Oncogenicity of Human Papillomavirus- or Adenovirus-Transformed Cells Correlates with Resistance to Lysis by Natural Killer Cells

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The reasons for the dissimilar oncogenicities of human adenoviruses and human papillomaviruses (HPV) in humans are unknown but may relate to differences in the capacities of the E1A and E7 proteins to target cells for rejection by the host natural killer (NK) cell response. As one test of this hypothesis, we compared the abilities of E1A- and E7-expressing human fibroblastic or keratinocyte-derived human cells to be selectively killed by either unstimulated or interferon (IFN)-activated NK cells. Cells expressing the E1A oncoprotein were selectively killed by unstimulated NK cells, while the same parental cells but expressing the HPV type 16 (HPV-16) or HPV-18 E7 oncoprotein were resistant to NK cell lysis. The ability of IFN-activated NK cells to selectively kill virally transformed cells depends on IFN's ability to induce resistance to NK cell lysis in normal (i.e., non-viral oncogene-expressing) but not virally transformed cells. E1A blocked IFN's induction of cytolytic resistance, resulting in the selective lysis of adenovirus-transformed cells by IFN-activated NK cells. The extent of IFN-induced NK cell killing of E1A-expressing cells was proportional to the level of E1A expression and correlated with the ability of E1A to block IFN-stimulated gene expression in target cells. In contrast, E7 blocked neither IFN-stimulated gene expression nor IFN's induction of cytolytic resistance, thereby precluding the selective lysis of HPV-transformed cells by IFN-activated NK cells. In conclusion, E1A expression marks cells for destruction by the host NK cell response, whereas the E7 oncoprotein lacks this activity.

Human papillomaviruses (HPV) and adenoviruses (Ad) are common human pathogens with a propensity to cause persistent infections. For example, at least 95% of adults are seropositive to one of the group C Ad (65), and in the United States alone, over 12 million sexually active adults are infected with genital tract HPV (38). Although evolutionarily distinct, Ad and HPV share one important feature: both viruses transform mammalian cells (including human cells) through the expression of viral oncoproteins (HPV E7 and E6; Ad E1A and E1B) that exhibit strikingly similar biological activities (18, 19, 49, 60, 70, 71, 75, 76).

In HPV-induced human malignancies (2, 61, 64) or Ad-transformed cells (29, 34), there is viral integration into the host genome, and the expression of two viral genes (HPV E6 and E7; Ad E1A and E1B) is consistently found. Although expression of E6 and E7 or E1A and E1B is required for the efficient transformation of primary cells, either the HPV E7 gene (32) or Ad E1A gene (29, 34) alone is sufficient for the immortalization of primary cells and therefore represents the major transforming gene of HPV or Ad, respectively.

The E1A oncoprotein exhibits both functional and amino acid homology with the E7 oncoprotein. Amino acids encoded within the homologous regions of E1A and E7 (conserved region 1 [CR1] and CR2) serve as sites of high-affinity binding to specific cellular proteins (p105 or the retinoblastoma gene product; p107, p130, and cyclin A) (18, 20), thereby transducing the many biological activities shared by these viral oncoproteins (e.g., transactivation of the E2 promoter through the generation of free E2F, cooperation with an activated *ras* on-

cogene to transform baby rat kidney cells, blocking transforming growth factor type β -induced transrepression of the *c-myc* promoter, and induction of cellular apoptosis) (35, 45, 48–50, 52).

Although genetically unrelated, E6 oncoproteins derived from the oncogenic HPV and Ad E1B p53 oncoproteins both inhibit the biological activities of the tumor suppressor gene p53. The HPV E6 oncoprotein complexes with the cellular protein p53, leading to its rapid degradation (60), whereas the E1B p55 binds to p53, sequestering p53 from the nucleus and blocking p53 transactivation function (75, 76).

However, despite their ubiquity and competence to transform human cells, Ad appear to be nononcogenic in humans (30), whereas HPV have been linked to as many as 90% of cervical carcinomas (77). The reasons for the dissimilar oncogenicities of Ad and HPV in humans are unknown, but an analogous situation exists with Ad-induced tumors in rodents. Although group A (Ad serotype 12 [Ad12]) and group C (Ad2 and Ad5) Ad are equally immunogenic and transform rodent cells with equal efficiency (25, 39), only group A Ad are tumorigenic in immunocompetent rodents. In studies comparing group A and group C Ad, the tumorigenicity of the different Ad groups in rodents appears to be related to the susceptibility (Ad2 and Ad5) or resistance (Ad12) of Ad-infected or Ad-transformed cells to elimination by natural killer (NK) cells, a process regulated by differences between Ad12 E1A and Ad2 and Ad5 E1A (11, 12, 14, 40, 59, 62).

On the basis of these studies in rodents, we speculated that NK cells would also be an important immunologic defense in limiting adenoviral infections and in preventing Ad-induced malignancy in humans. Studies performed to date using human cells are consistent with this hypothesis. Non-cytokine-activated NK cells selectively kill Ad-transformed cells but not

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Ad-infected cells (56, 57), whereas interferon (IFN) induces the discriminate killing of both infected and transformed cells by IFN-activated NK cells (53, 54). In both of these instances, the selective NK cell lysis of either Ad-infected or Ad-transformed cells is dependent on the expression of E1A gene products.

