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Adenovirus E1A Oncogene Expression in Tumor Cells Enhances Killing by TNF-Related Apoptosis-Inducing Ligand (TRAIL)[1]

John M. Routes,[2*†‡§¶||] Sharon Ryan,[*] Amanda Clase,[*] Tanya Miura,[*] Alicia Kuhl,[*] Terry A. Potter,[†§¶] and James L. Cook[||]

Expression of the adenovirus serotype 5 (Ad5) E1A oncogene sensitizes cells to apoptosis by TNF-α and Fas-ligand. Because TNF-related apoptosis-inducing ligand (TRAIL) kills cells in a similar manner as TNF-α and Fas ligand, we asked whether E1A expression might sensitize cells to lysis by TRAIL. To test this hypothesis, we examined TRAIL-induced killing of human melanoma (A2058) or fibrosarcoma (H4) cells that expressed E1A following either infection with Ad5 or stable transfection with Ad5-E1A. E1A-transfected A2058 (A2058-E1A) or H4 (H4-E1A) cells were highly sensitive to TRAIL-induced killing, but Ad5-infected cells expressing equally high levels of E1A protein remained resistant to TRAIL. Infection of A2058-E1A cells with Ad5 reduced their sensitivity to TRAIL-dependent killing. Therefore, viral gene products expressed following infection with Ad5 inhibited the sensitivity to TRAIL-induced killing conferred by transfection with E1A. E1B and E3 gene products have been shown to inhibit TNF-α- and Fas-dependent killing. The effect of these gene products on TRAIL-dependent killing was examined by using Ad5-mutants that did not express either the E3 (H5*dl327*) or E1B-19K (H5*dl250*) coding regions. A2058 cells infected with H5*dl327* were susceptible to TRAIL-dependent killing. Furthermore, TRAIL-dependent killing of A2058-E1A cells was not inhibited by infection with H5*dl327*. Infection with H5*dl250* sensitized A2058 cells to TRAIL-induced killing, but considerably less than H5*dl327*-infection. In summary, expression of Ad5-E1A gene products sensitizes cells to TRAIL-dependent killing, whereas E3 gene products, and to a lesser extent E1B-19K, inhibit this effect. ***The Journal of Immunology,* 2000, 165: 4522–4527.**

Human adenoviruses (Ad)[3] are common human pathogens. In immunocompetent people, Ad cause persistent, but self-limited infections (1, 2). Although not oncogenic in humans, Ad are capable of transforming human cells in vitro to a state where the cells are tumorigenic in immunocompromised rodents (3, 4). Cells transformed by Ad are virion free and only two viral genes (E1A and E1B) are consistently expressed (5). The primary immortalizing gene is E1A. E1B serves as a "helper" gene that increases the efficiency of viral transformation (5).

One possible explanation for the lack of oncogenicity of Ad in humans is the capacity of E1A to sensitize cells to destruction by components of the host cellular immune response (6–9). The expression of E1A sensitizes cells to lysis by components of the cellular antitumor immune response, including NK cells, activated macrophages, and CTL (6–9). These effector cells kill target cells by several mechanisms including the elaboration of secreted proteins, such as TNF-α and perforin, or by the interaction of Fas ligand on effector cells with Fas on target cells. The expression of E1A in epithelial or fibroblastic cells induces an increased susceptibility to lysis by TNF-α, perforin, and Fas ligand (10–12).

Recent studies have characterized a new member of the TNF family of cytokines, the TNF-related apoptosis-inducing ligand (TRAIL) (13, 14). TRAIL interacts with receptors that are distinct from the receptors for Fas ligand and TNF-α. To date, five receptors have been shown to bind TRAIL. The binding of TRAIL to either TRAIL-R1 (death receptor 1) or TRAIL-R2 induces apoptosis (15–17). In contrast, the binding of TRAIL to TRIAL-R3, TRAIL-R4, or osteoprotegerin does not induce apoptosis (15, 16, 18–21). TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and osteoprotegerin are all members of the TNF-receptor family. The cytoplasmic domains of TRAIL-R1 and TRAIL-R2, like those of the receptors for Fas ligand and TNF-α, contain cytoplasmic death domains and likely use the same or similar pathways of caspase activation to induce apoptosis (22). In contrast, TRAIL-R3 and TRAIL-R4 lack cytoplasmic death domains, and osteoprotegerin is a secreted protein. In contrast to TNF-α and Fas ligand, which are expressed primarily by cells of the immune system, TRAIL is produced by a wide variety of cell types (22). TRAIL preferentially induces apoptosis of certain tumor cell lines and virally infected cells, whereas nontransformed cells are generally resistant to TRAIL-induced killing (23, 24). TRAIL also contributes to the cytotoxicity mediated by $CD4^+$ T cells and monocytes (25).

