ABSTRACT

The E1A gene of human adenovirus (Ad) serotypes 2 and 5 induces susceptibility of cells from several species, including human, to lysis by natural killer cells, activated macrophages and a variety of other immunologic and nonimmune cellular injuries. This E1A activity is the rationale behind some treatment strategies using combined adenoviral vector infection and chemotherapy for cancer. This review will consider the evolution of the studies that have resulted in the current understanding of the cellular mechanisms of E1A-induced

tumor cell cytolytic susceptibility and sensitization to apoptotic injury. The translation of in vitro observations to experimental models testing E1A-induced tumor rejection in the context of the cellular immune response and E1A-induced sensitization of human tumor cells to therapeutic injuries will be discussed. Review of available information on the molecular mechanisms of E1A-induced cellular sensitivity to immune and nonimmune injuries will be used as a basis for consideration of possible future directions of this research.

2. INTRODUCTION

Adenovirus (Ad) vectors expressing the E1 region (E1A and E1B genes) of the viral genome are being used in cancer therapy protocols (1-4). The main concepts behind this are the goals of using the adenoviral E1A oncoprotein to directly induce apoptosis in tumor cells or to sensitize tumor cells to subsequent therapeutic injury. The premise behind the second strategy is based upon the evolving information about E1A-induced sensitivity of cells from several species to a variety of immunologic and nonimmunologic injuries. The purpose of this review is to consider the evolution of the concept of E1A-induced cellular susceptibility to injuries mediated by components of the host cellular immune response ("cytolytic susceptibility") and E1A-induced "sensitization to apoptotic injury." E1A expression has multiple effects on neoplastic cells that might reduce their tumor forming capacity. Some of these involve modulation of growth factor receptors, cellular interactions with extracellular matrix and other effects that may have marginal or indirect implications for E1A-induced cytolytic susceptibility and sensitization to apoptotic injury. These other E1A activities will only be mentioned as they relate to the primary theme. The goal of this review is to provide perspective about possible E1A mechanisms of action that may be useful in studies to define ways to improve the utility of this viral oncoprotein in viral vector therapy of cancer.

This review is divided into five major sections. The first considers the evidence for the association between E1A expression in neoplastic cells and their reduced tumorigenicity in the context of the host cellular immune response. The second considers the concept of E1A-induced cytolytic susceptibility as related to E1A-induced reduction in the tumorigenicity of neoplastic cells. The third considers the cytolytic mechanisms to which E1A oncoprotein expression sensitizes tumor cells. The fourth reviews the molecular mechanisms through which E1A converts cells from the cytolytic resistant to the cytolytic susceptible phenotype. The fifth reviews the translation of in vitro observations related to E1A-induced cytolytic susceptibility and sensitivity to therapeutic injuries that have been done using rodent cells to studies of E1A effects on human tumor cells in vitro and in tumorigenicity assays. These sections are followed by a summary of the concepts described and a consideration of possible future directions of this E1A-related research for clinical application.

3. REVIEW

3.1. Association between E1A oncogene expression in neoplastic cells and their reduced tumorigenicity in the context of the cell-mediated immune response of the host

3.1.1. Maturation of the cellular immune response related to Ad-transformed cell rejection

The first classification of Ad serotypes was based upon their ability to induce tumors in newborn hamsters (5). Group C, Ad serotypes 2 and 5 (Ad2, Ad5) were nononcogenic, whereas Group A, Ad12 was highly oncogenic. Studies comparing the tumorigenicity of group A and group C Ad-transformed cells revealed that their tumor inducing capacities reflected the tumorigenicity of the virus that had been used for cell transformation. Ad12-transformed cells were tumorigenic in immunocompetent rodents, but Ad 2-transformed cells were tumorigenic only in immunosuppressed or immunologically immature newborn rodents (6, 7). Because the transformation efficiency of group A and group C Ad were equivalent, other factors were sought to explain their different tumorigenicities.

Studies from several laboratories demonstrated the importance of the cellular immune response in defending rodents against tumor challenge. Age-related development of tumor resistance to Ad2-transformed cells paralleled the maturation of cellular immunity in rodents, and they could be rendered tumor-susceptible by thymectomy or lymphocyte depletion (6, 8, 9) Histopathological studies of tumors done over time after tumor challenge also demonstrated the association between tumor infiltration with lymphoid and histiocytic (macrophage-like) cells and tumor rejection (10).

3.1.2. The role of innate immunity in the rejection of E1A-expressing tumor cells

Antineoplastic cellular immune defenses can be divided into innate immunity, mediated primarily by natural killer (NK) cells and activated macrophages, and adaptive (or specific) immunity, mediated by tumor antigen-specific, cytotoxic T lymphocytes (CTL). One question that was addressed early in the course of studies of Ad-transformed cell rejection was whether CTL responses to virus-specific antigens were the key mediators of primary tumor rejection (i.e., tumor development in a nonimmunized animal). This hypothesis would predict that cells transformed by highly oncogenic Ad12 would be weakly immunogenic, whereas cells transformed by nononcogenic Ad2 would be highly immunogenic. Direct comparisons of virus-specific immunity induced by immunization with irradiated, DNA virus-transformed cells showed that cells transformed by both Ad serotypes were highly immunogenic and could induce virus-specific, protective immunity against tumor challenge (11). These results, along with the histopathological studies showing the importance of early appearing mononuclear inflammatory cells for tumor rejection, suggested that innate cellular immunity was the key host defense for primary rejection of Ad-transformed cells.

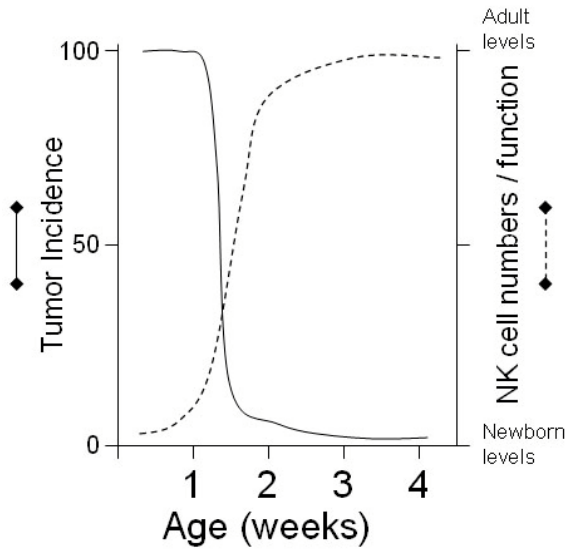

Figure 1. Graphic representation of the reciprocal relationship between susceptibility to development of tumors in nude rats of different ages following challenge with E1A-positive BHK-21 sarcoma cells (solid line) vs age-related maturation of the NK cell response (dashed line). Treatment with NK cell-depleting antibody renders 4-week-old nude rats susceptible to E1A-positive tumor challenge (92).

