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E1A Oncogene Enhancement of Caspase-2-Mediated Mitochondrial Injury Sensitizes Cells to Macrophage Nitric Oxide-Induced Apoptosis

Jay R. Radke,* Zeba K. Siddiqui,* Tanya A. Miura,† John M. Routes,‡ and James L. Cook²*

The adenovirus E1A oncogene induces innate immune rejection of tumors by sensitizing tumor cells to apoptosis in response to injuries, such as those inflicted by macrophage-produced TNF α and NO. E1A sensitizes cells to TNF by repressing its activation of NF-κB-dependent, antiapoptotic defenses. This suggested the hypothesis that E1A blockade of the NF-κB activation response might be the central mechanism of E1A induced cellular sensitivity to other proapoptotic injuries, such as macrophage-produced NO. However, creation of E1A-positive NIH-3T3 mouse cell variants with high-level, NF-κB-dependent resistance to TNF did not coselect for resistance to apoptosis induced by either macrophage-NO or chemical-NO, as the hypothesis would predict. E1A expression did block cellular recovery from NO-induced mitochondrial injury and converted the reversible, NO-induced cytostasis response of cells to an apoptotic response. This viral oncogene-induced phenotypic conversion of the cellular injury response of mouse and human cells was mediated by an E1A-related increase in NO-induced activation of caspase-2, an apical initiator of intrinsic apoptosis. Blocking caspase-2 activation or expression eliminated the NO-induced apoptotic response of E1A-positive cells. These results define an NF-κB-independent pathway through which the E1A gene of human adenovirus sensitizes mouse and human cells to apoptosis by enhancement of caspase-2-mediated mitochondrial injury. The Journal of Immunology, 2008, 180: 8272–8279.

Adenoviruses are common respiratory pathogens, which like many viruses commandeer cell cycle machinery to enhance the efficiency of viral replication. The central adenoviral gene in this activity is the early region 1A gene, E1A, which is a promiscuous modulator of viral and cellular gene transcription and the primary mediator of the cell cycle takeover (1). When expressed in abortive infection or ectopically during stable cellular transfection, E1A functions as an oncogene that immortalizes mammalian cells, similarly to functionally homologous oncogenes of other DNA tumor viruses and cellular oncogenes (2, 3). In addition to its cell cycle control and related cellular immortalizing activities, E1A expression has the side effect of sensitizing cells to apoptosis in response to a variety of immunological, chemical, and physical stimuli (reviewed in Ref. 4). It is partly for this reason that targeted E1A gene delivery has been used in various forms of cancer therapy, including those using oncolytic adenoviruses and liposomal delivery of E1A genes to tumor cells (reviewed in Ref. 5). Despite the well-described E1A-induced sensitivity of tumor cells to apoptosis in response to injury by immune killer cells (NK cells, activated macrophages, and CTL), TNF α, TRAIL, Fas ligand, chemotherapeutic drugs, and irradiation, there is an incomplete understanding of the cellular pathways and molecular mechanisms through which E1A mediates the conversion of cells from the apoptosis-resistant to the apoptosis-sensitive phenotype (reviewed in Ref. 4). One hypothesis is that E1A targets and modulates the activity of one or more apoptotic pathways in expresser cells, as a collateral effect of E1A cell cycle takeover. One candidate for such a pathway is the antiapoptotic, NF-κB activation response that is known to be repressed by E1A, resulting in increased cellular sensitivity to TNF α (reviewed in Ref. 4).

Activated macrophages, one component of the innate immune antitumor defense, repress cellular mitochondrial function, an effect that can result in either tumor cell cytostasis or apoptosis, depending on the cell being targeted (6, 7). NO is the main macrophage cytotoxin that kills E1A-positive tumor cells (8). One reported NO effect on cells is NF-κB activation (9–13), which can defend cells against NO-induced apoptosis (14–16). These observations suggested the hypothesis that the NF-κB activation response is the key pathway that is targeted by E1A to sensitize tumor cells to macrophage-NO-induced apoptosis. We report evidence in this study that this hypothesis is incorrect and that E1A sensitizes cells to NO-induced apoptosis through a mechanism that is independent of E1A-induced NF-κB repression. The data indicate that the alternative mechanism involves E1A-enhanced, NO-induced activation of caspase-2 that may directly injure mitochondria, resulting in triggering of the intrinsic apoptosis pathway (17, 18). These results, combined with those reported previously, indicate that there are two complementary effects of E1A that sensitize tumor cells to macrophage killing and that are involved in the antitumor cell effects of E1A in the context of the host innate immune response.
immune response. One is E1A repression of death receptor-triggered NF-κB activation that could be involved in E1A-induced tumor cell sensitivity to TNF α (19, 20), TRAIL (21–24), and Fas ligand (25). The other, reported in this study, involves NF-κB-independent enhancement of caspase-induced mitochondrial injury inflicted by killer cell cytolytic mechanisms such as NO.