The purpose of this study was to compare the abilities of the E1A and E7 oncoproteins to induce the discriminate lysis of Ad- or HPV-transformed human cells by either unstimulated or IFN-activated NK cells. Whereas the stable expression of Ad5 E1A oncoprotein in cytolytic resistant fibroblastic or keratinocyte-derived human cells induced an increased susceptibility to lysis by unstimulated (non-cytokine-activated) NK cells, the HPV type 16 (HPV-16) or HPV-18 E7 oncoprotein lacked this activity. IFN(α , β , and γ) increase the cytolytic capacity of NK cells. However, the selectivity of killing by IFN-activated NK cells depends on the induction of cytolytic resistance on to NK cell killing on normal (i.e., non-viral oncogene-expressing) but not virally transformed cells (58). Experiments reported here showed that IFN- γ treatment reduced the IFN-activated NK cell killing of both E7-expressing and non-E7-expressing cells to equivalent degrees, thereby precluding the selective lysis of HPV-transformed cells by IFN-activated NK cells. In contrast, E1A expression blocked IFN's induction of cytolytic resistance, thereby permitting the selective killing of E1A-expressing cells by IFN-activated NK cells. IFN's capacity to induce cytolytic resistance is likely secondary to its capacity to transcriptionally activate selected cellular genes (i.e., IFN-stimulated genes). Therefore, the differences in the capacities of E1A and E7 to block IFN's induction of cytolytic resistance may reflect dissimilarities in their abilities to block IFN-stimulated gene expression. In support of this hypothesis, we found that E1A but not E7 expression blocked the capacity of IFN- γ to increase IFN regulatory factor 1 (IRF-1) transcription. On the basis of these results, we speculate that the NK cell response would form an effective immunologic barrier against the escape and neoplastic progression of Ad- but not HPV-transformed cells in vivo. The differences in the capacities of the E1A and E7 proteins to target cells for destruction by the host NK cell response may contribute to the dissimilar oncogenicities of Ad and HPV in humans.

MATERIALS AND METHODS

Cells and cell lines. CaSki, SiHa, HeLa, and HeLa S3 are HPV-16 (CaSki and SiHa)- and HPV-18 (HeLa and HeLa S3)-transformed cervical carcinoma cell lines (74). M18 is an Ad5 E1A-transfected HeLa cell line provided by S. Frisch (La Jolla Cancer Research Center, La Jolla, Calif.) (23). A431 is a non-HPV-expressing vulvar epidermoid carcinoma cell line (74). HeLa c-env is a HeLa cell line which stably expresses the neomycin selection gene and envelope gene of human immunodeficiency virus type 1 and was provided by Miguel Gama Sosa and Ruth Reprecht (Dana-Farber Cancer Institute, Boston, Mass.) (26). HFK-1321 is a human epidermal keratinocyte cell line transformed by a plasmid containing the HPV-16 E6 and E7 genes expressed from a human β -actin promoter. 18NCO is a human epidermal keratinocyte cell line transformed by a plasmid containing the full HPV-18 genome linearized at a unique *NcoI* site. Both HFK-1321 and 18NCO were provided by Karl Munger (Harvard Medical School, Boston, Mass.) (44). IC4 is a HeLa S3 cell line transfected with the Ad2 E1A gene under the control of the murine mammary tumor virus promoter and was provided by A. Berk (University of California, Los Angeles) (4). IC4-D10 is a subclone of IC4 obtained by limiting dilution (58). WI-38 is a human embryonal diploid fibroblast cell line. A431, WI-38, HeLa S3, HeLa, SiHa, and CaSki were obtained from American Type Culture Collection (Rockville, Md.).

H4 is a subclone of the HT1080 fibrosarcoma cell line. P2AHT2A is an Ad5 E1A-transfected H4 cell line (23). H4-E7 clone 1 (CL1) and CL2 are H4 cells which stably express the HPV-16 E7 gene following transfection with pLSXN16E7. WI-38 E7 CL2 and CL4 are WI-38 cells transformed by the HPV-16 E7 and E6 genes following transfection with plasmid pLSXN16E7/16E6. pLSXN16E7 and pLSXN16E7/16E6 contain the HPV-16 E7 and HPV-16 E7 and E6 genes, respectively, under the control of the Moloney murine leukemia virus long terminal repeat and the neomycin selection gene (32). H4-E7 CL1 and

CL2 and WI-38 E7 CL2 and CL4 were cloned by limiting dilution from G418-resistant colonies selected from separate plates. pLSXN16E7/16E6 and pLSXN16E7 were provided by D. Galloway (Fred Hutchinson Cancer Center, Seattle, Wash.).

HFK-1321 and 18NCO were maintained in keratinocyte growth medium (Clenetics, San Diego, Calif.). SiHa and CaSki were maintained in RPMI 1640–10% fetal calf serum (FCS) plus antibiotics. A431, H4, H4-E7 CL1 and CL2, P2AHT2A, HeLa, HeLa S3, HeLa c-env, and IC4-D10 were maintained in Dulbecco modified Eagle medium supplemented with antibiotics, 15 mM glucose, and 5% FCS. WI-38 and WI-38 E7 CL2 to CL4 were maintained in Dulbecco modified Eagle medium supplemented with antibiotics, 15 mM glucose, and 10% FCS. IC4-D10 was incubated with dexamethasone added at the indicated concentrations to standard culture medium for 5 days to induce maximal E1A expression. Cell lines were periodically tested for mycoplasma contamination by the Mycotect assay (Bethesda Research Laboratories, Bethesda, Md.) and were negative.

