[CANCER RESEARCH 55, 5551–5555, December 1, 1995]

Advances in Brief

Adenovirus *E1a*-mediated Tumor Suppression by a c-*erb*B-2/*neu*-independent Mechanism[1]

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Abstract

We reported previously that the adenovirus *E1a* gene reversed the transformed phenotype of one human melanoma and one fibrosarcoma cell line (S. Frisch, Proc. Natl. Acad. Sci. USA, *88:* 9077–9081, 1991). To determine the generality of the tumor suppression effects of *E1a*, a diversity of tumor cell lines, including A204 rhabdomyosarcoma, RD rhabdomyosarcoma, Saos-2 osteosarcoma, NCI-H23 non-small cell lung carcinoma, MDA-MB435S breast carcinoma, and *ras*-transformed MDCK kidney epithelial cells, were infected with a retrovirus bearing the 12S *E1a* coding sequence. We demonstrate here that the expression of *E1a* severely reduced the anchorage-independent and tumorigenic growth of these cell lines without affecting their growth under normal culture conditions. The parental tumor cells used in this study did not overexpress c-*erb*B-2/*neu*, and *E1a* did not affect its expression in these cells. Thus, tumor suppression by *E1a* can operate in a wide variety of human tumor cells by c-*erb*B-2/*neu*-independent mechanisms. *E1a* also sensitized these cell lines to the cytotoxic effects of the anticancer drugs etoposide and cisplatin. The results suggest that *E1a* could prove useful for the gene therapy of a wide variety of human cancers.

Introduction

The adenovirus *E1a* gene products of 243 and 289 amino acids have numerous diverse effects on gene expression and cell physiology. These include: (*a*) transactivation of adenovirus promoters (1); (*b*) cooperation with oncogenes such as *ras* to transform certain rodent cell types, such as BRK cells (2); (*c*) immortalization of rodent fibroblasts (3); (*d*) induction of DNA synthesis in quiescent rodent cells (4); (*e*) regulation of the transcription of various cellular genes, both positively and negatively (1); and (*f*) induction of apoptosis when expressed at high levels (5). Although the literature usually refers to *E1a* as an oncogene, which is supported by the results with rodent cells, there is little or no evidence to suggest that *E1a* can transform human cells, the appropriate species for an adenovirus gene (see "Discussion"). In fact, we found that *E1a* actually behaves as a tumor suppressor gene in a human melanoma and a fibrosarcoma cell line (6). Whereas the mechanism underlying this effect is unclear, new insight arises from our recent observation that *E1a* has epithelial cell master-programming activity in diverse human tumor cells of mesenchymal origin (7). Because there is an apparent exclusivity between fully epithelial *versus* transformed phenotypes (8), these results raise the possibility that *E1a* is a generalized tumor suppressor gene.

In this study, we further characterized the tumor suppression effects of *E1a* in additional human tumor cell types. It was surprising that *E1a* reverse-transformed all of the cell lines tested. In contrast with a previous report dealing with different cell systems (9), *E1a* did not achieve this through the transcriptional repression of the c-*erb*B-2/*neu* oncogene. These results raise the prospect of developing applications of *E1a* for therapy of diverse human cancers. Such applications might be aided by the chemotherapeutic drug sensitization effects reported here as well.

Materials and Methods

Cell Lines. The construction of *E1a*-expressing cell lines was described previously (6, 7); all of the cell lines express the 12S *E1a* gene product alone, except for the E1a/ML cells, which express both the 12S and 13S products.

Soft Agar Colony Formation Assays. Soft agar (0.4%, SeaPlaque; FMC BioProducts) was inoculated with 2×10^5 FS[4] or BC cells, or 1×10^5 RM, OS, LC or v-Ha-*ras*-transformed Madin-Darby canine kidney epithelial cells (or their *E1a* derivatives) in 60-mm dishes as described previously (6). These plates were incubated for 14–17 days and photographed.