Materials and Methods

Cell lines and cell line characterization

NIH-3T3 cells expressing Ad5 E1A 12S proteins (MT12–1) and human H4 cells (a subclone of the well characterized human fibrosarcoma cell line, HT-1080) expressing genomic E1A (H4-E1A, P2AHT2A) (26, 27) and their derivatives were maintained in DMEM plus antibiotics and 5% calf serum (complete medium). TNF-resistant cell variants (20 ng/ml TNF; Biosource) were selected for at least 2 wk, and their incidence was estimated by colony counting. E1A-positive NIH-3T3 cells that stably over-express NF-κB, p65/RelA were created by retroviral transduction as follows. A murine stem cell virus retroviral vector was used to express GFP and p65/RelA from a bicistronic message, pMSCVRelA-ires-GFP (28), provided by William Sha (University of California, Berkeley, CA), was used (29) to generate the retroviral vector by cotransfection of 293 cells with pBABE-puro (provided by Inder Verma, The Salk Institute, La Jolla, CA), a packaging plasmid expressing structural proteins of Moloney murine leukemia virus (30). E1A, p65/RelA, Bax, cIAP2, Bcl-2 (Santa Cruz Biotechnology) and actin (Sigma-Aldrich) were detected by immunoblotting using ECL. Comparisons of cell protein expression were made by analysis of scanned images of immunoblots using Molecular Dynamics ImageQuant v. 5.1 software.

Bioenergetics assays

Mitochondrial membrane potential (MMP) was estimated by flow cytometry of cells stained with tetramethylrhodamine ethyl ester perchlorate (TMRE; 100 nM; Invitrogen). ATP levels were estimated using SigmaStat 3.11 software (Systat Software). Statistical analysis

Statistical analysis was done using Student’s t test or two-way ANOVA, using SigmaStat 3.11 software (Systat Software).

Results

E1A-positive cell variants exhibiting NF-κB-dependent resistance to TNF remain sensitive to macrophage NO and chemical NO

A few E1A-positive 3T3 cells survived apoptotic killing by recombiant TNF (4.5 ± 0.4 × 10^3 cells; mean ± SEM; n = 3). These TNF-resistant (TR) clones expressed parental-cell levels of E1A (TR1 and TRB1, Fig. 1A) but were as resistant to TNF-induced cytotoxicity as E1A-negative cells (Fig. 1C, untreated group). TR cell variant expression of selected proapoptotic and antiapoptotic proteins was investigated to understand their loss of TNF sensitivity. Loss of both Bak and Bax impairs TNF-induced apoptosis of E1A-positive cells, but expression of either protein is sufficient for apoptosis (33). Bax expression in TR variants at levels comparable to those of parental cells (Fig. 1A, upper panel) excluded Bak/Bax loss as the explanation for their TNF resistance. TRB1 overexpressed NF-κB p65/RelA (Fig. 1A, upper panel; 4-fold increase compared with parental, E1A-positive cells), suggesting that selection for TNF-resistant, E1A-positive cells could coselect for overexpression of antiapoptotic, NF-κB-related proteins.