NK cell cytotoxicity assays. Cytotoxicity assays using non-cytokine-activated NK cells were performed as previously described (13). Briefly, Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.)-separated peripheral blood mononuclear cells served as the source of NK cells. Target cells were labeled with ^{51}Cr (100 mCi/ml for 1 h; 1 Ci = 37 GBq). NK cells and target cells (10^4) were cosedimented at different effector-to-target cell ratios in flat-bottom microtiter plates by centrifugation at $500 \times g$ for 3 min, and after a 4-h incubation at 37°C in 5% CO_2 , half of the supernatant from each well was harvested and counted in a gamma counter. NK cell killing was determined by calculating the percentage of NK cell-induced release of radiolabel from target cells as described previously (13). The results presented represent the means $\pm$ standard errors of the means (SEM) of at least four separate experiments. The mean percentage spontaneous release from all target cells, including those infected with Ad, was less than 15%.

For cytotoxicity assays using IFN-activated NK cells, Ficoll-Paque-separated peripheral blood mononuclear cells were incubated for 16 h with 10,000 U of recombinant IFN- $\alpha 2$ (Schering Corp., Kenilworth, N.J.) per ml. We have previously shown that this concentration of IFN- $\alpha 2$ results in optimal activation of human NK cells (53). Other studies from this laboratory (data not shown) and by other authors (21, 68) demonstrate that the cytolytic activity in this population of cells as measured in 4-h cytotoxicity assays is mediated by CD-16 positive NK cells. Target cells were treated with 10,000 U of recombinant IFN- γ (Genentech, San Francisco, Calif.) per ml for 24 h. Prior studies from this laboratory have shown that this concentration of IFN- γ induces maximal cytolytic resistance (53). With the exception of IFN treatment, these cytotoxicity assays were then performed as described in the prior paragraph. In cytotoxicity assays which used cells incubated with dexamethasone, the cells were extensively washed with phosphate-buffered saline (PBS) prior to the labeling step. No exogenous dexamethasone was present during the cytotoxicity assay. The results presented represent the means $\pm$ SEM of at least four separate experiments. The mean percent spontaneous release from all target cells was similar and always less than 15%.

Western blot (immunoblot) analysis of viral proteins. For quantitation of E1A proteins, 60-mm-diameter plates of IC4-D10 cells were incubated with log dilutions of dexamethasone (10^{-6} , 10^{-7} , 10^{-8} , and 10^{-9} M) for 5 days and then incubated for 16 h with IFN- γ (10,000 U/ml) in the continued presence of dexamethasone. Cells were lysed in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 7.4], 150 mM NaCl), and protein concentrations of radioimmunoprecipitation assay supernatants were determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.). Equal amounts of protein from all cell lysates were separated on SDS–10% polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, Calif.). Each membrane was blocked in 5% nonfat milk solution and incubated with the anti-E1A monoclonal antibody M73 (33), supplied by E. Harlow (Massachusetts General Hospital, Charleston), for 1 h. Following several washes with PBST (PBS with 0.05% Triton X-100), the membranes were incubated for 1 h with rabbit anti-mouse antibody (Cappel, Durham, N.C.), washed extensively with PBST, and incubated with ^{125}I -labeled protein A (ICN, Costa Mesa, Calif.). Following several washes with PBST, the bands were visualized by fluorography.

Quantitation of E7 proteins was identical to the procedure used for E1A, with the following exceptions: the cell lysis buffer contained 20 mM Tris (pH 7.5), 50 mM NaCl, 0.5% SDS, 0.5% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, 1% aprotinin, and 1 μM phenylmethylsulfonyl fluoride; following incubation with the anti-E7 monoclonal antibody (Triton Diagnostics, Alameda, Calif.), the E7 protein was visualized as instructed by the manufacturer, using a Renaissance chemiluminescence kit (DuPont-NEN, Boston, Mass.).

Northern (RNA) analysis. Cells (5×10^6 to 10×10^6) were incubated with 5,000 U of recombinant IFN- γ (Genentech) per ml for 4 h. RNA was prepared by the NP-40 lysis method (43). Northern analysis was performed as described previously (67), with only slight modifications. Briefly, 10 μg of total RNA was separated by electrophoresis in a 1.5% agarose gel containing formaldehyde, photographed to assess RNA quantity and quality, and transferred to a nylon membrane (MSI hybridization transfer membrane) by capillary blotting. The membrane was baked at 80°C for 2 h and hybridized to a ^{32}P -labeled cDNA probe at 42°C in $5\times$ Denhardt's solution–50% formamide– $5\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.05 mg of salmon sperm DNA per ml,–50 mM NaPO_4 –1.0% SDS (pH 6.5). After overnight hybridization, the nylon

FIG. 1. Areas of conserved region homology between E1A and HPV E7. The first 140 amino acids of the E1A oncoprotein are shown, and the locations (amino acid numbers) of CR1 and CR2 are indicated. Areas of amino acid homology between E1A and E7 are indicated by black bars with corresponding amino acid numbers.

membrane was washed three times at room temperature in $3\times$ SSC-1% SDS and then subjected to a final wash in $0.1\times$ SSC-0.1% phosphate-1% SDS at 65°C . Autoradiography was carried out overnight at -70°C , using an intensifier screen.