Tumorigenicity Assays. Cells were injected s.c. into 4–5-week-old athymic nude mice (Harlan-Sprague-Dawley) in 0.3 ml of DMEM. Eight mice were injected per cell line, with 10^6 RM cells, 5×10^6 LC cells, 5×10^6 BC cells, and 5×10^6 RM2 cells, and tumors were dissected at 27, 18, 34, and 40 days, respectively, and weighed.

Drug Sensitivity Assays. Dishes (12-well) were inoculated with FS, ML, OS, and LC cells and their *E1a*-expressing counterparts. Subconfluent monolayers were treated with etoposide or cisplatin for 24 or 17 h, respectively. Viable cells were quantitated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays. These were performed as described by the manufacturer (Sigma Chemical Co.). Briefly, cells were incubated at 37°C in medium containing MTT for 1–3 h. The dye crystals were then solubilized with 0.1 N HCl in isopropyl alcohol. Viability was quantitated by measuring absorbance at 570 nm.

Northern Blots. Northern blots were performed using 10 μg of guanidine thiocyanate-cesium chloride gradient-purified RNA. The RNA was electrophoresed through a formaldehyde-agarose gel and blotted onto nitrocellulose. The probe consisted of a 0.67-kb *Bgl*II fragment from the human *neu* (*erb*B-2) gene [pHER2-436-1 (10)] labeled by random priming (Prime-It II; Stratagene) and purified by spun column chromatography on Sephacryl S-200 (Microspin columns, Pharmacia).

Growth Rates. Cells (1×10^5) were plated in 35-mm diameter wells. After incubation for various times, 0.1 ml of 5 mg/ml thiazolyl blue (MTT reagent, Sigma) was added to the well containing 1.0 ml of growth medium, and incubation at 37°C was continued for 1 h. The medium was removed, and the product crystals were redissolved in 1.0 ml of 0.1 N HCl in isopropyl alcohol and read in the spectrophotometer at 570 nm. The data were plotted, and doubling times were extrapolated.

Results

Broad Specificity of Tumor Suppression by *E1a*. A collection of diverse cell lines that stably express the adenovirus-5 243-amino acid form of *E1a* was generated previously by infection with a retroviral

Received 8/21/95; accepted 10/18/95.

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[1] The salaries of S. M. F. and K. E. D. were partially supported by the Elsa U. Pardee Foundation.

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[4] The abbreviations used are: FS, HT1080 fibrosarcoma (subclone H4); OS, Saos-2 osteosarcoma; RM, A204 rhabdomyosarcoma; RM2, RD rhabdomyosarcoma; BC, MDA-MB435S breast carcinoma; LC, NCI-H23 non-small cell lung carcinoma; mras, v-Ha-*ras*-transformed Madin-Darby canine kidney epithelial cells; ML, A2058 melanoma.

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Table 1 *Doubling times for parental and E1a-expressing cell lines (determined as described in "Materials and Methods")*

Cell line	Doubling time (h)
RM	18
E1a/RM	16
BC	15
E1a/BC	14
OS	23
E1a/OS	23

vector, as described (7). *E1a* altered gene expression in these cell lines such that epithelial phenotypes resulted, suggesting that *E1a* is a master programmer of epithelial differentiation.

The growth rates of the cell lines under normal culture conditions were not substantially affected by *E1a* at these expression levels (Table 1). However, anchorage-independent growth in soft agar was greatly reduced (Fig. 1).