We have reported that transient overexpression of NF-κB p65/RelA can block TNF-induced apoptosis of E1A-positive cells (20). We therefore tested the phenotypes of E1A-positive cells forced to stably overexpress p65/RelA (Fig. 1A, bottom panel). Two such clonal lines, A1 and B5, retained parental cell levels of E1A but expressed much higher levels of p65/RelA (Fig. 1A, bottom panel). These clones, along with TNF-selected, E1A-positive variants were compared with parental, E1A-positive cells for TNF-induced, NF-κB-dependent transcription responses and for sensitivity to TNF-induced cytotoxicity. Data for a representative clone from
FIGURE 1. Characterization of E1A-positive, NIH-3T3 cell variants selected for TNF resistance (TR1, TRB1) or forced to overexpress NF-κB p65/RelA (A1, B5), compared with E1A-positive (E1A) and E1A-negative (3T3) cell controls. A, Comparative immunoblots of cell lysates for the indicated proteins. B, TNF-induced, NF-κB-dependent transcription responses. Cells were transiently transfected with a κB-luciferase reporter and tested for response to TNF stimulation (20 ng/ml, 4 h). The numbers above bars represent the fold induction of NF-κB-dependent transcription that was significant for the TNF-resistant clones, TR1 and A1 (n = 3; p < 0.05), but not for the TNF-sensitive parental cells, E1A. C, NF-κB-dependent cellular sensitivity to TNF-induced cytotoxicity. TNF-resistant clones, TR1 and A1, were compared with E1A-positive, TNF-sensitive (E1A) and E1A-negative, TNF-resistant (3T3) control cells for sensitivity to TNF (20 ng/ml)-induced radiolabel release, before (“untreated”) and after blockade of cellular protein synthesis by treatment with cycloheximide (CHX) or of TNF-induced NF-κB activation by treatment with the IκB kinase inhibitor, Bay-117085 (Bay). The TNF-resistant, E1A-positive clones, TR1 and A1, were significantly more sensitive to TNF-induced cytotoxicity after repression of the NF-κB-dependent activation response, using both treatments, like E1A-negative 3T3 cells (n = 3, p < 0.05).

FIGURE 2. Sensitivity of TNF-resistant, E1A-positive cell variants to cytotoxicity by macrophage-NO and chemical-NO, compared with macrophage-sensitive, E1A-positive (E1A) and macrophage-resistant, E1A-negative (3T3) control cells as evidenced by radiolabel release. A, Macrophage-NO cytotoxicity. Radiolabeled target cells were incubated on monolayers of activated macrophages at a 50:1 macrophage:target ratio, in the absence or presence of 1 mM L-NAME to block macrophage NO-synthase activity. TNF-resistant TR1, A1, and B5 cells were highly sensitive to NO-dependent macrophage killing (n = 3, p < 0.05). B, Chemical-NO cytotoxicity. Radiolabeled cells were incubated with the NO generator, DETA-NONOate, at a concentration of 250 μM. All TNF-resistant, E1A-positive variants remained significantly more sensitive to NO cytotoxicity than E1A-negative control cells (3T3) (n = 3, p < 0.05).
TNF-resistant, E1A-positive cells was confirmed in experiments using the chemical NO generator, DETA-NONOate (Fig. 2B). These data indicated that the mechanism(s) through which E1A sensitizes cells to NO are independent of E1A repression of NF-kB activation to the level that is sufficient to induce TNF sensitivity.

**E1A sensitization to NO-induced apoptosis is associated with caspase-dependent mitochondrial dysfunction**

NO can induce either apoptosis or necrosis, depending on the cell type tested (35). NF-kB activation is an anti-apoptotic defense, but does not defend against necrotic cell death (36–38). Therefore, it was possible that the NF-kB-independent of the NO sensitivity of E1A-positive cells was because the cells were dying as a result of NO-induced necrosis, not apoptosis. However, flow cytometry analysis showed that the NO-treated, E1A-positive cells died by apoptosis (Fig. 3).

Classical flow cytometry traits of cells undergoing apoptosis—reduced cellular size, increased cellular granularity, and increased chromatin condensation before loss of cell membrane integrity (39)—were used to contrast the quality of the NO-induced, cell death responses of E1A-positive vs E1A-negative cells. Cells were stained with the DNA dye, Hoechst 33342, to measure the increased intensity of nuclear staining associated with chromatin condensation and with the vital dye, PI, to measure loss of cell membrane integrity. Increased NO-induced apoptosis of E1A-positive cells was evidenced by the typical flow cytometry traits: 1) decreased cell size and increased granularity of the majority of the cell population, as revealed by an NO-induced leftward shift in forward light scatter and upward shift up of side scatter, respectively (Fig. 3A, left panel) and 2) markedly increased chromatin condensation before loss of cell membrane integrity (Fig. 3A, right panel); increased percentage of Hoechst-positive/PI-negative cells among E1A-positive cells, upper right panel = 85% vs E1A-negative cells, lower right panel = 5%). In contrast, NO treatment of E1A-negative cells caused reduced size of a smaller subpopulation of cells (forward light scatter), little change in granularity (side scatter), and a simultaneous increase in Hoechst and PI staining of a minority (25%) of cells (Fig. 3A, lower right panel; double-positive cells = 25%), characteristics of necrotic death.