The human cDNA probe for the IFN- γ -regulated gene, encoding IRF-1, was provided by A. Deisseroth (M. D. Anderson Cancer Center, Houston, Tex.). The human cDNA probe for glyceraldehyde phosphate dehydrogenase (GAPDH) was provided by J. Domenico and E. Gelfand (National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.). Both cDNA probes were labeled by the random primer method.

RESULTS

HPV E7 expression does not block IFN's induction of cytolytic resistance in keratinocyte-derived cell lines. The discriminate killing of Ad-infected or Ad-transformed human cells by IFN-activated NK cells is dependent on IFN's dissimilar effects on normal (i.e., non-viral oncogene-expressing) cells in comparison with Ad-infected or Ad-transformed cells (53, 54). On normal cells, IFN induces an enhanced state of cytolytic resistance to killing by IFN-activated NK cells. In contrast, E1A expression blocks IFN's induction of cytolytic resistance, thereby focusing NK lytic activity exclusively on Ad-infected or Ad-transformed cells. E1A genetic mapping studies (54, 55) suggest that the capacity of E1A to block IFN's induction of cytolytic resistance depends on the expression of either or both CR1 and CR2 of E1A, regions homologous with E7 oncoprotein and linked to many of their shared biological activities (Fig. 1).

The first series of NK cytotoxicity assays determined if the E7 oncoprotein would also block IFN's induction of cytolytic resistance in manner similar to that of E1A. Because HPV appear to infect cells exclusively of keratinocyte origin, our initial studies used keratinocyte-derived cell lines. The cervical cancer cell lines CaSki, SiHa, and HeLa express the E7 and E6 genes derived from HPV-16 (CaSki and SiHa) or HPV-18 (HeLa). HFK-1321 and 18NCO are human epidermal keratinocytes transformed by a plasmid containing the HPV-16 E6 and E7 genes expressed from a human β -actin promoter (HFK-1321) or the full HPV-18 genome linearized at a unique *NcoI* site (18NCO).

Cells either naturally transformed by HPV (Fig. 2A) or transformed by transfection with HPV (Fig. 2B) were sensitive to lysis by IFN-activated NK cells. However, treatment of these HPV-transformed cell lines by IFN- γ resulted in a marked reduction of NK cell killing (Fig. 2). The magnitude of the IFN-induced reduction in NK cell killing of HPV-transformed cell lines was similar to the reduction measured with A431, a non-HPV-expressing vulvar carcinoma cell line (Fig. 2).

Inhibition of cytolytic resistance in keratinocyte-derived cell lines by E1A is expression level dependent. The next series of experiments compared the abilities of E7 and E1A to block cytolytic resistance in a keratinocyte-derived cell line. We also determined if there was a relationship between the level of E1A expression and the magnitude by which E1A blocks IFN's

FIG. 2. IFN-activated NK cell killing of HPV-transformed, keratinocyte-derived cells. Effector:target ratio indicates the ratio of IFN-activated peripheral blood mononuclear cells to targets cells. Target cell killing is expressed as the percentage (mean $\pm$ SEM) of NK cell-induced release of incorporated ^{51}Cr from target cells in four 4-h cytotoxicity assays. (A) NK cell lysis of IFN- γ -treated (open squares, circles, and triangles) and untreated (closed squares, circles, and triangles) HPV-16- (CaSki and SiHa) or HPV-18 (HeLa)-transformed cervical cancer cells. (B) Comparison of the induction of cytolytic resistance by IFN- γ on HPV-expressing (HFK-1321 and 18NCO) or non-HPV-expressing (A431) human keratinocyte-derived cell lines.

induction of cytolytic resistance. IC4 is a HeLa S3 cell line transfected with the Ad2 E1A gene under the control of the murine mammary tumor virus promoter. IC4-D10 is a subclone of IC4 obtained by limiting dilution in which the level of E1A induced is dexamethasone concentration dependent and which exhibits strict dexamethasone inducibility; there is no expression of E1A in the absence of exogenous dexamethasone, with 100% of the cells expressing E1A following treatment with 10^{-6} M dexamethasone for 24 h (58).

Initial experiments determined if IFN- γ treatment altered the levels of E1A proteins induced following stimulation of IC4-D10 cells with dexamethasone. Previous studies show that incubation of IC4-D10 cells with dexamethasone induces E1A expression in a time-dependent manner, with maximal levels of E1A protein present following 5-day dexamethasone treatment (58). Therefore, IC4-D10 cells were incubated in the presence of dexamethasone (10^{-6} , 10^{-7} , 10^{-8} , and 10^{-9} M) for 5 days and then incubated for an additional 16 h with IFN- γ (in the continued presence of dexamethasone). E1A expression was measured by Western analysis using monoclonal antibody M73. E1A protein levels in dexamethasone-stimulated IC4-D10 cells were unaffected by IFN- γ treatment (Fig. 3 and data not shown). Dexamethasone induced E1A protein expression in IFN- γ treated IC4-D10 cells in a concentration-dependent manner. Dexamethasone at 10^{-6} M (Fig. 3) or 10^{-7} M (data not shown) induced maximal, equivalent levels of E1A proteins in IC4-D10 cells, whereas only trace amounts of E1A could be detected following incubation with 10^{-9} M dexameth-

FIG. 3. Induction of E1A expression in IC4-D10 cells by dexamethasone. IC4-D10 cells were incubated in the presence of dexamethasone (DEX; 10^{-6} , 10^{-8} , or 10^{-9} M) for 5 days to induce E1A expression and then incubated for an additional 16 h with IFN- γ (in the continued presence of dexamethasone). The amount of E1A protein expressed in IC4-D10 cells was then assessed by Western analysis and compared with the level in Ad5 E1-transformed human embryonic kidney cell line 293.

asone upon prolonged exposure of the autoradiograph (data not shown). These data agreed with our previously published results for IFN-untreated, dexamethasone-stimulated IC4-D10 cells (58).