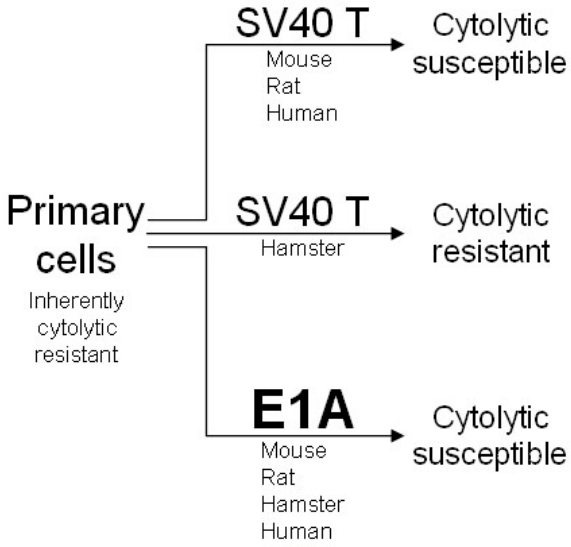

Figure 2. Relationship between expression of DNA virus tumor antigens during neoplastic transformation of primary cells from different species and the cytolytic susceptible or cytolytic resistant phenotypes of the tumor antigen-expressing, transformed cells. The studies of SV40-transformed cells from different species were done with activated macrophages. The studies of E1A-expressing cells from different species were done with NK cells (cells from all four species) and with activated macrophages (mouse, hamster and human cells).

Further studies of the manipulation and maturation of immunological responses supported the importance of innate immunity for Ad-transformed cell rejection. Among the most informative of these studies were those involving athymic (nude) mice and rats incapable of mounting CTL defenses. It was observed that nude mice were more susceptible to challenge with Ad 2-transformed cells, and with cells transformed by other DNA viruses, than were nude rats (12). Initial tumor challenge studies were done with DNA virus-transformed hamster cells. Comparative studies of the cytolytic activities of NK cells from these two types of nude rodents revealed a possible explanation for their different susceptibilities to tumor challenge. Nude rats had greater NK activity than nude mice, and mouse NK cells were defective for killing Ad 2-transformed hamster and rat cells (12). Immunologically immature, newborn nude rats were more susceptible to challenge with hamsters sarcoma cells expressing E1A oncoproteins, and the age-related increase in tumor challenge resistance was correlated with maturation of their NK cell responses (12) (Figure 1). Furthermore, NK cell depletion from immunologically mature nude rats rendered them susceptible to tumor challenge with E1A-positive cells (9, 13). These studies indicated the importance of the NK cell defenses of the host at the time of tumor challenge for rejection of Ad 2-transformed cells and tumor cells expressing Ad2/5 E1A oncoproteins.

3.2. The concept of E1A-induced cytolytic susceptibility related to E1A-induced changes in the tumorigenic phenotype of neoplastic cells

3.2.1. DNA virus tumor antigens and cytolytic susceptibility

A question that arose during studies of the immune-related tumorigenicity of Ad-transformed cells was how to study the cellular phenotype of increased susceptibility of Ad transformed cells (or cells expressing Ad early genes) to destruction by host NK cells, activated macrophages and other antitumor defenses. One of the earliest observations about the differential susceptibility to host killer cells of neoplastic cells expressing DNA virus tumor antigens involved studies of SV40-transformed mouse cells and activated macrophages. John Hibbs first reported that SV40-transformed mouse cells exhibited increased susceptibility to killing by activated macrophages, compared with SV40-negative control cells (14). Based upon this observation, a model was developed to test the relative susceptibility of other DNA virus-transformed cells to the cytolytic effects of activated macrophages. The results provided the first evidence that differences in the capacity of activated macrophages to kill virus-transformed cells contribute to the species-related differences in the tumorigenicity of SV40-transformed cells (15). Thus, nontumorigenic, SV40-transformed mouse and rat cells were susceptible to macrophage-induced killing, whereas highly tumorigenic, SV40-transformed hamster cells were resistant (Figure 2). This concept was expanded to studies of Ad-transformed cells and to comparative studies of the cytolytic effects of activated macrophages and NK cells (16). The results extended the correlation

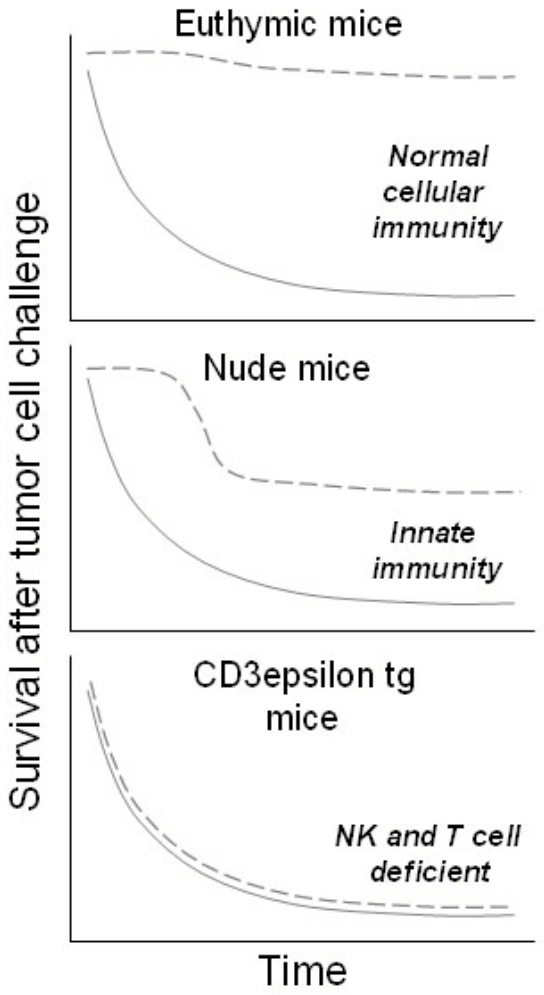

Figure 3. Graphic representation of the tumorigenicity of E1A-positive (dashed lines) versus E1A-negative (solid lines) mouse sarcoma cells in three different types of syngeneic (C57/BL6) mouse tumor challenge recipients (29). Tumor development is represented as relative survival (tumor-free status) with time after tumor cell challenge.

found using SV40-transformed cells. Nontumorigenic, Ad2/5-transformed cells were highly susceptible to killing by NK cells and activated macrophages, whereas cells transformed by highly oncogenic DNA tumor viruses were cytolytic resistant (11). This correlation between the resistance or susceptibility of cells transformed by different DNA tumor viruses to lysis by NK cells and activated macrophages (cytolytic susceptibility) *in vitro* and their respective tumorigenicities *in vivo* was reproduced in different laboratories (17-19).

3.2.2. E1A oncogene-induced cytolytic susceptibility to innate immune-effector cells

Next a series of studies established the role of the Ad E1A oncogene in actively inducing the cytolytic susceptible phenotype. First, it was shown that expression of Ad-early genes during transformation of primary cells induced cytolytic susceptibility (18-20). Additionally, expression of Ad early genes in highly oncogenic, SV40-transformed hamster cells also induced cytolytic susceptibility *in vitro* and eliminated the tumorigenicity of cells co-expressing both tumor antigens *in vivo* (21). Expression of Ad early genes during viral infection also induced conversion of cells from the cytolytic resistant to the cytolytic susceptible phenotype (20). Ad infection-induced cytolytic susceptibility was observed with both NK cells and activated macrophages and was greater with increasing multiplicity of infection and associated increases in viral early gene expression. This relationship between the level of Ad early gene product expression and the induction of cytolytic susceptibility was confirmed in studies of Ad-transformed cells (22). Subsequent studies established that the E1A oncoprotein and no other Ad early gene product was responsible for inducing cytolytic susceptibility (23-26). Other studies indicated that expression of all or part of the E1A second exon, in addition to the E1A first exon, is required for induction of cellular susceptibility to NK killing (23, 25, 27). These studies laid the groundwork for the evaluation of the mechanisms by which E1A expression induced conversion of cells to the cytolytic susceptible phenotype. Two nonexclusive, general mechanisms were proposed: (1) E1A-induced alterations in the neoplastic cell surface that could trigger the cytolytic activity of killer cells and (2) E1A-induced "physiological changes" in cells that would render them more susceptible to the cytolytic mechanisms of killer cells (23). Although both mechanisms are likely to be involved, most data have been developed regarding the second hypothesis.