These results motivated experiments to test the tumorigenic

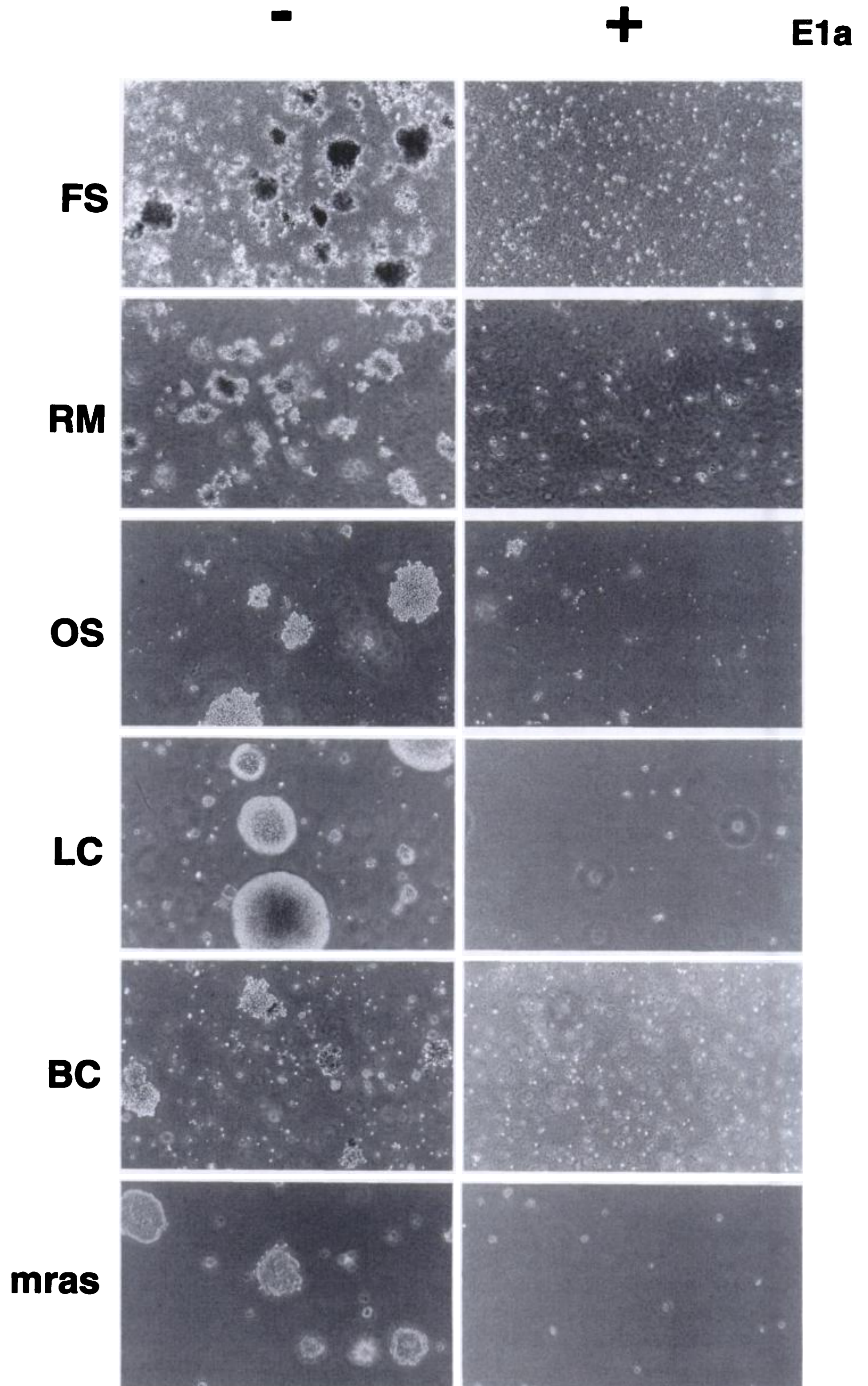

Fig. 1. Soft agar colony formation assays of parental tumor cell lines or *E1a*-expressing derivatives. Photomicrographs (final magnification, approximately ×100) show colonies after 14–17 days. *mras*, v-Ha-*ras*-transformed derivative of Madin-Darby canine kidney cells.