NK cell cytotoxicity assays showed that dexamethasone treatment (10^{-6} M for 5 days) of HeLa S3 cells did not alter the ability of IFN to induce cytolytic resistance to NK cell lysis (Fig. 4A). Control experiments were also performed with dexamethasone-treated and untreated G418-resistant HeLa S3 cells (HeLa S3-neo) or HeLa cells stably expressing both the HIV-1 envelope gene and G418 resistance (HeLa c-env), with equivalent results (data not shown). In contrast, the expression

FIG. 4. IFN-activated NK cell killing of E1A-expressing, keratinocyte-derived cells. (A) HeLa or IC4-D10 cells were treated with 10^{-6} M dexamethasone (DEX) for 5 days and then incubated for an additional 16 h with IFN- γ (in the continued presence of dexamethasone). The cells were then used as targets in standard 4-h cytotoxicity assays. (B) IC4-D10 cells were treated with different concentrations of dexamethasone (10^{-6} , 10^{-8} , and 10^{-9} M) for 5 days and then incubated for an additional 16 h with IFN- γ (in the continued presence of dexamethasone). The cells were then used as targets in standard 4-h cytotoxicity assays.

FIG. 5. IFN-activated NK cell killing of E7- or E1A-expressing fibroblastic cell lines. (A) Comparison of the cytolytic resistance induced by IFN- γ on the fibrosarcoma cell line H4, E1A-transfected H4 cells (P2AHT2A), or HPV-16 E7-transfected H4 cells (H4-E7 CL1 and CL2). (B) Induction of cytolytic resistance by IFN- γ on the normal diploid fibroblast cell line WI-38 and HPV-16-transformed WI-38 cells (CL2 and CL4).

of E1A gene products in dexamethasone-treated IC4-D10 cells blocked IFN's induction of cytolytic resistance (Fig. 4A). The magnitudes by which IFN reduced NK cell killing were compared in IC4-D10 cells stimulated with 10^{-6} , 10^{-8} , or 10^{-9} M dexamethasone. These cytotoxicity assays (Fig. 4B), in conjunction with E1A levels measured by immunoblotting (Fig. 3), demonstrated that the extent to which E1A inhibited cytolytic resistance was proportional to the level of E1A expression.

E1A but not E7 expression blocks IFN's induction of cytolytic resistance in fibroblastic cell lines. The previous experiments used either cancer cell lines naturally transformed by HPV or primary human keratinocytes transformed through the use of plasmids expressing the E7 and E6 genes. In both cases, the parental cell is of keratinocyte origin. Therefore, it was possible that the E6 oncoprotein or keratinocyte-specific factors interfered with the capacity of the E7 oncoprotein to block cytolytic resistance. To address this question and to further compare the abilities of E1A and E7 to block IFN's induction of cytolytic resistance, cytotoxicity assays were performed on the fibrosarcoma cell line H4, E1A-transfected H4 cells (P2AHT2A), and HPV-16 E7-transfected H4 cells (H4-E7 CL1 and CL2) (see Materials and Methods for construction of the H4-E7 cell lines). IFN- γ treatment of H4 cells and E7-transfected H4 cells resulted in equivalent, approximately 50% reductions in killing by IFN-activated NK cells in comparison with untreated controls. In contrast, there was no consistent reduction in NK cell killing of IFN- γ -treated P2AHT2A cells (Fig. 5A).

Finally, to determine if HPV E7 expression had any effect in blocking cytolytic resistance in normal, diploid fibroblastic cells, WI-38 cells were transformed by transfection with pLSXN16E6/16E7. Two colonies from separate plates were randomly selected, cloned by limiting dilution, and used in subsequent NK cytotoxicity assays (see Materials and Methods for

FIG. 6. HPV-16 E7 protein levels in HPV-16-transformed keratinocyte-derived and fibroblastic cell lines. CaSki, HFK-1321, and WI-38-E7 CL1 and CL2 (A) or SiHa and H4-E7 CL1 and CL2 (B) cells were treated with IFN- γ for 16 h, and levels of HPV-16 E7 oncoprotein were measured by Western analysis. The use of CaSki cells in each immunoblot allowed for comparison of E7 levels between different cell lines.

construction of this cell line). As illustrated in Fig. 5B, E7 expression did not effect the capacity of IFN to induce cytolytic resistance, with equivalent reductions in NK cell killing of IFN- γ -treated, parental WI-38 cells and HPV-transformed WI-38 cells.

