Enhanced c-erbB-2/neu Expression in Human Ovarian Cancer Cells Correlates with More Severe Malignancy That Can Be Suppressed by E1A

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ABSTRACT

Amplification or overexpression of c-erbB-2/neu protooncogene, or both, occur frequently in many different types of human cancers and have been shown to correlate with decreased survival in ovarian cancer patients. We have previously found that the ovarian carcinoma cell line SK-OV-3 overexpresses c-erbB-2/neu mRNA. To further study the biological effect of c-erbB-2/neu overexpression in SK-OV-3 cells, we injected such cells i.p. into female nu/nu mice and found that this cell line forms extensive abdominal tumors and ascites. From the ascites in an injected mouse, we established the SKOV3.ipl cell line and found that it expressed 2-fold more c-erbB-2/neu-encoded p185 proteins than the parental SK-OV-3 cells. When transformation phenotypes of SK-OV-3 and SKOV3.ipl cells were compared, SKOV3.ipl cells showed higher cell growth and DNA synthesis rates, formed more colonies in soft agar, produced larger s.c. tumors, and resulted in shorter survival of nu/nu mice after i.p. injection. These data indicate that the level of c-erbB-2/neu overexpression may correlate with the degree of malignancy in these ovarian carcinoma cells. Since we had previously shown that the adenovirus 5 E1A gene product can suppress transformation and metastatic properties induced by mutation-activated rat neu oncogene in mouse embryonic fibroblasts, we further examined whether E1A can abrogate malignancy in c-erbB-2/neu-overexpressing human ovarian cancer cells. We introduced the E1A gene into c-erbB-2/neu-overexpressing SKOV3.ipl cells and found that the E1A-expressing ovarian cancer cell lines had decreased c-erbB-2/neu-encoded p185 expression and reduced malignancy, including a decreased ability to induce tumors in nu/nu mice. Therefore, we concluded that E1A is a tumor suppressor gene for c-erbB-2/neu-overexpressing human ovarian cancer cells and may be useful in developing therapeutic reagents for these human cancers.

INTRODUCTION

The c-erbB-2/neu oncogene (also known as HER-2 or NGL) encodes a M, 185,000 epidermal growth factor receptor-related transmembrane protein (p185) with intrinsic tyrosine kinase activity (1–5). The mutation-activated rat neu oncogene (the rat equivalent of human c-erbB-2/neu), which contains a single point mutation in the transmembrane domain of the p185 protein (6), possesses higher tyrosine kinase activity than its normal counterpart and can transform fibroblasts (7, 8). Amplification of the normal rat neu protooncogene facilitates oncogenic activation by the single point mutation (9), which suggests that the single point mutation in the transmembrane region of the neu-encoded p185 is a hot spot for activation of the rat neu oncogene. Instead of mutation-activation, amplification or overexpression of the normal human c-erbB-2/neu protooncogene, which can lead to higher overall tyrosine kinase activity, is a frequent event in many types of human cancers, including cancers of the breast (10–12), ovary (13–16), lungs (17–19), stomach (20, 21), and colon (22). So far, no mutation equivalent to that of rat neu oncogene has been found in the c-erbB-2/neu gene from human tumors (23, 24). Interestingly, though, overexpression of the c-erbB-2/neu gene has been shown to correlate with the number of lymph node metastases in breast cancer patients (10) and decreased survival in both breast and ovarian cancer patients (13). Although there have been controversial clinical reports on the correlation between lymph node metastasis and the overexpression of c-erbB-2/neu in breast cancer patients (25–27), we have recently provided experimental evidence that the mutation-activated rat neu oncogene can induce metastatic potential in mouse 3T3 cells by promoting multiple steps in the metastatic cascade (28, 29), which is consistent with the notion that the c-erbB-2/neu gene is involved in the process of tumorigenesis and cancer metastasis.

Ovarian carcinoma is the most lethal tumor of the female genital tract and continues to be the major cause of mortality in female cancer deaths, largely as a function of early abdominal seeding of this neoplasm producing carcinomatosis (30). Therefore, studies investigating this process are necessary to determine molecular mechanisms that may induce such aggressive phenotypes and to develop a novel means of predicting and possibly treating these aggressive malignancies. We have found that amplification/overexpression of the c-erbB-2/neu gene in primary ovarian cancer is a common phenomenon in such tumors across different populations (14, 16). In addition, we have detected c-erbB-2/neu gene overexpression in the SK-OV-3 ovarian carcinoma cell line (16). Based on these observations, we deemed it important to study the biological consequences of c-erbB-2/neu gene overexpression in ovarian cancers. Therefore, in the present study, we investigated the relationship between c-erbB-2/neu gene overexpression and transformation potential in SK-OV-3 cells. We compared transformation phenotypes of SK-OV-3 and derivative SKOV3.ipl cells, which were isolated from ascites in the mouse given i.p. injections of SK-OV-3 and which expressed 2-fold higher c-erbB-2/neu. Our results indicate that a higher level of c-erbB-2/neu expression in SKOV3.ipl ovarian cancer cells correlates with rapid and increased peritoneal carcinomatosis.