3.2.3. E1A oncogene-induced reduction of tumorigenicity

The observation that E1A oncogene expression was necessary and sufficient for induction of cytolytic susceptibility was used to develop correlative studies of the ability of E1A to convert highly tumorigenic cells into cells that could be rejected by immunocompetent hosts. Stable E1A expression after transfection of highly tumorigenic sarcoma cells (BHK-21) induced cytolytic susceptibility and eliminated sarcoma cell tumorigenicity in a manner that depended on the competence of the host NK cell response to kill E1A-positive cells (28). These studies also demonstrated that activated macrophages from athymic animals could kill E1A-positive cells and therefore might play a complementary role with NK cells in the innate immune defense against E1A-positive tumors.

The relationship between the immunocompetence of the host and the ability to reject E1A-expressing tumor cells was subsequently confirmed. To avoid potential problems in interpretation resulting from cross-species tumor challenges (e.g., hamster tumor cells inoculated into nude mice or rats), a mouse tumor model was developed to test the interactions between host cellular immune defenses and E1A-expressing sarcoma cells in a single species (29) (Figure 3). Tumor induction experiments were done using a quantitative method that allowed independent measurement of differences in tumor latency and tumor inducing efficiency (30). Adult, immunocompetent mice were highly susceptible to tumor formation by E1A-

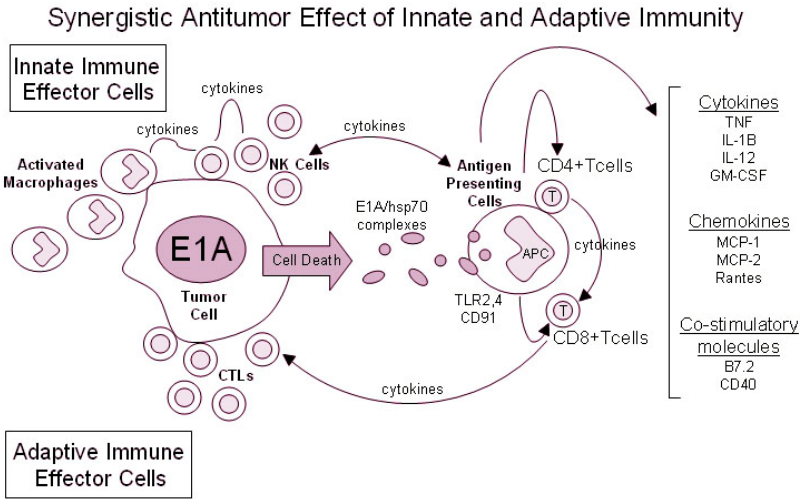

Figure 4. Representation of the hypothetical synergistic antitumor effect of innate and adaptive immunity against E1A-expressing tumor cells. It is postulated that innate immune effector cells (activated macrophages and NK cells) play multiple roles in tumor cell rejection. They kill cytolytic susceptible, E1A-positive tumor cells. By killing the cells, they enhance tumor antigen presentation to antigen presenting cells (APC), thereby triggering a tumor-specific, adaptive immune response. NK cells participate in reciprocal stimulatory interactions with APC through contact and cytokine-mediated (IL-12 and IL-18) signals. The innate immune effector cells also elaborate proinflammatory cytokines during their activation. APC and subsequent T-cell (CD4 + and CD8 +) activation results in a cascade of stimulatory effects through cytokines, chemokines and co-stimulatory molecules. Late appearing CTL further enhance this anti-inflammatory effector cell loop by continuing tumor cell killing.

negative, syngeneic sarcoma cells but were almost completely resistant to challenge with E1A-positive sarcoma cells. Nude mice (NK cell competent for E1A-expressing mouse cells, but T-cell deficient) exhibited some resistance to E1A-positive cells, but less than euthymic mice. CD3 epsilon transgenic mice (deficient in both NK cells and T cells) (31) were highly susceptible to tumor formation and could not discriminate between E1A-positive and E1A-negative tumor cells. This confirmation of the importance of the host NK cell response for rejection of E1A-positive tumor cells was reinforced by studies of NK cell depletion, which increased host susceptibility to E1A-positive tumors. The observation that euthymic mice were more resistant to E1A-positive sarcoma cell challenge than nude mice indicated that T-cell-dependent immune responses complement innate immune defenses during primary rejection of E1A-positive cells.

3.2.4. E1A oncoprotein effects on adaptive (E1A-specific) cellular immune defenses

E1A is an endogenously expressed viral protein. Consequently, E1A is a source of antigenic peptides that are presented on class I MHC molecules leading to the recognition of E1A-expressing cells by E1A-specific CTL. Immunization studies in which animals were primed by Ad infection or immunization with E1A-expressing tumor cells indicated that E1A was highly immunogenic and efficiently induced CTL responses (32-34). Adoptive transfer of E1A-specific CTL also eradicated E1A-positive tumors in mice (35). Generation of E1A-specific CTL following Ad-infection is MHC specific, so that in some cases E1A was not the dominant epitope following viral infection in different inbred strains of mice (36). Thus, in a manner similar to the generation of antigen-specific CTL following other viral infections, the efficiency of generating of E1A-specific CTL can be influenced by the major histocompatibility antigens expressed by the individual. Unlike highly inbred strains of mice, however, humans express a variety of highly polymorphic MHC class I antigens, Therefore, the likelihood that an individual would lack an MHC class I molecule unable to bind at least one E1A antigenic peptide would be predicted to be low.

The ability of E1A to interact with and increase the expression of heat shock proteins (such as hsp70) (37-39), may further enhance the immunogenicity of E1A-expressing tumor cells. Hsp70 binds endogenously expressed, antigenic peptides (40). Hsp70 is also a ligand for CD91 and the toll-like receptors, TLR2 and TLR4, that are highly expressed on antigen presenting cells (APC) (41, 42). Therefore, release of hsp70-E1A complexes following tumor cell killing by NK cells and activated macrophages could result in efficient delivery of E1A to APC for CTL activation. Conversely, hsp70 can also protect tumor cells against a variety of apoptosis inducing stimuli, including lysis by activated macrophages (42, 43). However, it has been shown that E1A induces cytolytic susceptibility, despite hsp70 overexpression (43). These observations are consistent with the concept that the ability of E1A to simultaneously induce cytolytic susceptibility and upregulate hsp70 expression could result in synergistic antitumor interactions between innate and adaptive immune responses.

3.2.5. Integrated model of the synergistic antitumor effect of innate and adaptive immunity

These data on the interactions between the host cellular immune response and E1A-expressing tumor cells suggest a model of synergistic antineoplastic effects of innate and adaptive immunity (Figure 4). The studies

linking the ontogeny of the NK cell response to host resistance to challenge with E1A-positive tumor cells and the mouse studies of E1A-related tumor rejection by euthymic vs nude or CD3 epsilon transgenic mice indicate the combined importance of NK cell defenses and T-cell-dependent cellular immune responses for optimal tumor rejection (Figure 3). Early studies in this series showed that irradiated tumor cells transformed by both nononcogenic and highly oncogenic DNA tumor viruses were equally able to protect animals from tumor challenge in a virus-specific manner (11). Those data were interpreted initially as showing the importance of innate immunity but the lack of importance of CTL-dependent adaptive immunity in the differential rejection of a primary challenge with DNA virus-transformed cells. We now propose a new interpretation of these data, in light of the demonstrated importance of both NK cell- and T-cell-dependent host antitumor defenses and new information about the coordinating and activating role of antigen presenting cells (APC) in both NK cell and T-cell responses (44).