In summary, treatment of E1A-expressing keratinocyte-derived (Fig. 4) or fibroblastic (Fig. 5) cells with IFN- γ did not result in a reduction in target cell killing by IFN-activated NK cells. In contrast, IFN induced equivalent, two- to fourfold reductions in the NK cell lysis of E7-expressing and non-HPV (non-viral oncogene)-expressing keratinocyte-derived or fibroblastic cells. Because the ability of IFN-activated NK cells to selectively lyse virally transformed cells depends on a preferential induction of cytolytic resistance on nontransformed cells, the inability of E7 to block cytolytic resistance precludes the selective lysis of HPV-transformed cells by IFN-activated NK cells.

Effect of IFN- γ treatment on E7 expression in E7-expressing fibroblastic and keratinocyte-derived cell lines. IFN- γ treatment of some HPV-transformed human cervical cancer cell lines can decrease the expression of the E7 and E6 oncoproteins (46, 73). Therefore, given the previously established relationship between the level of E1A expression and the magnitude of E1A's inhibition of cytolytic resistance, it seemed possible that the IFN- γ -induced downregulation of E7 expression could explain the inability of E7 to block IFN's induction of cytolytic resistance in the cell lines used in this study. However, this explanation appeared unlikely because many of these cell lines (HFK-1321, WI-38 E7 clones, and H4-E7 clones) were transformed by plasmids in which E7 gene expression is driven by the IFN-insensitive promoters. To directly test this possibility, E7 oncoprotein levels were measured by immunoblotting in the HPV-16 E7-expressing human cell lines (CaSki, HFK-1321, SiHa, WI-38 E7 CL1 and CL2, and H4-E7 CL1 and CL2). HPV-16 cell lines were exclusively examined because of the lack of commercially available E7 antibodies for HPV-18. IFN- γ did not decrease E7 expression in the E7-transfected H4 or WI-38 cells, CaSki cells, and HFK-1321 cells, whereas a small reduction was seen in SiHa cells (Fig. 6). However, E7 levels differed considerably among the cell lines tested. CaSki cells and the H4-E7 clones expressed extremely high levels of E7, with lower but still easily detectable amounts in the WI-38

FIG. 7. E1A but not E7 blocks the capacity of IFN- γ to transcriptionally activate IRF-1. H4, E1A-transfected H4 (P2AHT2A), and E7-transfected H4 (H4-E7 CL1 and CL2) cells were treated for 4 h with IFN- γ (5,000 U/ml), and IRF-1 and GAPDH mRNA levels were determined by Northern analysis.

E7 clones, SiHa cells, and HFK-1321 cells (Fig. 6). In conjunction with the previously shown cytotoxicity assays, these immunoblots demonstrated that IFN's induction of cytolytic resistance was unaffected by even high levels of E7 expression.

E1A but not E7 expression blocks IFN-stimulated gene expression. Previous studies show that E1A blocks IFN-stimulated gene expression, a process dependent on the inability to form an activated IFN-stimulated gene factor 3 complex in E1A-expressing cells (31, 36). We speculate that the capacity of IFN to induce cytolytic resistance is related to its capacity to transcriptionally activate selected cellular genes (IFN-stimulated genes). Therefore, the difference in the capacities of E1A and E7 may be secondary to dissimilarities in their respective capacities to block IFN-stimulated gene expression.

As one test of this hypothesis, the capacity of IFN- γ to transcriptionally activate the IRF-1 gene was measured in H4, P2AHT2A, and H4-E7 CL1 and CL2. The H4-derived cell lines were used for these studies because they allowed a direct comparison of E1A and E7 functions in the same parental background. Furthermore, high levels of the E7 oncoprotein were present in the H4-E7 clones, levels unaffected by IFN- γ treatment (Fig. 6B). IRF-1 was chosen because its expression is tightly regulated at the level of transcription by IFN- γ , with low mRNA levels expressed in the uninduced, basal state (63). mRNA levels for GAPDH, a constitutively expressed housekeeping gene, were also measured and served as an internal control.

For these studies, H4, P2AHT2A, and H4-E7 CL1 and CL2 cells were incubated with IFN- γ (5,000 U/ml) for 4 h, and IRF-1 and GAPDH mRNA levels were measured by Northern analysis. As illustrated in Fig. 7, the expression of E1A gene products in H4 cells (P2AHT2A) markedly diminished the capacity of IFN- γ to increase IRF-1 gene expression, whereas E7 expression had no effect. Similarly, we have found that IFN- γ markedly increases IRF-1 gene expression in CaSki cells, SiHa cells, and the WI-38 E7 clones (55).

E1A but not E7 expression sensitizes cells to lysis by unstimulated NK cells. Previous studies show that stable expression of the Ad5 E1A gene in human cells results in an increased cytotoxic susceptibility to lysis by unstimulated NK cells or cloned NK cell lines (57). Therefore, we next determined if HPV-16 or HPV-18 E7 expression in either keratinocyte-derived or fibroblastic cell lines would sensitize these cells to lysis by unstimulated NK cells and compared the capacities of E1A and E7 to induce this effect. Because dexamethasone altered

FIG. 8. Lysis of E7- or E1A-expressing keratinocyte-derived cell lines by unstimulated NK cells. Shown are comparisons of NK cell killing (mean $\pm$ SEM of four experiments) of HPV E7-expressing cell lines (CaSki, SiHa, HeLa, HFK-1321, and 18NCO), E1A-transfected HeLa cells (M18), or the non-HPV-expressing vulvar carcinoma cell line A431. Non-cytokine-activated peripheral blood mononuclear cells served as the source of NK cells for these assays.