The E1A mechanism(s) of sensitization to NO were considered in the context of NO effects on mitochondrial function because of its central role in the cell death response (Fig. 3B). MMP is the main determinant of mitochondrial respiration and ATP synthesis. NO-induced loss of MMP causes progression to apoptosis (40). The net mitochondrial injury and recovery response of NO-treated, E1A-positive vs E1A-negative cells was contrasted using TMRE fluorescence as an indicator of MMP at 18 h after injury (Fig. 3B). The MMP of NO-injured, E1A-positive cells was reduced in a manner that was caspase dependent, because loss of MMP was blocked by the pan-caspase inhibitor, zVAD (Fig. 3B, left panel); compare shaded histogram (NO-treated) vs dashed-line histogram (zVAD-blocked, NO-treated); solid line histogram = control). In contrast, there was no reduction of MMP of most E1A-negative cells at 18 h after injury (Fig. 3B, lower histogram). This observation of the caspase dependence of the E1A-related blockade of cellular recovery of MMP was observed in repeated experiments (Fig. 3B, right panel; n = 3). A small fraction of NO-injured, E1A-positive cells exhibited increased TMRE fluorescence (Fig. 3B, right-shoulders on histograms), consistent with caspase-independent mitochondrial swelling or hyperpolarization (41, 42). zVAD blockade of a net loss of MMP by E1A-positive cells increased survival and changed the quality of the NO response to one that resembled E1A-negative cells, as assessed by flow cytometry (compare the patterns of NO plus zVAD-treated, E1A-positive cells in Fig. 3C with those of NO-treated, E1A-negative cells in Fig. 3A). This suggested that the E1A-related sensitivity to NO-induced apoptosis resulted from caspase-dependent, irreversible mitochondrial dysfunction (loss of MMP), in contrast to NO-injured, E1A-negative cells that sustained their MMP.

**E1A blocks cellular recovery from NO-induced repression of mitochondrial respiration**

Macrophage-NO represses mitochondrial respiration, resulting in a transient cystostatic effect on tumor cells (6, 43). This same NO-induced, reversible cytostasis was observed with E1A-negative mouse cells in our studies (data not shown). NO reversibly inhibits...
mitochondrial respiration by competing with oxygen at cytochrome oxidase, complex IV in the electron transport chain (44, 45). Cellular recovery from this respiration blockade depends on NO-induced compensatory glycolysis and reversal of F1F0 ATP synthase activity that extrudes protons from the mitochondrial matrix, thereby sustaining the MMP (40, 46). We compared NO effects on cellular ATP levels to investigate the reasons for E1A-related progression to apoptosis. NO caused identical reductions in intracellular ATP levels in E1A-negative cells (p < 0.05). There was a time-related recovery of ATP levels in E1A-negative cells (p < 0.05), but not in E1A-positive cells. ATP levels were repressed at 4 h in E1A-negative cells treated with either 2-deoxyglucose or oligomycin (p < 0.05). B, Comparison of the stimulus-induced glycolytic responses of E1A-positive vs E1A-negative cells at 3 h after treatments. Responses to NO and oligomycin were not significantly different for these two cell types (n = 3).