We propose the hypothesis that innate immune effector cells play a critical dual role in the early defense against E1A-expressing tumor cells (Figure 4). First, NK cells and activated macrophages would kill cytolytic susceptible, E1A-positive tumor target cells and would elaborate proinflammatory cytokines during activation. The killer cell-induced cell death, combined with the aforementioned interaction between E1A and hsp70, would increase delivery of E1A (and other tumor antigens) to APC. Hsp70 interaction with APC would also induce production of a cascade of cytokines and chemokines along with the expression of co-stimulatory molecules (Figure 4). This amplification loop initiated by innate immune effector cells would augment T-cell-dependent, antigen-specific (and other tumor antigen-specific) CTL responses directed against E1A-expressing tumor cells (and possibly also against E1A-negative tumor cells expressing other tumor antigens whose recognition could be increased). NK cell-induced APC activation would also result in a reciprocal APC-induced NK cell amplification and possibly macrophage activation response that would further increase the antitumor effect of innate immune effector cells. These multiple, stimulatory loops would continue, as long as E1A-positive tumor cells were available to serve as targets for destruction by NK cells and activated macrophages and as a source for tumor-specific antigen presentation to APC. The later appearing, E1A-specific (and other tumor antigen-specific) T cell activation and CTL killer responses would also contribute to this antitumor stimulatory loop mechanism through cytokine amplification and apoptotic tumor cell destruction. This hypothetical model is consistent with available information from E1A tumor systems but will require further experimental analysis to determine whether the postulated cell-cell interactions and mediator-induced regulatory cascades apply to E1A-positive tumor cell interactions with these components of the host cellular immune defense.

3.3. Cytolytic mechanisms to which E1A sensitizes tumor cells

To understand the E1A-induced mechanisms that result in increased tumor cell cytolytic susceptibility, it was necessary to consider both the types of injuries that are selectively active in the lysis of E1A-positive cells and the E1A-induced target cell changes that render the cells more susceptible to these injuries. One question is What are the cytolytic mechanisms used by host antitumor, cellular immune defenses to destroy E1A-positive cells? Activated macrophages and cytolytic lymphocytes, such as NK cells and CTL, have multiple cytotoxic mechanisms that can be used independently or more likely in collaboration to kill susceptible tumor target cells. Some of these cytotoxic mechanisms are unique to cytolytic lymphocytes, including those involved in degranulation-dependent killing (perforin and granzymes) (45). Depending upon the cell systems tested, other types of cytotoxic mechanisms may be used to a variable extent by both cytolytic lymphocytes and activated macrophages, including Fas ligand, TNF related apoptosis-inducing ligand (TRAIL), TNF alpha, nitric oxide, and reactive oxygen species (46-58). E1A expression has been observed to sensitize different types of cells to several different cytotoxic mechanisms of both cytolytic lymphocytes and activated macrophages.

3.3.1. E1A-induced sensitization of target cells to both degranulation-dependent and degranulation-independent killing by cytolytic lymphocytes at a "post-recognition" stage in the interaction

When matched E1A-positive and E1A-negative rodent cells were tested for susceptibility to lysis by different types of cytolytic lymphocytes, the E1A positive member of the pair always exhibited greater cytolytic susceptibility (59). This preferential killing of E1A-positive cells was observed irrespective of the type of recognition mechanism involved in cytolytic lymphocyte interaction with the target cell. For example, allospecific (MHC class I antigen-specific) rat CTL directed against different pairs of E1A-positive and E1A-negative cells, with comparable levels of cell surface MHC class I antigens, preferentially killed E1A-positive cells in each case (59). The same E1A-specific target cell killing was observed using xenogeneic CTL that could only recognize target cells in the presence of lectin. That killer cell system minimized the possibility that E1A was controlling target cell recognition, because the killer cell interaction with the target cell was created artificially by the "lectin glue" phenomenon. These observations suggested that some E1A mechanisms of sensitizing target cells to killer cell injury are unrelated to E1A-induced changes in cell surface targeting structures.

Cytolytic lymphocytes use two main mechanisms to kill targeted cells (Figure 5): (1) the collaborative cytotoxic interaction between perforin and granzymes that requires calcium-dependent killer cell degranulation during target cell binding and (2) calcium-independent killing by the triggering interaction between Fas ligand expressed on killer cell surfaces and Fas antigen expressed on target cells (45, 60). E1A expression sensitizes target cells to both of these cytolytic mechanisms when tested separately, even when target cells express comparable levels of cell surface Fas antigen (59). It also has been reported that E1A-positive, but not E1A-negative, cells are sensitive to killing when directly exposed to cytotoxic granules obtained from

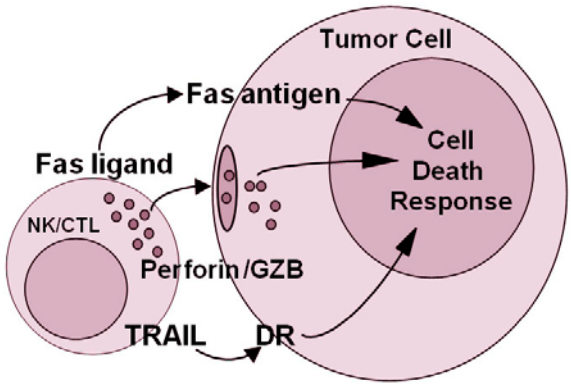

Figure 5. Graphic depiction of the main cytolytic mechanisms of killer lymphocytes. These mechanisms include calcium-dependent degranulation and killing through the collaborative interaction between perforin and granzymes (e.g., granzyme B, GZB) and calcium-independent, degranulation-independent killing through the interaction between Fas ligand and TRAIL on cytolytic lymphocyte surfaces and cognate death receptors (Fas antigen and TRAIL-Death Receptor, respectively) on target cell surfaces. All three cytolytic mechanisms trigger apoptotic cell death in sensitive tumor target cells. Other reported cytolytic mechanisms not shown include nitric oxide and TNF alpha.

NK cells (61). These observations further support the conclusion that the mechanism of E1A-induced cytolytic susceptibility is unrelated to E1A-mediated changes in tumor cell surfaces but instead is induced by one or more E1A-related changes in the cellular response to different injuries.

3.3.2. E1A oncoprotein expression-level-dependence of cytolytic susceptibility

E1A-induced cytolytic susceptibility of target cells in both mouse and hamster NK cell systems and in the aforementioned lectin-dependent CTL assays required high-level E1A oncoprotein expression (26, 62). Cells expressing low E1A levels remained cytolytic resistant. The inability of low-level E1A oncoprotein expression to induce cytolytic susceptibility suggested that this E1A activity is different from others such as E1A-induced cellular immortalization and E1A-related viral and cellular transcriptional control, for which low-level oncoprotein expression is sufficient (63-67). There are other reports of E1A activation of cellular gene expression in which E1A expression level was a factor, however (38, 67, 68). Similar to E1A-induced cytolytic susceptibility, E1A-induced, NK cell-dependent rejection of hamster sarcoma cells also depends on high-level E1A oncoprotein expression (26). These results indicate that there could be a critical threshold level of E1A expression that is required to induce cytolytic susceptibility, possibly through titration of cellular activities that control the cytolytic phenotype.