FIG. 9. Lysis of E7- or E1A-expressing fibroblastic cell lines by unstimulated NK cells. Shown are comparisons of NK cell killing of the fibrosarcoma cell line H4, E1A-transfected H4 cells (P2AHT2A), and HPV-16 E7-transfected H4 cells (H4-E7 CL1 and CL2) (A) and of WI-38 cells and HPV-16-transformed WI-38 cells (WI-38 CL2 and CL4) (B).

the cytolytic susceptibility of untransfected HeLa S3 cells to lysis by unstimulated NK cells, HeLa cells and E1A-transfected HeLa cells (M18 cells) were substituted for IC4-D10 cells in these cytotoxicity assays. HPV-16 or HPV-18-expressing cervical cancer cell lines (Fig. 8A) or keratinocytes transformed by plasmids containing the HPV-16 or HPV-18 E6 and E7 genes (Fig. 8B) were resistant to lysis by NK cells, with levels of cytotoxicity similar to those of the non-HPV-expressing epidermoid carcinoma cell line A431. Furthermore, H4-E7 CL1 and CL2 and WI-38 E7 CL2 and CL4 exhibited the same extent of cytotoxic resistance as control parental cells (Fig. 9). In contrast, the expression of E1A gene products in either HeLa cells (Fig. 8A) or H4 cells (Fig. 9A) results in a more than threefold increase in cytotoxic susceptibility compared with parental cells.

DISCUSSION

Experiments reported here showed differences in the capacities of E1A and E7 oncoproteins to target cells for lysis by NK cells. In contrast to E1A, E7 expression neither increased the cytotoxic susceptibility of target cells to lysis by NK cells nor blocked IFN's induction of cytotoxic resistance. We speculate that both of these functions, which are present in E1A and absent in E7, represent unique activities encoded by distinct regions of E1A. This conclusion is based on the following observations.

Studies using rodent cells show that the expression of the first exon of E1A is insufficient to induce susceptibility to NK cells lysis (14). In contrast, expression of E1A first exon in human cells blocks IFN's induction of cytotoxic resistance (54). Human cell lines transformed by Ad or stably transfected with the E1A gene are susceptible to lysis by unstimulated NK cells,

whereas Ad-infected human cells are resistant (56, 57). In contrast, the expression of E1A gene products in either Ad-infected, Ad-transformed, or E1A-transfected human cells blocks IFN's induction of cytotoxic resistance (53, 54).

Finally, we believe that the ability of E1A to block IFN's induction of cytotoxic resistance is related to E1A's capacity to block IFN-stimulated gene expression (1, 31, 36). It seems unlikely that the capacity of E1A to block IFN-stimulated gene expression, which is based on the inability to form an activated IFN-stimulated gene factor 3 complex in IFN-stimulated cells (31, 36), would also be related to E1A's induction of cytotoxic susceptibility.

It is this latter activity of E1A, the ability to block IFN-stimulated gene expression, that likely explains the dissimilar capacity of IFN to induce the selective NK cell lysis of Ad- but not HPV-transformed cells. The capacity of IFN-activated NK cells to kill virally transformed cells rests on the ability of IFN to induce cytotoxic resistance on normal but not virally transformed cells (54). Because E7 does not block IFN-stimulated gene expression, cytotoxic resistance is induced to the same extent as in normal cells. E1A genetic mapping studies suggest that the ability of E1A to block IFN-stimulated gene expression and cytotoxic resistance (55). To date, we and other investigators have been unable to show that E7 binds p300. On the basis of these findings, we believe that the inability of E7 to block cytotoxic resistance and IFN-stimulated gene expression is secondary to the inability of the E7 oncoprotein to bind p300.

Previous results determining the sensitivity of HPV-transformed cells to lysis by unstimulated NK cells have been contradictory. The cell line Sk-v, an HPV-16-transformed vulvar carcinoma, is sensitive to NK cell lysis, a process dependent on activation of NK cells secondary to tumor cell release of IL-6

(41, 42). In contrast, Furbert-Harris et al. reported that human cervical epithelial cells isolated from two separate individuals and then immortalized by HPV-16 remained resistant to lysis by non-cytokine-activated NK cells (24). Our results are in agreement with those of Furbert-Harris et al. and suggest that the observation regarding the Sk-v cell line is the exception rather than the rule.

The lack of a consistent relationship between the capacity of a virus to transform cells in vitro and the oncogenicity of either the virus or the virally transformed cells in vivo is a well-known but poorly understood phenomenon. For example, the papovavirus simian virus 40 (SV40) can transform cells from a number of species, including rodents, primates, and humans, but is oncogenic in only Syrian hamsters, mastomys, and perhaps humans (3, 7, 8). Group A (Ad12) and group C (Ad2 and Ad5) Ad can transform cells from a variety of species, including rodents and humans; however, only group A Ad is oncogenic in immunocompetent rodents, and neither group is oncogenic in humans.