E1A ENHANCED CASPASE-2 ACTIVATION IN RESPONSE TO NO

The caspase dependence of MMP loss in E1A-positive cells (Fig. 3B) suggested that the E1A effect on the cellular bioenergetic response might involve enhancement of caspase-induced mitochondrial injury in response to NO. NO induces activation of caspase-2 (49, 50), which is unique among initiator caspases, because it directly injures the mitochondria and does not process other caspase zymogens (17, 18, 51). E1A induces increased basal expression of selected caspases, as a result of E1A activation of E2F transcription factors (52). Because the effects of E1A on caspase-2 function and activation were unknown, we tested the requirement for caspase-2 activity in NO-induced apoptosis of E1A-positive mouse and human fibroblast cells (Fig. 5). Initial experiments were used to define optimal concentrations and times of incubation with the NO generator, DETA-NONOate, to detect induction of caspase-2 activity in lysates of the E1A-positive mouse (250 μM, 4 h) and human (750 μM, 6 h) cells. Under these conditions, E1A-positive cells from both species exhibited increased NO-induced caspase-2 activity when contrasted with E1A-negative cells, which showed little or no change in activity compared with that detected in untreated controls (Fig. 5A).

In addition to these E1A-related increases in NO-induced caspase-2 activity in cells from both species, immunoblotting analysis revealed increases in caspase-2 protein expression and processing in mouse cells, under both control and NO-induced conditions (Fig. 5B). There was a 5- to 9-fold increase in the expression of full length and intermediate (p31) forms of caspase-2, respectively, in E1A-positive vs E1A-negative NIH-3T3 cells, in the absence of NO treatment (Fig. 5B, lane 3 vs lane 1, respectively). In addition, there was a barely detectable level of complete processing of caspase-2 to the p12 form in untreated, E1A-positive cells (Fig. 5B, lane 3 vs lane 1; 9-fold increase in intensity of the p12 band). NO treatment of E1A-negative NIH-3T3 cells resulted in no detectable change in the pattern of caspase-2 expression or processing (Fig. 5B, lane 2 vs lane 1, respectively). In contrast, there was increased NO-induced processing of caspase-2 in E1A-positive cells, as evidenced by a 7-fold increase in the fully processed, p12 form, accompanied by a 2-fold decrease in both the full-length and intermediate (p31) forms of caspase-2 in NO-treated (NO+) compared with untreated (NO−), E1A-positive cells (Fig. 5B, lane 4 vs lane 3, respectively). These patterns of caspase-2 expression and processing are similar to those reported previously for NIH-3T3 cells transiently overexpressing caspase-2 (53). There was no consistent change in basal caspase-2 protein expression detected in E1A-positive human cells, when compared with E1A-negative control cells (data not shown). The observations that E1A expression in human fibroblasts induces increased caspase-2 activity in response to NO stimulation (Fig. 5A) in the absence of an E1A-induced increase in caspase-2 protein expression suggests that these may be two independent E1A activities.

To investigate the requirement for E1A-enhanced caspase-2 activation in NO-induced apoptosis, E1A-positive mouse and human
cells were treated, before injury, with the caspase-2-selective, cell permeable pentapeptide inhibitor, zVDVAD-fmk (54) (Fig. 5C). This treatment protected E1A-positive cells from NO-induced apoptosis. Complementary experiments were done using a caspase-2 knockdown, E1A-positive cell line, E1AiC2, created using shRNA technology (Fig. 5D). These cells retained normal E1A expression but had barely detectable caspase-2 expression (Fig. 5D, left panel). The specificity of caspase-2 repression in these cells was confirmed by their continued expression of caspase-3 and caspase-9 (Fig. 5D, left panel). Caspase-2 knockdown significantly reduced E1AiC2 sensitivity to both macrophage killing and chemical-NO-induced apoptosis (Fig. 5D, right panel). These complementary studies with zVDVAD inhibition of caspase-2 and shRNA knockdown of caspase-2 expression indicate that caspase-2 activity is required for E1A-induced enhancement of cellular sensitivity to NO-induced apoptosis, whether mediated by activated macrophages or by injury from chemically generated NO that is independent of any other macrophage cytotoxic mechanisms.

Discussion

These data indicate that E1A enhancement of NO-triggered, caspase-2-mediated mitochondrial injury is a key initiating event in E1A-induced tumor cell sensitization to killing by macrophage-NO. These results define a new pathway through which E1A sensitizes cells to apoptotic injuries. E1A induces an NF-κB-independent, gain-of-function effect, resulting in increased caspase-2 activation, caspase-dependent blockade of mitochondrial respiration recovery after NO injury and increased mitochondria-triggered “intrinsic” apoptosis in response to NO. This E1A activity complements the previously reported E1A-induced loss-of-function effect—repression of the NF-κB activation response—that sensitizes cells to death-receptor-triggered “extrinsic” apoptosis (20).