3.3.3. Role of E1A-induced cellular sensitivity to TNF family ligands in cytolytic susceptibility

E1A expression in both rodent and human cells has been reported to sensitize the cells to the cytotoxic effects of recombinant TNF alpha (62, 69-73). E1A-induced cellular sensitivity to TNF can be cell type-dependent (70) and can be observed with E1A expressed during viral infection of either rodent or human cells (74, 75) and following stable E1A transfection. Like E1A-induced susceptibility to cytolytic lymphocytes, E1A-induce sensitivity to TNF is dependent upon high levels of E1A oncoprotein expression in target cells (62). TNF might mediate some of the cytolytic activity of both NK cells (9, 62) and activated macrophages (62, 76, 77), since both types of killer cells can produce this cytokine. Other cytolytic mechanisms are likely to be more important than TNF for killing of E1A positive cells by both of these killer cell types, however (62, 77). The importance of degranulation- and Fas-dependent killing by cytolytic lymphocytes has been discussed. Activated macrophages use nitric oxide as the predominant mechanism of killing E1A-positive cells (77). TNF plays a minor role, whereas Fas ligand and reactive oxygen intermediates are less important for macrophage killing. In addition to TNF and Fas ligand, E1A expression also sensitizes certain types of human tumor cells to killing by TRAIL, a third member of the TNF family (78). TRAIL is expressed on both NK cells and activated macrophages and can be used by both killers to trigger cell death in sensitive targets (53-55, 57, 79-81) (Figure 5). This E1A-induce sensitivity to apoptotic injury by TNF family ligands does not require E1A induction of the relevant receptor on the target cell surface (59). Collectively, these observations further reinforce the conclusion that there are probably multiple mechanism(s) through which E1A sensitizes cells to diverse injuries and that this E1A activity does not rely on a single mechanism of enhancement of recognition of E1A-positive cells.

3.3.4. Blockade of immune-mediated killing of E1A-positive cells by expression of the other Ad early genes

Several studies have assessed the Ad mechanisms that have evolved to block or repress immune-mediated killing of virally infected cells. These are mostly beyond the scope of this review, but consideration of the key observations provides some perspective about the limitations of E1A-induced cytolytic susceptibility and sensitization to apoptotic injury in the context of viral infection. Expression of the Ad E3 gene region can block TNF killing of E1A-sensitized mouse and human cells (82-84), as well as other forms of death receptor-mediated killing - e.g., Fas and TRAIL (78, 85). E3 expression does not prevent E1A-induced cytolytic susceptibility to NK cells or activated macrophages. Thus, rodent cells infected with wild type Ad (and coexpressing E1A and E3 genes) are susceptible to killing by both NK cells and activated macrophages (20, 25). Whether E1A-positive cells coexpressing E3 gene products are less susceptible to killing by E1A-specific CTL is controversial and may depend upon the cell systems studied (86-90). Expression of Ad E1B 19 kD protein blocks human, but not mouse, cell killing by TNF (83). However, E1B 19 kD expression does not block NK cell or activated macrophage killing of rodent or human cells (23, 25, 26, 28, 91) and does not independently affect cellular cytolytic susceptibility (26). Moreover, E1B gene expression does not prevent innate

immune response-dependent rejection of either rodent or human tumor cells (26, 28, 92).

3.3.5. E1A induced cellular sensitization to both immune-mediated and nonimmune-induced apoptotic injuries

As noted previously, several observations indicate that E1A sensitizes cells to diverse types of immunological injuries that do not share common triggering mechanisms. Most of these injuries do, however, share the ability to activate the cellular apoptotic death response. Therefore, it is possible that E1A expression sensitizes cells to diverse types of killer cells by increasing cellular sensitivity to apoptotic injury. The relationship between E1A and cellular apoptosis can generally be divided into (1) a "Direct" activity where E1A expression per se, in the context of viral infection or during attempted cellular immortalization, triggers cellular apoptosis without any further stimulus and (2) an "Indirect" activity where E1A expression that is otherwise tolerated by a cell that is selected to be resistant to the direct effect is, however, still sensitized to subsequent injuries that have the potential to trigger apoptosis.

3.3.6. "Direct" induction of apoptosis by E1A during viral infection or attempted cellular immortalization

Direct induction of apoptosis in virally infected cells as a result of E1A oncoprotein overexpression can be either p53-dependent and p53-independent, depending upon which other Ad early genes (e.g., E4) are expressed by the infecting virus (93-96). p53-triggered apoptosis by E1A expressed in virally infected cells may be partly explained by activation of the proapoptotic Bcl-2 family members, Bak and Bax (97). Direct induction of apoptosis during viral infection can be blocked by coexpression of E1B 19 kD (or Bcl-2) (94, 97-100). Direct induction of apoptosis during attempted cellular immortalization has usually been reported to be p53-dependent (101-103) and blocked by coexpression of the E1B 19 kD or 55 kD proteins (104, 105). This E1A effect is also blocked by coexpression of activated ras (106) or Rb (107).

3.3.7. "Indirect" sensitization of E1A-expressing cells to apoptotic injuries

The increased sensitivity of cells (especially tumor cells) that are forced to express E1A and are subsequently injured by some form of external stimulus is the primary focus of this review. There is evidence that cytolytic lymphocyte-induced killing of E1A-positive cells is the consequence of a cellular apoptotic death response (108). E1A-induced sensitivity to TNF-triggered apoptosis has been reviewed above. There have been studies from several laboratories indicating that E1A expression in cells from several species and tissue origins also induces "chemosensitization" - defined as an increased apoptotic response following exposure to chemotherapeutic drugs (72, 109-118). E1A-induced chemosensitization has been reported to be either p53-dependent or p53-independent, which may be related to the cell species or system tested (102, 111, 118-122). Similar cellular sensitization to apoptosis and variability of p53-dependence have been reported for radiation-induced injury of E1A-expressing cells, although it appears that p53-mutant human cells are sensitized by E1A to this injury, as well as many chemotherapeutic drugs (109, 113, 120, 123-125). Sensitization of E1A-expressing cells has also been reported with other cellular stresses, including serum withdrawal (126) or E1A repression of growth factor receptor expression (127-129) and cell removal from substrate adherence (130, 131). All of these observations do not exclude the possibility that E1A could also sensitize cells to injury-induced necrotic cell death. However, with limited testing, E1A-positive cells were no more sensitive to necrosis-inducing cellular injury that E1A-negative cells (108).

3.4. Molecular mechanisms through which E1A mediates the conversion of cells from the cytolytic resistant to the cytolytic susceptible phenotype

3.4.1. Lack of a correlation between E1A induced cytolytic susceptibility and modulation of MHC class I antigen control of NK cell cytolytic activity

NK cell recognition of target cells involves a balance between activation of "killer activating receptors" and "killer inhibitory receptors" (132, 133). Class I MHC molecules expressed on the surfaces of tumor cells stimulate inhibitory receptors, thereby blocking activating signals and NK-cell-mediated killing. In the absence of class I molecules on tumor cells, signals transduced through activating receptors are not blocked, resulting in NK cell-induced killing. This model known as the "missing self hypothesis" predicts that NK cells form a defense against target cells with deleted or reduced expression of self-MHC antigens (134). Observations from the adenovirus system have not fit well with this hypothesis. Cells transformed by highly oncogenic Ad12 (and expressing Ad12 E1A) exhibit low levels of cell surface MHC class I antigens (135-138) but are NK-resistant. Cells transformed by nononcogenic Ad2/5 (and expressing Ad2/5 E1A) exhibit variable expression of cell surface MHC class I antigens - from levels as low as Ad12-transformed cells to levels as high as nontransformed cells (138) - but are NK-sensitive. These patterns are the reverse of what would be predicted by the missing self hypothesis.