These observations suggest that the adequacy of the host immune response to eliminate virally transformed cells must be critically important in determining the oncogenicity of a transformation-competent virus. This hypothesis is supported by numerous studies demonstrating that certain viruses and virally transformed cells are oncogenic only in immunodeficient animals. The cellular immune response appears to be the dominant host defense involved in the recognition and elimination of virally transformed cells. There are two components of cellular immunity that likely act synergistically in clearing tumor cells: specific cellular immunity, mediated primarily by cytotoxic T lymphocytes, and nonspecific cellular immunity, consisting of both macrophages and NK cells.

Nonspecific cellular immunity appears to play a particularly important role in the elimination of Ad- and SV40-transformed cells. A number of studies have correlated the cytolytic sensitivity or resistance of either SV40- or Ad-transformed cells to lysis in vitro by either macrophages and NK cells or NK cells alone with their respective tumor-inducing capacities. For example, SV40-transformed mouse and rat cells, which are not tumorigenic, are susceptible to killing by both activated macrophages and NK cells, whereas highly tumorigenic SV40-transformed hamster cells are resistant to both (9, 10, 22). Rodent cells either infected or transformed by the nononcogenic group C Ad are sensitive to both macrophage and NK cell lysis, whereas cells infected or transformed by the oncogenic Ad are resistant to NK cell lysis but sensitive to killing by activated macrophages (10, 11, 40, 59, 62). Direct evidence for the importance of NK cells is provided by observations in rodents showing that NK cells can reject E1A-expressing sarcoma cells in vivo in the absence of thymus-dependent responses (15, 72). The results of this study, therefore, are consistent with and extend to HPV the prior observations regarding SV40 and Ad correlating NK cell, cytolytic susceptibility of virally transformed cells, and the oncogenicity of the transforming virus. In addition to HPV, SV40 and many polyomaviruses (16, 17) express viral oncoproteins with CR1/CR2 homology to E1A and thereby interact with cellular proteins (p105 and p107) that are key regulators of cell proliferation. We speculate that the adequacy of NK cells to mount an effective immunologic defense against this group of DNA tumor viruses is an important factor in determining the oncogenicity of these viruses in their immunocompetent hosts.

The relationship between the selective killing of virally infected or virally transformed cells by unstimulated or IFN-activated NK cells in vitro and the actual biological effects of these NK cells in vivo is unknown. Studies using murine cyto-

megalovirus and lymphocytic choriomeningitis virus infection of rodents showed that the IFN-induced, selective killing of virally infected cells in vitro mirrors the antiviral activity of NK cells mediated in vivo (5). On the other hand, unstimulated NK cells were used in studies which correlated the oncogenicity of group A and group C Ad in vivo with the cytolytic susceptibility of group A and group C Ad-transformed cells in vitro. We used both IFN-activated and unstimulated NK cells in our cytotoxicity assays in an effort to best approximate the lytic activities of NK cells that may be present in vivo. In part on the basis of the results of this study, we propose the following model to help explain the dissimilar oncogenicities of Ad and HPV in view of their proclivity to cause persistent infections and capacity to transform cells.

NK cells are a component of the inflammatory infiltrate in HPV cervical infections and cervical epithelial neoplasia (66). Because E7 expression does not induce an increase in cytolytic susceptibility, unstimulated NK cells would be ineffective in the rejection of these virally infected or transformed cells. HPV-transformed cells can induce IFN production by inflammatory cells in vitro (69); therefore, it is likely that IFN is produced locally in vivo at the inflammatory interface between host leukocytes (including NK cells) and HPV-infected or transformed cells. Locally produced IFN would increase the lytic activity of NK cells at this inflammatory site. However, this effect on NK cells would be negated because IFN would induce equivalent levels of cytolytic resistance on HPV-infected, HPV-transformed cells and normal cells. Persistence of both HPV-infected and HPV-transformed cells would be favored because of the subversion of the host NK cell response. Furthermore, if not eliminated by other mechanisms, HPV-transformed cells could then acquire additional somatic mutations leading to increased virulence and frank malignancy.

In contrast to HPV-transformed cells, Ad-transformed cells are susceptible to killing by both IFN-activated and unstimulated NK cells. Therefore, IFN induced in response to Ad infection (51) or constitutively produced in Ad-transformed cells (47) would be expected to enhance the NK cell-mediated lysis of Ad-infected or -transformed cells. Therefore, the capacity of Ad to transform cells may be insufficient to cause tumors in humans because the gene that is essential for transformation (E1A) also targets the cell for destruction by the host NK cell response. However, the capacity of Ad to cause persistent infections may be explained, in part, by the resistance of Ad-infected cells to lysis by unstimulated NK cells. This cytolytic resistance may allow for the replication and spread of Ad prior to local IFN production and concomitant acquisition of an effective NK cell response. This mechanism may complement other strategies attributed to Ad that undermine the antiviral immune response of humans (1, 6, 27, 28, 37).

In summary, human cells expressing the E7 oncoprotein were not discriminately lysed by either IFN-activated NK cells or unstimulated NK cells, whereas the same cells expressing the E1A oncoprotein were differentially killed. We believe that these differences in NK cell killing of Ad- and HPV-transformed cells reflect two separate functions which are present in E1A and absent in E7: the capacity to induce cytolytic susceptibility to NK cell lysis and the ability to block the induction of cytolytic resistance. We speculate that the disparity in the NK cell-mediated destruction of Ad-transformed compared with HPV-transformed human cells in vivo contributes to dissimilar oncogenicities of Ad and HPV.

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