Based on the ability of TRAIL to selectively kill virally infected cells and the similar molecular mechanisms of TRAIL-, Fas-, and TNF-α-induced apoptosis, we postulated that E1A expression in Ad-infected and E1A-transfected tumor cells might also sensitize

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[3] Abbreviations used in this paper: Ad, adenovirus; TRAIL, TNF-related apoptosis-inducing ligand.

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cells to TRAIL-induced killing. To test this hypothesis, we determined the effect of E1A expression on TRAIL-induced killing of A2058 and H4 cells. A2058 cells, a human melanoma cancer cell line, and H4, a human fibrosarcoma cell line, were chosen because they are resistant to TRAIL-induced killing and are permissive for infection with Ad5 (26). We found that the expression of E1A gene products sensitizes human tumor cells to TRAIL-dependent killing. The ability of E1A to sensitize cells to TRAIL-induced killing following Ad5 infection was inhibited by E3 gene products, and to a lesser extent E1B-19K.

Materials and Methods

Cells and cell lines

A2058 is a human melanoma cell line. H4 is a human fibrosarcoma cell line. A2058-E1A (also known as 1A558C8) and H4-E1A (also known as P2AHT2A) are Ad5-E1A-transfected A2058 and H4 cells, respectively (27). Cell lines were maintained in DMEM supplemented with 5% calf serum, glucose (15 mM), antibiotics, and glutamine. The absence of *Mycoplasma* in the cell lines was established by using the Mycotect Assay (Life Technologies, Gaithersburg, MD).

Viruses

H5*dl327* is an Ad5 mutant virus that does not express any E3 proteins (28). H5*dl250* is an Ad5 mutant that does not express the E1B-19K protein (29). Wild-type Ad5 and H5*dl327* were grown in A549 cells. H5*dl250* was grown in 293 cells. Each virus was titered by plaque assay using the cell line in which it was grown.

TRAIL cytolysis assays

Target cells were infected with the different Ad (multiplicity of infection = 100) for 16 h and labeled with ^{51}Cr (100 mCi/ml for 1 h; 1 Ci = 37 gBq). Initial studies showed that equivalent amounts of E1A were expressed following infection of cells at a multiplicity of infection of 100 with wild-type or mutant forms of Ad5 (data not shown). Target cells (1×10^4 cells) were then incubated with different concentrations of recombinant human TRAIL (R&D Systems, Minneapolis, MN). After a 16-h incubation at 37°C in 5% CO_2, half of the supernatant from each well was harvested and counted in a gamma counter. TRAIL-dependent killing was determined by calculating the percentage of TRAIL-induced release of radiolabel from target cells as described (9). The results shown represent the mean ± SEM of at least four separate experiments. The mean percentage spontaneous release from all types of target cells was <30%. The significance of the differences in TRAIL-induced killing of control and infected cell lines was estimated using Student's *t* test.

Western analysis

For quantitation of E1A proteins, 60-mm plates of Ad-infected or E1A-transfected A2058 or H4 cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl), and protein concentrations of RIPA supernatants were determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL). An equal amount of protein from each cell lysate was separated on 10% SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked in 5% nonfat milk solution and incubated with the anti-E1A mAb, M73 (supplied by E. Harlow, Massachusetts General Hospital, Charleston, MA) for 1 h (30). Following several washes with PBST (PBS with 0.05% Triton X-100), membranes were incubated for 1 h with rabbit anti-mouse IgG Ab (Cappel, Durham, NC) and washed extensively with PBST. The E1A protein was then visualized as per manufacturer instructions using the Renaissance Chemiluminescence Kit (DuPont-NEN, Boston, MA).