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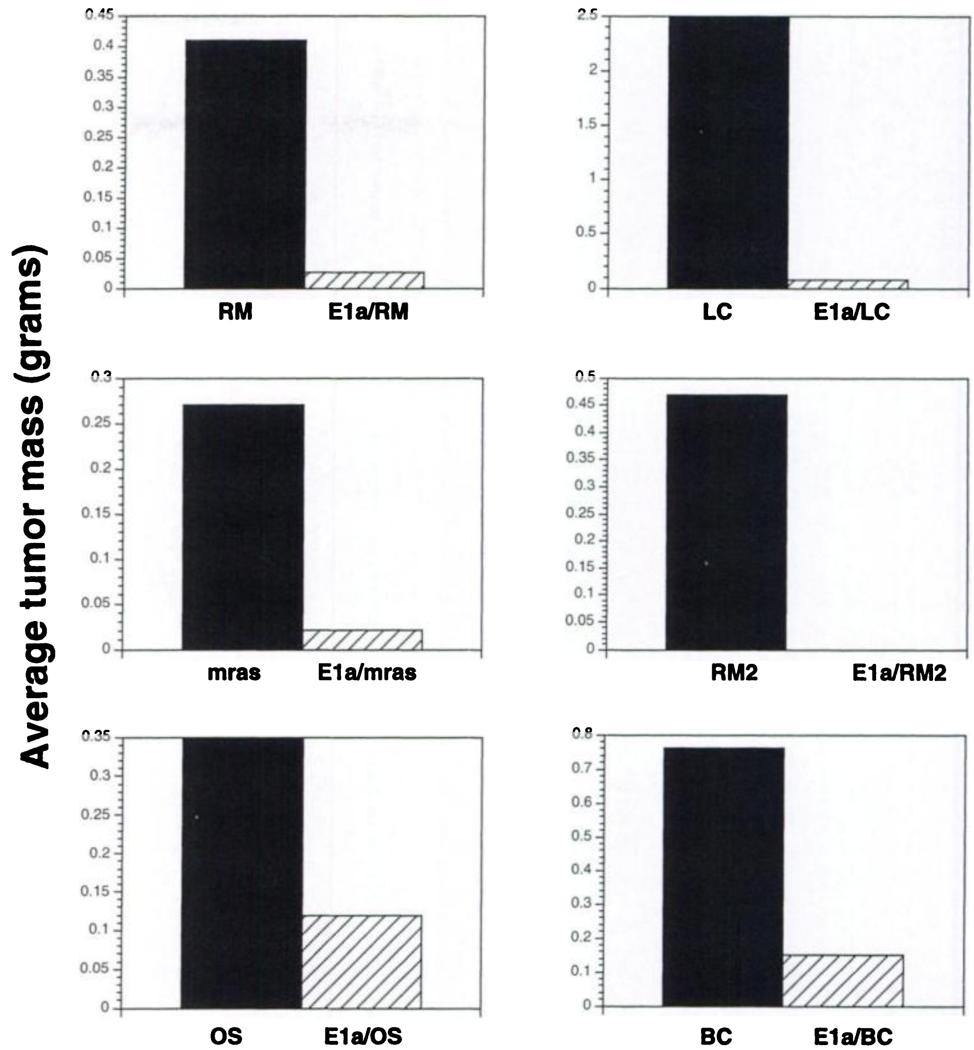

Fig. 2. *In vivo* tumor formation by parental cell lines or *E1a*-expressing derivatives. Average tumor mass from eight nude mice given injections of each cell line is shown for each pair of cell lines. Cell numbers and incubation times are cited in "Materials and Methods."

potential of the cells *in vivo*. Fig. 2 shows that, in each case, the expression of *E1a* reduced the tumor size dramatically.

Thus, by the standard functional criteria of anchorage dependence and reduced *in vivo* tumorigenicity, *E1a* is a tumor suppressor gene in a wide variety of human tumor cell types.