We have previously shown that the adenovirus 5 E1A gene product can repress c-erbB-2/neu oncogene expression and suppress the tumorigenic and metastatic potential of mutation-activated rat neu oncogene-transformed mouse 3T3 cells (29, 31, 32). Since enhanced c-erbB-2/neu overexpression correlates with a higher degree of malignant transformation in human ovarian cancer cells, we questioned in the present study whether E1A can suppress c-erbB-2/neu expression and function as a tumor suppressor gene for c-erbB-2/neu-overexpressing human cancer cells. The introduction of the E1A gene into SKOV3.ipl ovarian cancer cells resulted in decreased cell growth and DNA synthesis rates, reduced colony formation in soft agar, suppressed s.c. tumor growth in nu/nu mice, and longer survival of nu/nu mice when the cells were injected i.p. Therefore, the E1A gene can be considered a tumor suppressor gene for c-erbB-2/neu-overexpressing human cancer cells.
MATERIALS AND METHODS

Cell Lines and Culture. The SK-OV-3 human ovarian carcinoma cell line was purchased from the American Type Culture Collection. The SKOV3.ip1 cell line was established from ascites that developed in a nu/nu mouse given an i.p. injection of SK-OV-3 cells. Cells were grown in Dulbecco’s modified Eagle’s medium/F12 medium supplement (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum. The ipl.E1A1 and ipl.E1A2 transfectants and ipl.E1B transfectants were grown under the same conditions, except that G418 (800 µg/ml) was added to the culture media.

DNA Transfection. All DNA transfections were carried out using the modified calcium phosphate precipitation procedure (33). SKOV3.ip1 cells (10⁶ cells in 10-cm dishes) were seeded 24 h before transfection. The cells were transfected with 10 µg of either the E1A-expressing pE1A plasmid DNA or pE1A-dl343, which contains a 2-base pair frameshift deletion in the E1A coding sequences (adenovirus nucleotide sequence positions 621 and 622), along with 1 µg of pSV2-neo plasmid DNA carrying the neomycin resistance marker gene (34). Approximately 10 h posttransfection, cells were washed and cultured in fresh medium for 24 h and split at a 1:10 ratio. The cells were then grown in selection medium containing 800 µg/ml of G418 for 4–6 weeks, after which individual G418-resistant colonies were cloned using cloning rings and expanded to mass culture.

Immunoblotting. Immunoblot analyses were performed as previously described (31, 35). The primary monoclonal antibodies used were M73 against the E1A proteins (a generous gift from Dr. L. S. Chang, Ohio State University, Columbus, OH) and c-neu-Ab-3 against the new-encoded p185 protein (purchased from Oncogene Science, Inc., Manhasset, NY). The blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Bio-Rad Laboratories, Richmond, CA) and detected with ECL chemiluminescence Western blotting detection reagents (Amersham, England).

Southern Analysis. To measure c-erbB-2/Neu gene copy number, Southern blot analyses were performed as previously described (14). Genomic DNAs extracted from cultured cells were digested overnight at 37°C with a 2-fold excess of the BamHI restriction endonuclease. Ten µg of each sample were then resolved by electrophoresis on a 0.8% agarose gel and transferred to Nitran membrane (Schleicher & Schuell, Inc., Keene, NH) using 10X standard saline citrate (1.5 µM sodium chloride, 0.15 µM sodium citrate). The blotted DNAs were then hybridized under highly stringent conditions (68°C) with a full-length (4.4-kilobase) human c-erbB-2/neu cDNA probe purified from the plasmid (4,4-kilobase) human c-erbB-2/neu cDNA probe purified from the plasmid pSV2erB-2 (a generous gift from Dr. Tadashi Yamamoto) (36) digested with HindIII restriction endonuclease and labeled with ³²P (1-5 × 10⁸ cpm ug⁻¹) using a random primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The blots were washed, dried, and then exposed to Kodak X-OMAT AR film at -80°C for 1 to 3 days.

In Vitro Growth Rate Analysis. The in vitro growth rates of the cell lines were assessed by measuring increases in cell number with the MTT assay (37). Cells (2 × 10⁵/well) were plated in 96-well culture plates in 0.2 ml of culture medium. A total of 5 plates (9 wells/cell line/plate) were used. One of the plates was analyzed at 24-h intervals after the addition of 40 µl MTT (Sigma Chemical Co., St. Louis, MO) stock solution (1.25 mg MTT/ml of phosphate-buffered saline) to each well on the plate. Cells were harvested, and cellular DNA was bound to fiberglass filters. The radioactivity of each filter was counted with a scintillation counter.