The NK cytolytic susceptibility of cells expressing Ad2/5 E1A suggested either that E1A might sensitize target cells to multiple killing mechanisms that override the inhibitory effects of MHC class I antigen expression or that E1A might block the NK-repressive effect of MHC class I molecules. The latter hypothesis has been tested. E1A-positive mouse sarcoma cells expressing an MHC inhibitory ligand, H-2D^d, for the NK cell inhibitory receptor, LY49A, were tested against an NK cell clone and NK cell subpopulations expressing LY49A (139). The expression of H-2D^d blocked NK killing of these E1A-positive cells, indicating that E1A expression does not prevent signaling of NK cell killer inhibitory receptors. The same E1A-positive sarcoma cells were killed by polyclonal NK cell populations and were rejected during tumor challenge of immunocompetent mice. These results indicate that E1A does not have to interfere with

signaling by killer inhibitory receptors to induce cytolytic susceptibility and tumor rejection.

The net effect of E1A on NK cell signaling remains unclear. A reasonable proposal, given the existing data, is that E1A expression increases expression of activating NK cell ligands on target cells, tipping the balance in favor of enhanced killer cell activity. Any mechanism that is defined must be consistent with the observation that NK killing of E1A-positive target cells is effective across species barriers (12, 23, 26, 28, 92). The reason for the NK resistance of Ad12-infected and Ad12-transformed cells expressing low levels of MHC class I molecules on their surfaces remains to be defined. One possibility that has not been tested is that Ad12 E1A might repress expression or signaling by killer activating receptors in addition to its well known repression of MHC class I molecules so that the balance of the interaction between these NK cell regulatory molecules favors inhibition of NK cell triggering.

3.4.2. Definition of post-recognition mechanisms of induction of cytolytic susceptibility and sensitization to apoptotic injury – E1A Gene mapping studies

One approach to understanding the E1A mechanisms of induction of cytolytic susceptibility and sensitization to apoptotic injury is to map the E1A gene regions required for these oncoprotein-induced cellular activities. Defining such associations might provide links to studies of the molecular pathways that control these cellular phenotypes. E1A mediates the majority of its other cellular activities through interactions with transcriptional regulatory proteins involved in cell cycle control, including p300/CBP (CREB-binding protein) and the retinoblastoma (Rb) family of proteins. Studies of E1A-induced NK cell cytolytic susceptibility of hamster and rat cells expressing E1A during viral infection or stable transfection, respectively, indicated that the E1A first exon regions that bind p300/CBP (termed the E1A N-terminus and conserve region 1, CR1) are essential for this activity (27, 140). The main E1A first exon region required to bind Rb family proteins (termed conserve region 2, CR2) was not needed for induction of cytolytic susceptibility. Whereas the E1A-p300/CBP binding domain was necessary, it was not sufficient for induction of cytolytic susceptibility. Coexpression of E1A second exon sequences was required. This requirement for E1A second exon coexpression was reproduced with both infected and transformed cells.

Recent studies using human tumor cells expressing E1A-E7 chimeric molecules have confirmed the importance of the collaboration between the E1A N-terminus and second exon for induction of NK cell cytolytic susceptibility (43). These studies also extended the original observations using NK cells to include analysis of the E1A gene expression requirements for induction of cytolytic susceptibility to activated macrophage killing, where differences from NK killing were observed. These results indicate that, in contrast to NK cell cytolytic susceptibility, E1A induction of susceptibility to killing by activated macrophages requires expression of only the E1A N-terminus and CR1 without collaboration of E1A second exon-encoded components of the molecule. These results provide a basis for further studies of the differences in E1A-controlled cellular pathways for sensitization of tumor cells to killing by these two different types of innate immune effector cells. Other reports have implicated an analogous collaboration between E1A first exon and second exon regions for other E1A activities, including E1A-cell protein interactions (141-147) and transcriptional activation (148-150) and repression (147, 151-153) of cellular and viral genes. This requirement for collaborative interaction between E1A first and second exon sequences for several E1A activities might reflect structural needs for molecule stability rather than mechanistic similarities among different E1A activities. It may, however, continue to be interesting to analyze these genetic mapping requirements as more is learned about the effects of E1A on cellular mechanisms that control the response to injury.

Variable results have been obtained from E1A mapping studies of sequence requirements for cellular sensitization to other types of injuries. Some studies have indicated that expression of E1A binding domains for either p300/CBP or Rb family proteins are involved in sensitization to apoptotic injury by TNF or chemotherapeutic drugs (71, 111), whereas other studies have indicated that only the E1A binding domain for Rb family proteins is required for these two different types of cellular injury or irradiation (124, 154, 155). Whether these differences are related to variations in cell systems or study methods remains to be determined. It is also important to acknowledge that most of these studies, including those on E1A-induced cytolytic susceptibility, are correlative in nature and do not prove that E1A interactions with p300/CBP or Rb actually mediate the activities tested or exclude that other E1A-cell protein interactions through these putative binding domains are involved in the various E1A activities. Further mechanistic studies testing the specific roles of cell proteins interacting with E1A will be required to make progress in this area.

3.4.3. Definition of molecular mechanisms of E1A-induced cytolytic susceptibility and sensitization to apoptotic injury - Apoptosis pathway studies

As previously noted, E1A expression has been associated with cellular apoptosis in several different experimental settings. Therefore, another approach to understanding the cellular mechanisms involved in this E1A-induced phenotypic change is to identify the effects of E1A expression on the cellular apoptosis pathway that renders cells more susceptible to proapoptotic injuries. Most other cellular E1A activities studied to date are mediated by redundant mechanisms that presumably evolved to ensure viral persistence. Therefore, the most likely possibility is that E1A-induced cellular sensitization to apoptotic injury will also involve multiple, redundant cellular pathways. It is tempting, however, to seek an "Achilles heel" that might explain the entire phenomenon of E1A induced cellular sensitivity to diverse injuries.

3.4.3.1. p53 family members

The p53 tumor suppressor is a pivotal component in many cellular apoptotic responses (156, 157). Most

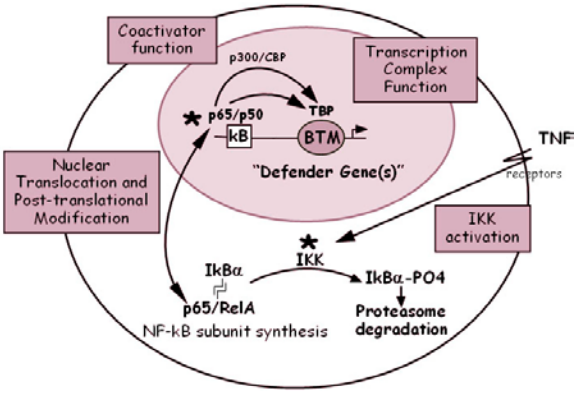

Figure 6. Simplified conceptual model of the main steps in TNF-induced triggering of the cellular, NF-kappa B-dependent defense against apoptosis. The first step is signaling through the trimeric TNF receptor and an activating kinase cascade that triggers I kappa B kinase (IKK) activation. IKK activation results in phosphorylation of the I kappa B alpha "retention" molecule that retards NF-kappa B (e.g. , p65/Rel A) subunit translocation to the nucleus. The second step is freeing of NF-kappa B subunits for nuclear translocation, phosphorylation and binding to the kappa B enhancer. The third step is the complex interaction among NF-kappa B, transcriptional coactivators (e.g. p300/CBP) and components of the basal transcriptional machinery (BTM), such as TATA binding protein (TBP) (among other intermolecular interactions). In E1A-negative cells, this series of activation steps results in triggering of transcription of putative NF-kappa B-dependent "defender genes" whose products block cell death at several steps in the apoptosis pathway. In E1A-positive cells, TNF-induced NF-kappa B activation can be strongly repressed proportionately with increasing E1A oncoprotein expression level. There are at least two reported steps at which E1A repression of the NF-kappa B activation response may occur (asterisks) - repression of the function of IKK and qualitative and functional alteration of the enhancer-bound NF-kappa B in the nucleus.

reports indicate that neither expression nor activation of normal p53 is necessary for E1A-induced cytolytic susceptibility or cellular sensitization to immune-mediated apoptosis as previously discussed. However, most of the studies were done before it was appreciated that there are other p53 family members such as p73 that can be involved in triggering cellular apoptosis. Therefore, a more complete understanding of the role of p53 family-related signaling mechanisms will be required to determine whether or not they are involved in E1A-induced changes in cellular cytolytic or apoptotic phenotypes.