In our experiments, NO-induced comparable, initial inhibition of respiration of E1A-negative and E1A-positive cells (Fig. 4A). The E1A-related difference in the response to NO involved the cellular response that followed this initial repression of respiration. E1A-negative cells exhibited a glycolysis-dependent recovery of mitochondrial function after NO injury (Fig. 4) and resumed normal growth after a period of cytostasis (data not shown). This is comparable to what has been reported for macrophage-NO and chemical-NO injury of various types of tumor cells (6, 43). In contrast, E1A-positive cells never recovered from NO-induced mitochondrial injury but proceeded to apoptosis, despite unlimited glucose and an intact, compensatory glycolytic response (Fig. 3 and 4B). These results are consistent with the reported relationship between the cellular bioenergetic response to NO and survival and suggest the existence of a caspase-dependent mechanism through which E1A represses the ability of mitochondria to benefit from the MMP-restoring effect of NO-induced compensatory glycolysis (40, 46).

These data suggest that E1A-enhancement of NO-induced caspase-2 activation may be the direct cause of irreversible mitochondrial injury, which then triggers apoptosis. Caspase-2 is unique among initiator caspases (17, 18, 55, 56). It can permeabilize mitochondria, uncouple mitochondrial respiration, and directly trigger release of mitochondrial apoptotic factors. These caspase-2 effects can be independent of Bid cleavage, do not require caspase-2 cleavage of other caspase zymogens, do not require expression of either Bax or Bak, and are unaffected by Bcl-2 overexpression. The E1A-related caspase-2 activation in our assays, detected within the few first hours after NO injury (Fig. 5A), was temporally associated with the failure of recovery of mitochondrial respiration of E1A-positive cells (Fig. 4). In contrast, respiration recovered quickly in E1A-negative cells, most of which survived NO injury and resumed normal growth, after a period of cytostasis.

E1A expression was observed to induce increased caspase-2 activation in both mouse and human cells following NO stimulation (Fig. 5A). However, we could only detect an E1A-related increase in caspase-2 protein expression in mouse fibroblasts (Fig. 5B) and not in human H4 fibroblasts. It has been shown previously that E1A induces overexpression of other caspase proteins (i.e., caspases 3, 7, 8, and 9) in primary mouse fibroblasts and a different human fibroblastic cell line (IMR-90) through the Rb-binding and E2F-enhancing activities of E1A (52). Therefore, whereas one mechanism by which E1A might enhance caspase-2-dependent
cellular activity is induction of increased caspasezymogen expression, our data suggest the existence of another mechanism by which E1A enhances the caspase-2 activation response, independently of effects on caspase protein expression. The question of whether these two, possibly different activities, map to the same E1A domain and cell protein binding activity is the focus of ongoing study.

In addition to its cell sensitizing effect for macrophage-NO injury, E1A enhancement of caspase-2-mediated triggering of the intrinsic, mitochondria-mediated, apoptosis pathway might also be mechanistically involved in E1A-induced sensitization of tumor cells to killing by other proapoptotic injuries that trigger intrinsic apoptosis, as such chemotherapeutic drugs and irradiation (reviewed in Refs. 57 and 58). This E1A effect on caspase-2 activation might also partly explain the increased sensitivity of E1A-expressing tumor cells to perforin/granzyme B-mediated killing by cytolytic lymphocytes (25, 59), because granzyme B can activate caspase-2 (60, 61) and kill tumor cells in a caspase-2-dependent manner (62). Further studies of the molecular mechanisms through which E1A enhances caspase-2 activation in response to diverse proapoptotic stimuli might provide a basis for development of strategies to optimize the use of E1A-expressing, oncolytic adenoviruses for adjuvant therapy of cancer (63). Because most E1A effects are mediated by modulation of cellular gene transcription, studies of the cellular mechanisms through which E1A enhances caspase-2 activation in response to proapoptotic stimuli might also provide a better understanding of the regulatory control of the activation of this initiator caspase.

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Disclosures

The authors have no financial conflict of interest.

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