Results

E1A-transfection, but not infection, with wild-type Ad5 sensitizes tumor cells to TRAIL-induced killing

To determine whether E1A sensitized cells to TRAIL, cytolysis assays were performed on parental and E1A-transfected melanoma (A2058, A2058-E1A) and fibrosarcoma (H4, H4-E1A) cells. In agreement with prior reports, we found that both H4 and A2058 cells were resistant to TRAIL-induced killing (Fig. 1). The resistance of A2058 or H4 cells to TRAIL-induced killing was abrogated by the stable transfection with E1A (Fig. 1). Cell lines have been considered sensitive to TRAIL-induced killing if incubation of 300 ng/ml of recombinant TRAIL results in >20% killing (22, 26). We found that the addition of 12.5–25 ng/ml of TRAIL killed >25% of A2058-E1A and H4-E1A cells. The ability of E1A expression to sensitize tumor cells to TRAIL-dependent killing was not restricted to melanoma or fibrosarcoma cells. TRAIL-dependent killing of breast cancer and cervical cancer cell lines was also increased by stable E1A-transfection (data not shown). Next, we ascertained whether the expression of E1A gene products following Ad5 infection would also induce TRAIL-dependent killing. In contrast to transfection with E1A, infection of A2058 or H4 cells with Ad5 did not confer sensitivity to TRAIL-dependent killing (Fig. 1).

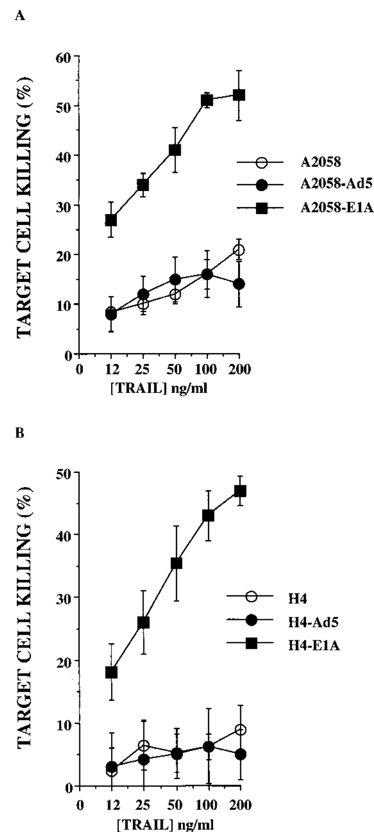

FIGURE 1. E1A-transfected, but not Ad5-infected, human tumor cells are sensitive to TRAIL-induced lysis. A2058 and A2058-E1A are parental and E1A-transfected, human melanoma cells, respectively. H4 and H4-E1A are parental and E1A-transfected fibrosarcoma cells. *A,* TRAIL-induced lysis of A2058 (○), Ad5-infected-A2058 (●), or A2058-E1A (■) cells. *B,* TRAIL-induced killing of H4 (○), Ad5-infected H4 (●), or H4-E1A (■) cells.

E1A oncoprotein expression is equivalent in E1A-tranfected or Ad5-infected A2058 cells

The ability of E1A gene products to increase the susceptibility to lysis by NK cells and macrophages is dependent on the level of E1A expressed (11). Prior studies from our laboratory showed that equivalent amounts of E1A protein are expressed in Ad5- compared with E1A-transfected H4 cells (6). However, it was possible that the levels of E1A produced following Ad5-infection of A2058 cells were insufficient to induce sensitivity to TRAIL-dependent killing. To test this possibility, the levels of E1A oncoprotein in Ad5-infected and E1A-transfected A2058 cells were compared. Western blot analysis of cell lysates demonstrated that the amounts

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of E1A in Ad5-infected and E1A-transfected A2058 cells were comparable (Fig. 2). Therefore, the resistance of Ad5-infected A2058 or H4 cells to TRAIL-dependent killing could not be explained by quantitative differences in the expression of E1A gene products.

Inhibition of TRAIL-dependent killing by E1B and E3 gene products

Expression of E1A gene products in immortalized cells induces susceptibility to NK cell lysis. In contrast, infection of human cells with Ad does not render the cells susceptible to NK cell lysis (6). Thus, it was possible that the transient expression of E1A gene products following Ad5-infection was insufficient to induce sensitivity to TRAIL-dependent killing. Alternatively, other viral gene products produced following Ad infection could be responsible for the inability of Ad5 infection to sensitize cells to TRAIL-dependent killing. To test the latter possibility, we examined whether Ad5-infection inhibited TRAIL-dependent killing of A2058-E1A cells. As shown in Fig. 3, Ad5-infection of A2058-E1A cells reduced the killing by TRAIL compared with that obtained with uninfected cells.