3.4.3.2. Bcl-2 family members

Possible E1A interactions with Bcl-2 family members, as related to the cellular apoptotic response, have been considered in several studies. Most information derives from studies of the functional similarities of the Ad E1B 19 kD protein and Bcl-2 during "direct" induction of apoptosis during either cellular immortalization or viral infection (100, 104, 126, 158, 159). There are conflicting reports about whether Bcl-2 itself can block the "indirect" effect of E1A during induction of sensitivity to apoptotic injury (158, 160). Our reports and unpublished data indicate that comparable cytolytic susceptibility and sensitization to apoptotic injury can be detected with human, mouse and hamster cells expressing E1A + E1B 19 kD vs E1A alone in the context of NK cell, activated macrophage or TNF injury (23, 25, 28, 91, 161). These observations suggest that there are one or more major mechanisms through which E1A sensitizes cells to immune-mediated apoptosis that are not blocked by the Bcl-2-like antiapoptotic effects of E1B 19 kD.

There are limited data on the possible interactions between the proapoptotic Bcl-2 family members and E1A. These molecules have been defined primarily by their ability to bind and block the antiapoptotic effect of E1B 19 kD protein. For example Bak is an E1B 19 kD binding protein defined as "Bcl-2 homologous antagonistic/killer" (162). Bax (163) and Bik (164) are other members of this proapoptotic group of molecules. Available evidence indicates that these proapoptotic family members mediate part of the p53-dependent cellular apoptotic response (121) and may also induce apoptosis in some p53 mutant cells (163). Furthermore, Bik can be upregulated during the "direct" apoptosis induced by E1A during viral infection (165). The role of these proapoptotic molecules in E1A-induced cytolytic susceptibility and sensitization to apoptosis remains to be determined.

3.4.3.3. E1A-induced repression of the NF-kappa B-dependent cellular defense against apoptosis

NF-kappa B activation of one more "antiapoptotic genes" provides one line of cellular defense against injury-induced apoptosis (Figure 6). This activity has been best studied as a defense against apoptosis triggered by TNF and is most closely related to the function of the p65/RelA NF-kappa B subunit (166-168). E1A sensitizes certain types of cells to TNF-induced apoptosis (62, 69, 74, 169). E1A also represses NF kappa B-dependent transcription in other contexts - e.g., HIV promoter repression (146, 170, 171). Together, these observations suggested the possibility that E1A induced repression of the cellular NF-kB-dependent defense against apoptosis could explain E1A-induced sensitization to TNF. In an analogous early report, it was shown that E1A expression altered the quality of NF-kappa B dimeric transcription factor species binding to the nuclear kB enhancer, thereby blocking TNF-induced transcription of the interleukin-6 (IL-6) gene (172). Other studies indicated that E1A expression could repress the function of I kappa B kinase (IKK) in cells stimulated by either irradiation or TNF (123, 169). This signal-induced kinase induces phosphorylation and subsequent degradation of I kappa B alpha, resulting in nuclear translocation of NF-kappa B subunits such as p65/RelA (Figure 6). Therefore, E1A induced repression of IKK activity provides a second possible mechanism of repression of the NF-kappa B-dependent response to injury. These reports suggested the existence of multiple possible mechanisms through which E1A could repress stimulus-induced activation of NF-kB-dependent cellular defenses that might be cell system specific.

We used a well characterized NIH-3T3 cell system to evaluate the relationships between E1A-cell protein interactions, NF-kappa B activation and cellular sensitization to TNF-induced apoptosis (155). High level E1A repression of TNF-induced NF-kappa B activation was detected, despite apparently normal I kappa B alpha turnover and p65/RelA nuclear translocation. Selective E1A repression of NF-kappa B-dependent transcription and the associated sensitization of cells to TNF-induced apoptosis were relieved by overexpression of p65/RelA NF-kappa B. E1A gene mapping studies indicated an association between the integrity of the Rb binding domain of E1A and repression of the NF-kappa B activation response, but showed no requirement for the E1A-p300/CBP binding domain for NF-kappa B repression. Other observations about apoptosis triggered by irradiation (124) or chemotherapeutic drugs (154) in E1A-expressing human tumor cells are consistent with this association between the E1A-Rb binding domain and cellular sensitization to injury. Other reports of cells treated with TNF (71) or chemotherapeutic drugs (111) have indicated that E1A binding to either Rb family proteins or p300/CBP might mediate this sensitizing activity of E1A. Therefore, further studies will be needed to resolve the relative roles of E1A-cell protein interactions in different cells and with different proapoptotic injuries and to define the molecular pathways through which these putative E1A-cell protein interactions might control cellular sensitivity. One such study of an NF kappa B-dependent cellular mechanisms that increases cellular sensitivity to TNF-induced apoptosis has suggested a role for E1A repression of c-FLIP(S) (an inhibitor of caspase 8 activation) and possibly other NF-kappa B-dependent genes that are implicated in the antiapoptotic cellular defense (75).

These observations indicate that E1A repression of the cellular NF-kappa B activation response to injury is one mechanism of in E1A-induced cytolytic susceptibility and sensitization to apoptotic injury. Considering the history of redundancy of E1A mechanisms involved in the control of cellular pathways, it is likely that E1A repression of NF-kappa B activation is only one of several pathways through which E1A sensitizes cells proapoptotic injury. This concept is consistent with the reported interactions between E1A and the Akt signaling pathway in cells undergoing apoptosis during growth factor starvation or injury with chemotherapeutic drugs (173, 174, 175).

3.5. Translation of observations regarding E1A-induced cytolytic susceptibility and sensitization to apoptotic injury to studies of human tumor cells and *in vivo* assays of tumorigenicity

E1A expression sensitizes human tumor cells to a diverse array of cytolytic injuries during viral infection, neoplastic transformation and stable transfection. E1A expression during viral infection of human cells "focuses" killing by interferon-activated NK cells on E1A-positive targets (176, 177). The ability of interferon to induce selective NK killing of Ad-infected or E1A-transfected human cells is independent of target cell class I MHC antigen expression but dependent on the expression of E1A and correlates with E1A-p300 binding. The dynamics of this E1A-induced cytolytic susceptibility are different than with E1A-infected rodent cells, but the outcome is the same - selective killing of E1A-positive cells. In contrast to human cells, Ad-infected, E1A-positive rodent cells are sensitive to unstimulated (non-cytokine activated) NK cells. Stable E1A expression in human tumor cells induces cytolytic susceptibility to killing by both NK cells and activated macrophages (23, 77, 118) and also sensitizes human tumor cells to TRAIL (78, 118), TNF (72, 77, 83), chemotherapeutic drugs (72, 110-112, 114, 115, 117, 118) and irradiation (120, 123).