Transcription of the c-*erb*B-2/*neu* Gene Is Not Repressed in These Cell Lines. Published reports have proposed that *E1a* may act as a tumor suppressor in certain cell lines by repressing the expression of the c-*erb*B-2/*neu* gene (9, 11, 12). These studies were limited to cell lines that were known to be transformed by the overexpression of this oncogene (either ectopic or through gene amplification). Nevertheless, it is formally possible that basal expression of c-*erb*B-2/*neu* is generally required for the maintenance of transformation, and that *E1a* represses this basal expression. The effect of *E1a* on c-*erb*B-2/*neu* was shown previously to occur at the RNA level. Thus, to explore this possibility for the cell lines investigated here, RNAs from the parental and *E1a*-expressing tumor cell lines were analyzed by Northern blotting for c-*erb*B-2/*neu* expression. It was unexpected that the expression of this gene was not significantly affected by *E1a* (Fig. 3). The c-*erb*B-2/*neu* protein could be detected neither on Western blots of parental tumor cells nor *E1a* derivative cells (data not shown). These data render the possibility of tumor suppression via repression of c-*erb*B-2/*neu* quite unlikely in these cell lines.

***E1a* Sensitizes Human Tumor Cells to Killing by Etoposide and Cisplatin.** *E1a* expression in these human tumor cell lines did not cause spontaneous apoptosis; however, the cells became sensitized to "anoikis," defined as apoptosis in response to disrupted cell-matrix interactions (13). These results suggested that *E1a* might affect apoptosis in response to other stimuli, such as DNA damage.

Parental or *E1a*-expressing cell lines were exposed to etoposide or cisplatin, anticancer drugs that cause DNA damage, thereby eliciting an apoptotic response in sensitive cells. *E1a* caused a remarkable degree of sensitization to killing by these drugs (Fig. 4).

Discussion

The oncogenic activities of adenovirus type 5 *E1a* in rodent cells are widely documented and studied (*e.g.*, Ref. 14). However, in human cells, there is no significant evidence for oncogenic activity of this gene [although the substantially different adenovirus type 12 *E1a*

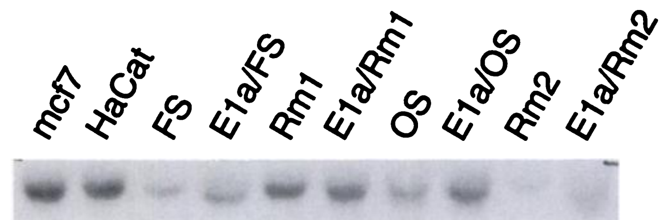

Fig. 3. Expression of c-*erb*B-2/*neu* mRNA in parental tumor cells or *E1a*-expressing derivatives. Levels of c-*erb*B-2/*neu* mRNA were assessed by Northern blotting as described in "Materials and Methods." MCF-7 breast carcinoma and HaCat keratinoctyes were used as positive controls.