Adenoviral E1B and E3 gene products inhibit apoptosis induced by Fas ligand and TNF-α The effect of these gene products on TRAIL-dependent killing was examined by using mutant strains of Ad5 that did not express either the E3 (H5*dl327*) or E1B-19K (H5*dl250*) coding regions. A2058 cells infected with H5*dl250* (which does not express E1B-19K, but does express E1A and E3 proteins) resulted in a small increase in TRAIL-induced killing compared with uninfected cells (Fig. 4*A*). In contrast, infection of A2058 cells with H5*dl327* (which does not express E3 proteins, but does express E1A and E1B-proteins) increased the sensitivity of A2058 cells to TRAIL-dependent killing to a level that was similar to that observed with A2058-E1A cells (Fig. 4*A*). Furthermore, infection of A2058-E1A cells with H5*dl327* did not inhibit their TRAIL sensitivity (Fig. 4*B*). The ability of *H5dl250* infection to block TRAIL-induced killing in A2058-E1A cells could not be assessed because infection of these cells with H5*dl250* produced a high spontaneous release of ^{51}Cr. In total, these data indicated that E3 proteins inhibited the ability of E1A gene products, produced following transfection or infection, to sensitize cells to TRAIL-dependent killing. The E1B-19K protein inhibited TRAIL-induced killing to a lesser extent than E3 gene products.

Discussion

In this study, we demonstrate that expression of the Ad5-E1A oncoprotein following transfection of the E1A gene sensitized melanoma (A2058) and fibrosarcoma (H4) tumor cells to lysis by TRAIL (Fig. 1). Similar data were obtained with E1A-transfected breast cancer and cervical cancer cells (data not shown). These data are similar to reports that E1A gene products sensitize human and murine cells to TNF-α and Fas-dependent killing (10–12). However, E1A gene products do not always sensitize human or rodent cells to lysis by immune mediators or effector cells (8, 31–33). Furthermore, the molecular basis for the increased susceptibility of E1A-expressing cells to TNF-α and Fas-dependent killing is unknown. Therefore, the ability of E1A to sensitize human tumor cells to TRAIL-dependent killing needed to be directly ascertained.

TRAIL-dependent killing of A2058 cells was also increased by the expression of E1A gene products produced in the context of Ad-infection. However, because E1B and E3 gene products block TRAIL-induced killing, this effect of E1A could not be detected by infection of A2058 cells with wild-type Ad5. For example, in contrast to A2058-E1A cells, A2058 cells infected with wild-type Ad5 were resistant to lysis by TRAIL (Fig. 1*A*). This difference between Ad5-infected and E1A-transfected cells was not due to differences in E1A expression. A2058-E1A cells and A2058 cells infected with Ad5 expressed equivalent levels of E1A oncoprotein (Fig. 2). Similar to results on A2058 cells, Ad5-infected H4 cells also were resistant to TRAIL-dependent killing, despite high levels of E1A expression (Fig. 1*B*, and Ref. 6). Proteins encoded by the E3 regions were predominately responsible for the resistance of Ad5-infected A2058 cells to lysis by TRAIL. A2058 cells infected with H5*dl327* (which does not express E3 proteins, but does expresses E1B proteins) were nearly as sensitive to TRAIL-dependent killing as A2058-E1A cells (Fig. 4*A*). TRAIL-dependent killing was also blocked by E1B-19K, although they were less effective than proteins encoded by the E3 region. Thus, H5*dl250* (which does not express E1B-19K, but expresses E3 proteins) infection of A2058 cells increased TRAIL-dependent killing, but to a lesser extent than infection with H5*dl327* (Fig. 4*A*). Furthermore, E1B gene products failed to inhibit killing of A2058-E1A cells following infection with H5*dl327* (Fig. 4*B*). In contrast, TRAIL-dependent killing of A2058-E1A cells was blocked following infection with Ad5 (expresses E1B and E3; Fig. 4). In total, these studies indicate that E3 gene products were more effective at inhibiting TRAIL-dependent killing than the E1B-19K proteins.