Several observations support the conclusion that E1A-induced sensitivity of human tumor cells to immune-mediated and chemotherapy drug-induced apoptosis renders the cells less tumorigenic in the context of the innate immune response and chemotherapy in vivo. Several studies have shown that E1A-positive tumor cells of different types are more sensitive to chemotherapeutic agents and irradiation than their E1A-negative counterparts (113, 118, 178-181). E1A-positive human tumor cells of different tissue origins exhibit reduced tumor growth in nude mice (110, 118, 182). This in vivo effect of E1A is probably multifactorial and may also have a bystander inhibitory effect on E1A-negative tumor cells in the microenvironment (178).

Since all of these human tumor studies were done in nude mice, it is likely that they underestimate the potential contribution of the host cellular immune response as a factor in E1A-positive tumor cell rejection. Nude mice lack T cells; therefore, the potential synergistic antitumor defense between innate and adaptive (T cell-dependent) immune responses (Figure 4) are lacking. Primates and rodents express unique NK receptors that are triggered by distinct ligands on tumor cells. Therefore, it is likely that murine NK cells would be less effective in mediating rejection of E1A-expressing human tumor cells than human NK cells. Thus, the effect of E1A expression on human tumor cells in immunocompetent patients is likely to be greater than predicted using tumorigenicity assays in nude mice. Despite these theoretical limitations of nude mouse studies of E1A-expressing human tumor cells, our studies indicate that E1A expression in human tumor cells can both prolong the latent period for tumor development and reduce tumor inducing efficiency (118) (Figure 7). A similar conclusion has been reached in studies of mouse carcinoma cells (183).

There may be other effects of E1A expression on human tumor cells *in vivo*. In some cell types, E1A overexpression can reduce cell growth rates. In others, E1A expression represses tumor formation in the absence of any detectable changes in cell doubling (118). Finally, it is likely that E1A-induced human tumor cell sensitivity to killer cell injuries and other potentially therapeutic injuries is additive or possibly synergistic (184-187).

4. PERSPECTIVE

E1A expression sensitizes tumor cells from several species, including human, and from different tissue

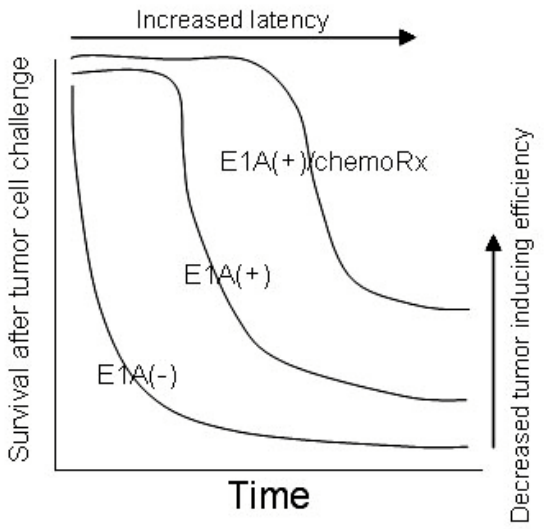

Figure 7. Graphic representation of the changes in the nude mouse tumorigenicity of human tumor cells resulting from E1A expression or combined E1A expression and chemotherapy. This quantitative assay of tumor induction can measure two independent variables of tumorigenicity - tumor latency and tumor inducing efficiency. E1A expression and combined E1A + chemotherapy can increase the tumor latency and decrease the tumor inducing efficiency of human tumor cells.

origins to the cytolytic effects of killer lymphocytes and activated macrophages. These killer cells use overlapping cytolytic mechanisms to kill sensitive target cells, suggesting the existence of redundant cellular immune defenses against E1A-positive cells. Therefore, whereas the main mechanism through which E1A induces sensitization to NK cells or activated macrophages may vary with the cell type and species of the target cell tested, it is possible that the net result will be the same E1A-related increase in susceptibility to cytolytic activity and rejection by the immunocompetent host.

E1A, as a foreign, endogenous viral protein, is also capable of sensitizing cells to adaptive (E1A-peptide-specific) cellular immune responses. E1A-specific CTL are generated, with the epitope specificity being dependent upon the haplotype of the host. This is consistent with the central dogma of MHC class I-restricted CTL generation and predicts that there will be variations in individual human CTL responses to E1A-expressing tumor cells depending upon genetic regulation of antigen presentation. The E1A-induced increase in hsp70 expression might enhance antigen processing and presentation and therefore might increase the immunogenicity of E1A and other tumor-specific antigens. At the same time, E1A expression blocks the ability of hsp70 overexpression to protect tumor cells against potentially cytotoxic molecules such as nitric oxide and TNF. *In vivo* tumorigenicity data from mouse studies suggest that E1A-induced cytolytic susceptibility to components of the innate immune response is complemented by T-cell-dependent rejection mechanisms for highly efficient resistance to E1A-positive tumor cells. Whether these observations on the efficiency of immune-mediated rejection of E1A-expressing tumor cells can be translated to humans remains to be determined.

E1A expression induces sensitivity of a wide variety of tumor cell types to apoptotic death in response to treatment with a different classes of chemotherapeutic drugs and with therapeutic irradiation. It is possible that these tumor cell sensitizing effects of E1A to immune destruction and to the proapoptotic effects of other therapeutic strategies might be additive or synergistic.

The mechanisms through which E1A mediates these diverse sensitizing effects on tumor cells remain to be completely defined. There are some possibilities that currently seem unlikely. E1A-induced changes in cellular expression of MHC class I antigens do not appear to explain increased sensitivity to NK killing. Whether E1A increases expression of cellular ligands that trigger NK stimulatory receptors is an interesting possibility that remains to be tested. Studies of TNF- and Fas-induced cytotoxicity for E1A expressing cells indicate that E1A-induced sensitization to apoptotic injury triggered by these TNF family ligands is not explained by overexpression of death receptors, or at least can occur independently of any such effect on death receptor expression.

Given the available data, it is difficult to postulate a single E1A mechanism that would explain all types of E1A induced cytolytic susceptibility and sensitization to diverse proapoptotic injuries. There are either published or unpublished data that indicate that E1A sensitizes cells to injuries through different triggering pathways, through pathways that are p53-dependent and p53-independent, NF-kappa B-dependent and NF-kappa B-independent and that involve modulation of Akt activity. This apparent redundancy of E1A activities that affect cellular susceptibility to proapoptotic injuries is consistent with what has been observed for other E1A activities that control cellular and viral transcription and cell cycle regulation.

There are several possible future directions for studies of the mechanisms and applications of E1A-induced cytolytic susceptibility and sensitization to apoptotic injury. It will be interesting to seek linkages among different cellular pathways that regulate these cellular phenotypes and E1A control mechanisms that regulate cell cycle and viral gene expression. It will be important to determine whether there are additive or synergistic interactions between different E1A activities that could enhance the combined effects of various immunological and nonimmunological therapies against tumor cells. It will be useful to learn more about the limits of these E1A effects for tumor rejection *in vivo* and to seek ways to increase the effectiveness of this activity. At present, it appears that E1A can retard tumor formation and decrease tumor inducing efficiency (as tested in nude mice). Whether these activities are even more impressive in the context of the intact cellular immune response in humans remains to be determined. It also will continue to be important to seek improved methods for targeting E1A for tumor-specific

expression and for the use of E1A as a mechanistic model to develop viral gene-independent methods of triggering tumor cell sensitivity to host immunological defenses and other therapeutic injuries.

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