Colonies Formation in Soft Agarose. The ability of different cells to grow in soft agarose was determined as previously described (35). Cells (1 × 10⁵ cells/well) were plated in a 24-well plate in culture medium containing 0.35% agarose (BRL, Gaithersburg, MD) overlying a 0.7% agarose layer. The cells were then incubated at 37°C for 5 weeks, after which the plates were stained with p-iiodonitrotetrazolium violet (1 mg/ml) for 48 h at 37°C. Colonies greater than 100 µm were counted for each dish and cell line. Experiments were repeated four times for each cell line.

Animals. Four- to 6-week-old athymic homozygous nu/nu mice were purchased from the Animal Production Area, National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD) or from Harlan Sprague Dawley, Inc., (Indianapolis, IN). The care and use of the animals was in accordance with institutional guidelines.

Tumorigenicity Assay. The cells in log-phase growth were trypsinized, washed twice with phosphate-buffered saline, and centrifuged at 250 × g. The viable cells were counted; of those, 3 × 10⁶ cells in 0.1 ml of phosphate-buffered saline were injected s.c. into both the right and left flanks of female mice under aseptic conditions. Tumor volumes were estimated as the product of three-dimensional caliper measurements (longest surface length and width; tumor thickness). The growth of tumors was monitored for a minimum of 80 days and a maximum of 160 days.

Formation of Malignant Ascites after I.p. Injection. Suspensions of cells (harvested as above) at concentrations of 1 × 10⁶ or 5 × 10⁶ in 0.2 ml of Hank’s balanced salt solution were injected i.p. into individual female nu/nu mice. For each cell line, 8 to 10 mice were given injections. Mice were initially observed twice a week for signs of tumor development and then daily when any or all of the following tumor symptoms appeared: abdominal bloating, loss of subcutaneous fat, hunched posture, and decreased movement. Mice were killed when they appeared moribund or, judging from our previous experience, would not survive more than 24–48 h. Symptom-free mice were killed 120 days after injection. Autopsies were performed on all mice killed.

RESULTS

Isolation of the SKOV3.ip1 Cell Line, a More Cancerous Derivative of the SK-OV-3 Cell Line. Since the ovarian carcinoma cell line SK-OV-3 was previously found to overexpress c-erbB-2/neu mRNA at least 100-fold higher than other cancer cell lines from the female genital tract (16), SK-OV-3 was considered suitable for further studies on the biological effect and the molecular mechanisms of c-erbB-2/neu overexpression in human cancer cells. Furthermore, since we have shown that the mouse fibroblasts transformed by mutation-activated rat neu oncoprotein can induce tumors and metastatic lung nodules in nu/nu mice (28, 32), we decided to determine whether the c-erbB-2/neu-overexpressing SK-OV-3 cell line induces tumour formation and colonization upon i.p. injection into nu/nu mice, which was chosen as an appropriate experimental approach for studying advanced human ovarian cancer. In our initial study, either 1 × 10⁴ or 5 × 10⁴ viable SK-OV-3 cells were injected i.p. into female nu/nu mice. Signs of tumor development were noted at about 53 days after injection. Mice were killed when they appeared moribund and developed extensive ascites. Autopsies revealed typical tumor patterns, i.e., colonized tumor nodules on peritoneal surfaces and surfaces of the diaphragm. Approximately 20–30 bowel, uterine-ovarian mesenteric, subserosal, and subhepatic nodules were found as well as large omental cakcs of tumor. At the time the mice were sacrificed, no liver invasion or tumor formation outside of the peritoneal cavity was detected. These results clearly demonstrated that nu/nu mice that received i.p. injection of the c-erbB-2/neu-overexpressing SK-OV-3 cell line could develop tumors and eventually die of disseminated intraabdominal tumors. Therefore, the SK-OV-3 cell line is a highly malignant ovarian cancer cell line with overexpression of the c-erbB-2/neu gene.

Each mouse given an injection of the SK-OV-3 cell line usually developed 0.97 ± 0.21 (SD) ml of ascites, and from the ascites in one mouse the SKOV3.ip1 cell line was established. To compare the transformation potential of the SKOV3.ip1 cell line with its parental SK-OV-3 cell line, we performed i.p. injection experiments with these two cell lines. Mice developed similar tumor symptoms with the two cell lines. However, mice given injections of the SKOV3.ip1 variant showed a significant decrease in survival compared with mice given
4 of 13 mice were alive until 92 days postinjection (Fig. 1). These exhibited a more aggressive tumor pattern than the SK-OV-3 cells that died with extensive tumor growth within 51 days after injection; on operated tumor symptoms at 33 days postinjection, and all of the mice 9 mi/nu mice given injections with 1 X 10^6 SKOV3.ipl cells developed tumor symptoms until 53 days postinjection, and all of the mice 9 mi/nu mice given injections with 1 X 10^6 SKOV3.ipl cells developed tumor symptoms until 53 days postinjection, and all of the mice 9 mi/nu mice given injections with 1 X 10^6 SKOV3.ipl cells developed tumor symptoms until 53 days postinjection, and all of the mice 9 mi/nu mice given injections with 1 X 10^6 SKOV3.ipl cells developed tumor symptoms until 53 days postinjection. Potential of SKOV3.ipl Cells. Since the SKOV3.ipl variant cells overexpress level might be involved in the enhanced malignancy. Results indicate that the SKOV3.ipl variant cells were phenotypically more aggressive, more cancerous, than their parental SK-OV-3 cells.