Ad encode numerous proteins that facilitate the evasion of the host immune response and contribute to viral persistence (34). Several proteins encoded by the E1B and E3 regions have been previously shown to inhibit TNF-α and Fas-induced killing and

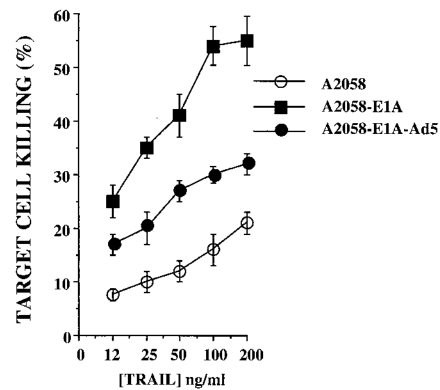

FIGURE 3. Ad5-infection inhibits TRAIL-dependent killing of E1A-transfected cells. Parental and E1A-transfected A2058 cells were mock or Ad5-infected for 16 h and their sensitivity to TRAIL-induced killing was determined in a 16-h cytolysis assay. TRAIL-induced lysis of A2058 (○), Ad5-infected A2058-E1A (●), or uninfected A2058-E1A cells (■).

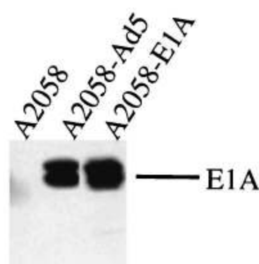

FIGURE 2. Expression of E1A in Ad5-infected compared with E1A-transfected human tumor cells. A2058 cells were infected with Ad5 for 16 h and the levels of E1A oncoprotein expression were determined by Western analysis (see *Materials and Methods*). A2058-E1A is an E1A-transfected A2058 cell line.

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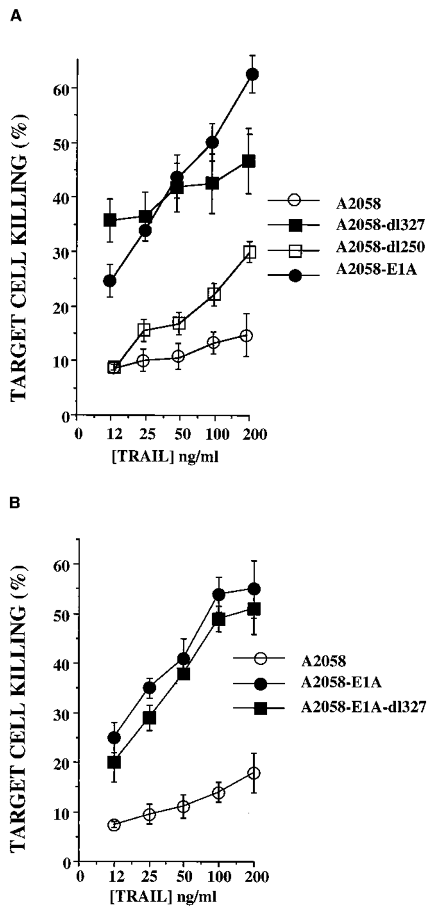

FIGURE 4. E1B and E3 gene products inhibit TRAIL-dependent killing. H5*dl250* and H5*dl327* are Ad5-mutants that delete the E1B-19K and E3 open reading frames, respectively. Tumor cell lines were mock-, H5*dl250-,* or H5-*dl327*-infected for 16 h and their sensitivity to TRAIL-induced killing was determined in 16-h cytolysis assays. *A,* TRAIL-induced killing of A2058 (○), A2058-E1A (●), H5*dl327*- (■), or H5*dl/250*-infected (□) A2058 cells. *B,* TRAIL-induced killing of A2058 (○), H5*dl327*-infected (■), or unifected-A2058-E1A cells (●).

thus are candidates to inhibit TRAIL-induced killing (35–37). For example, the E1B 19K protein, which is functionally homologous to the protoncogene Bcl-2, inhibits the activation of Procaspace-8 (FLICE) (36). In addition, the E3 10.4K and 14.5K proteins (also known as E3-RID) bind to and reduce the surface expression of Fas (39, 40). Another E3 protein, E3–14.7K, blocks apoptosis by its ability to bind and alter the function of proteins involved in NF-κB-apoptosis regulatory pathway (41). Studies are ongoing to determine the molecular mechanism by which the E1B and E3 proteins inhibit TRAIL-induced killing.