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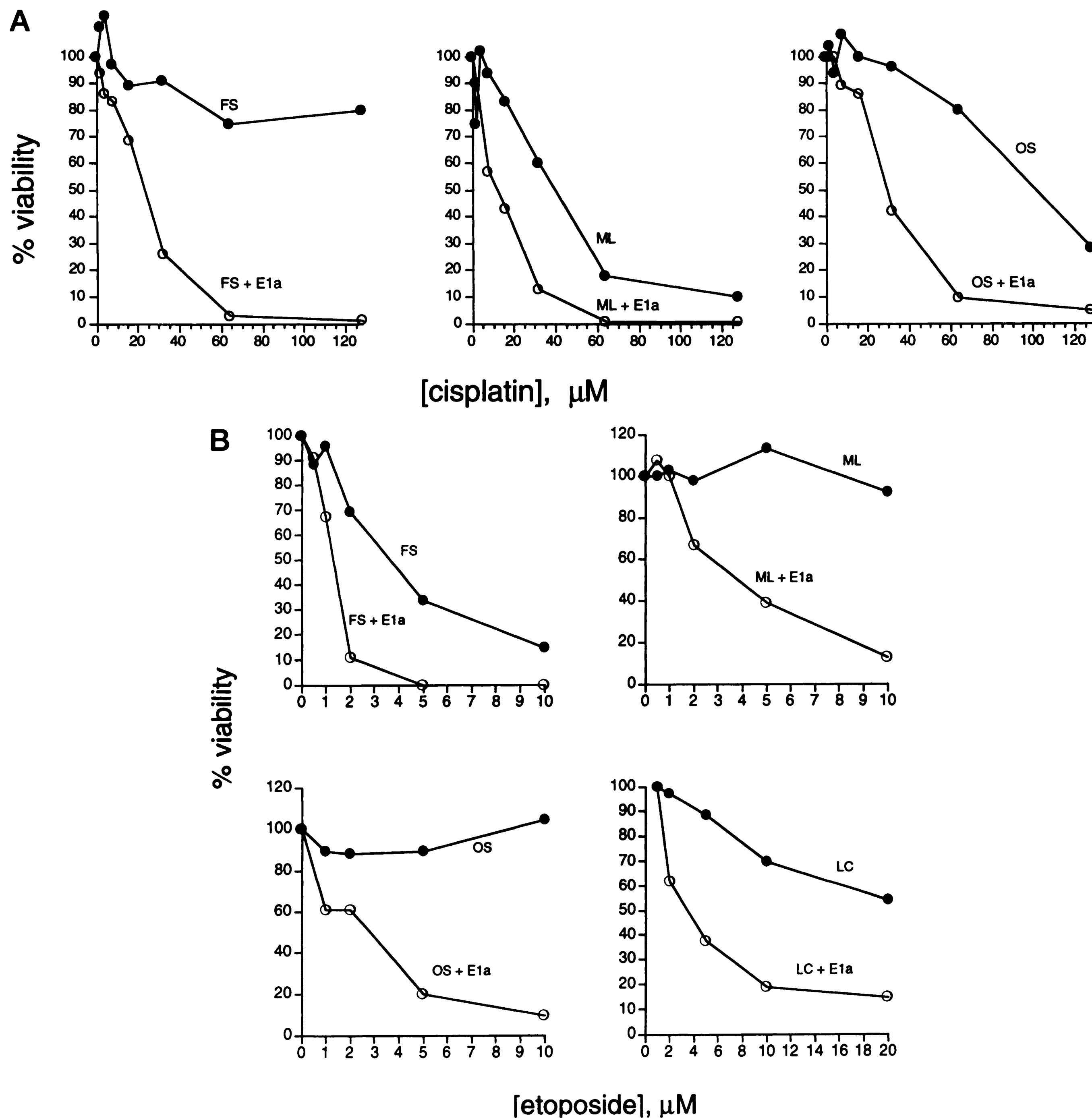

Fig. 4. Etoposide and cisplatin cytotoxicity dose-response curves of tumor cell lines or *E1a*-expressing derivatives. Viable cells were measured by MTT assay as described in "Materials and Methods."

gene has transformed human cells (15, 16)]. Adenoviral sequences are equally prevalent in normal and tumor cell genomes of humans (17). The cell line 293, which was derived from human embryonic kidney cells by transfection with adenovirus E1a+E1b (18), are immortalized but nontumorigenic. A number of cell lines were established (19) from human embryonic retinoblast cultures that express E1a+*ras* or E1a+E1b sequences; their tumorigenicity is quite marginal (*e.g.*, 185-day latency period).

The results presented here indicate that *E1a* is a broad specificity tumor suppressor gene for human cells, and they reveal several new facets of this phenomenon that indicate its unique potential for the gene therapy of cancer.

E1a reverse-transformed tumor cells of multiple origins (sarcomas, melanomas, and carcinomas). The degree of malignancy of the original parental cell line apparently did not affect the outcome, in that the highly malignant and dedifferentiated rhabdomyosarcoma cell line RM and the less malignant, more differentiated RM2 were reverse-transformed equally well.