Other adenoviral proteins are implicated in the evasion of host immune responses. Certain Ad-encoded proteins inhibit the biological activities of IFNs and CTL recogntion of Ad-infected cells (42–44). Therefore, it is puzzling that Ad rarely cause disseminated infections and do not appear to be oncogenic in humans, despite their well-described ability to transform human cells (4, 45–48). We speculate that the biological behavior of Ad in humans is best explained by the incomplete inhibition of the host cellular innate (NK cells, macrophages) and acquired (T cell) immune responses by adenoviral immunomodulatory proteins during infection as well as minimal effects of these viral proteins on immune defenses following viral transformation. For example, Ad-transformed human cells are selectively killed by resting and IFN-activated NK cells (6, 49). In contrast, Ad-infected human cells are only selectively killed by IFN-activated NK cells (33, 50). In mouse models, a robust CTL response is induced following injection of E1A-expressing tumor cells, and, as a result, CTL are highly effective at eliminating Ad-transformed cells in vivo (51, 52). We speculate that an effective CTL response would also be generated against E1A-expressing, Ad-transformed cells in humans. CTL are also induced following Ad-infection (53–55). However, CTL recognition of Ad-infected cells appears to be partially subverted by the Ad-E3-19K protein, a protein that is usually not expressed in Ad-transformed cells (44, 56, 57). The inhibition of TRAIL-dependent killing by the E3 proteins provides another mechanism that could enable Ad to escape innate immunity and favor viral persistence. However, our data suggest that TRAIL-dependent killing would be highly effective in eliminating E1A-expressing human cells that became immortalized following a transforming viral infection. For example, E1A-transfected A2058 and H4 cells and A2058 cells infected with H5*dl327* (which express the E1A and E1B proteins) were highly susceptible to TRAIL-dependent killing (Fig. 4). Therefore, we predict that Ad-transformed, E1A and E1B expressing human cells would also be highly sensitive to TRAIL-dependent killing. Thus, the immunomodulatory proteins encoded by Ad may prevent a rapid sterilizing immune response and favor viral-persistence, but are insufficient to fully evade host immunity. Furthermore, in Ad-transformed cells, these immunomodulatory proteins are either not present (E3) or are ineffective (E1B) in blocking killing by host effector killer mechanisms (5, 58).

The results from this study are also highly relevant in the selection of the optimal adenovirus mutant to be used in the treatment of human malignancy. Phase I clinical trials are presently underway using ONYX-015, an Ad-mutant that does not express the E1B-55K protein (59). E1B-55K partially inhibits the p53-dependent apoptosis induced by E1A-expression (60). The deletion of E1B-55K may enable ONYX-015 to replicate and induce apoptosis in p53-deficient human cancer cells but may leave normal (p53 positive) cells intact (61–63). For several reasons, we speculate that adenoviral mutants that contain deletions in both the E1B and E3 coding regions would be superior to ONYX-015 for the treatment of human malignancies. The first reason is that immune mechanisms likely participate in mediating tumor regression following Ad-infection. For example, several reports indicate that the ability of E1A to reduce tumorigenicity is dependent on NK and T cells (64–67). The ability of E1A to sensitize cells to immune-mediated apoptosis is independent of p53 (66, 68), insensitive to E1B-19K. The second reason is that prior studies indicate that combining chemotherapeutic agents with ONYX-015 is more effective in mediating tumor destruction than either agent alone (63). This effect is likely due to the ability of E1A to sensitize cells to apoptosis induced by chemotherapeutic agents (69). However, in E1A-expressing tumor cells, the p53-dependent induction of apoptosis induced by chemotherapeutic agents is also inhibited by E1B-19K (68). A third reason is that recent studies indicate that TRAIL may be useful in the treatment of human malignancies. By inducing the expression of TRAIL-R1 and TRAIL-R2, chemotherapeutic agents such as etoposide synergize with TRAIL to mediate tumor cell apoptosis (70). Studies reported here indicate that TRAIL-resistant tumors can be sensitized by the expression of E1A gene products. In contrast, E3 gene products block TRAIL-induced killing of E1A-expressing tumor cells. The use of mutant Ad that have deleted both E1B (19K and 55K) and E3 gene products may optimize the synergistic interactions involving chemotherapeutic agents, TRAIL and E1A. For all these reasons, we believe that mutant adenoviruses that contain deletions of both the

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E1B and E3 open reading frames would be more effective in the treatment of human malignancies than ONYX-015, regardless of whether the virus was used alone or in combination with other chemotherapeutic agents. Studies are ongoing using murine models to directly test this hypothesis.

In summary, we show that the expression of E1A gene products sensitizes tumor cells to TRAIL-dependent killing. The gene products of the E3 region, and to a lesser extent E1B1-19K, inhibit this effect. Studies are ongoing to determine the molecular basis for the ability of E1A gene products to sensitize cells to TRAIL-dependent killing.

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