It has been proposed that *E1a* suppresses tumorigenicity by repressing the transcription of c-*erb*B-2/*neu* in tumor cells that overexpress this oncogene (9, 11, 12). However, the parental tumor cells used in this study did not overexpress c-*erb*B-2/*neu*, and *E1a* did not affect its expression in these cells. Thus, this mechanism apparently does not pertain to the cell lines described here. The basis for the discrepancy

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between previous reports showing the repression of this gene by *E1a* and this report showing the lack of repression is unclear. However, the previous reports utilized cell lines in which the c-*erb*B-2/*neu* gene was amplified, possibly subjecting it to novel transcriptional regulation. In addition, the *E1a* gene used in this study was introduced on a retrovirus, perhaps yielding lower expression levels than those obtained by others using transfection protocols. In any event, *E1a*, delivered via adenovirus vectors, causes the regression of tumors that were derived from c-*erb*B-2/*neu*-transformed cell lines (20). Our present results suggest that a similar approach may be effective in other tumors that do not involve this oncogene.

E1a has previously been reported to induce apoptosis in certain cell lines after transient transfection (reviewed in Ref. 21). However, this effect appears to be restricted to certain cell lines and/or apoptotic stimuli because 12S *E1a* has been shown to prevent cytokine-induced apoptosis in one system (22). In the current cell lines, *E1a*-induced apoptosis was neither observed in DNA ladder assays (13) nor indicated by a decreased apparent growth rate in the stable *E1a*-expressing cell lines described here. A lower level of *E1a* expression resulting from retroviral transduction as opposed to transfection could perhaps explain this discrepancy. Furthermore, *E1a*-induced apoptosis is mediated by a stabilization of the wild-type form of p53 occurring in response to *E1a* (23). However, because some of the cell lines used in this study (OS, RM2, and LC) do not contain wild-type p53 (24–26), this mechanism would not appear to be relevant in these cell systems either.

Thus, the tumor suppression observed in this study appears to result from a mechanism that is not yet obvious from the literature. We have reported that *E1a* is a master programmer of the epithelial phenotype (7). We have further reported that epithelial cells are sensitive to a particular form of apoptosis, called anoikis, that is induced by the disruption of integrin-mediated, cell-matrix interactions (13). Cells that are sensitive to anoikis must logically be anchorage dependent, which would restrict their tumorigenicity. This would seem to be a compelling model to explain the tumor suppression effect in sarcomas. In fact, *E1a* rendered FS cells anoikis sensitive. In the case of carcinoma cells, *E1a* actually converted a poorly differentiated, anoikis-resistant carcinoma (BC) into well-differentiated, anoikis-sensitive cells (data not shown). It will be interesting in the future to test the role of anoikis in the tumor suppression effect of *E1a* by the use of apoptosis inhibitors such as *bcl-2* or E1b.

For the gene therapy of cancer, it is currently not feasible to deliver a gene to every cell within a tumor *in vivo*. Thus, it may be preferable to utilize a gene that converts tumor cells into viable normal cells (as opposed to a gene that kills tumor cells). In the context of a tumor, these normal cells may have the potential of exerting growth-inhibitory effects on the surrounding tumor cells. For example, focus formation after transfection with the *ras* oncogene requires the elimination of surrounding normal cells by drug selection (27). This was proposed to reflect dominant growth inhibition of the tumor cells by the surrounding normal cells. In addition, it may be possible to codeliver genes together with *E1a*, such as cytokine genes, that would be stably expressed in the tumor, targeting it for immune surveillance. Finally, the results suggest the application of *E1a* to convert partially or totally drug-resistant tumors (*e.g.*, ML) into drug-sensitive tumors. This may ensure a particularly efficient tumor eradication if bystander effects operate (28).

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Cancer Res 1995;55:5551-5555.

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