Increased Expression of p185 and Increased Transformation Potential of SKOV3.ipl Cells. Since the SKOV3.ipl variant cells were more aggressive, more cancerous, than their parental SK-OV-3 cells. It is interesting to note that the higher level of c-erbB-2/neu expression in SKOV3.ipl ovarian cancer cells seems to correlate with their more severe malignant behavior when these cells were injected i.p. into nu/nu mice. To further investigate the correlation between the increased c-erbB-2/neu expression and enhanced malignancy in the SKOV3.ipl cell line, we compared other transformation phenotypes between SK-OV-3 and SKOV3.ipl cells in vitro and in vivo, including cell growth and DNA synthesis rates, anchorage-independent growth, and the ability to induce tumors in nu/nu mice following s.c. injection.

Uncontrolled cell growth is a typical feature of malignant cells. Therefore, the growth characteristics of SK-OV-3 and SKOV3.ipl cells were analyzed by measuring growth curves with an MTT reaction. Growth curves of the SK-OV-3 and SKOV3.ipl cells revealed that SKOV3.ipl cells had significantly increased cell growth rates compared with SK-OV-3 cells (P < 0.01) (Fig. 3A). Another parameter frequently measured in growth-stimulated cells is [3H]thymidine incorporation into DNA, which generally correlates well with the overall DNA synthesis rate. The parental c-erbB-2/neu-overexpressing SK-OV-3 cells had a high level of [3H]thymidine incorporation; however, the SKOV3.ipl cells had an even higher DNA synthesis rate (Fig. 3B), which coincided with their higher cell growth rate. These data suggest that the increased expression of c-erbB-2/neu-encoded p185 protein may lead to accelerated cell growth and DNA synthesis rates.

Anchorage-independent growth in soft agar is generally considered a marker of transformation and tumorigenicity. To test the effect of increased expression of p185 proteins on anchorage-independent growth, we assayed SK-OV-3 cells and SKOV3.ipl cells for their ability to grow in soft agar. As shown in Fig. 4A, the SKOV3.ipl cells exhibited 3-fold higher colony-forming efficiency in 0.35% soft agar than their parental SK-OV-3 cells.

An additional experimental test of neoplastic behavior is the ability of s.c. injected cells to form tumors in nude mice. Therefore, the parental SK-OV-3 cells and the SKOV3.ipl cells were injected s.c. into nude mice to determine their tumorigenic potential. Small solid tumors developed by 8 days in mice given injections of SKOV3.ipl cells; however, the same inoculum of SK-OV-3 cells did not form tumors in nude mice until 13 days after injection. Notably, in every case, tumor volumes in mice given injections of SK-OV-3 cells were much smaller than those in mice given injections of SKOV3.ipl cells.

Fig. 1. Decreased survival time of mice given injections of SKOV3.ipl human ovarian cancer cell lines versus parental SK-OV-3. Mice were given i.p. injections of 1 X 10^6 viable SK-OV-3 or SKOV3.ipl cells suspended in 0.2 ml of Hanks' balanced salt solution. Mice were initially examined two times a week for signs of tumor development and then daily when any tumor symptoms (described in "Materials and Methods") developed.
EIA SUPPRESSION OF OVARIAN CANCER MALIGNANCY

A. B.

Fig. 3. A. increased growth rate of derivative SKOV3.ip1 versus SK-OV-3 parental cells. MTT assays were performed as described in "Materials and Methods" at the indicated days after plating. Results were analyzed by regression analysis. Experiments were repeated for each cell line at least twice. B. increased [3H]Thymidine incorporation of the SKOV3.ip1 cells versus SK-OV-3 parental cells. [3H]Thymidine (1 μCi/well) was added to cells at the indicated times to label those cells that were synthesizing DNA prior to harvest. Radioactivities of individual samples were counted by scintillation counter. Average cpm were calculated from ten replicate samples. Experiments were repeated three times for each cell line.

(Fig. 4B), which agrees with the c-erbB-2/neu gene expression level in these cell lines. Taken together, these data indicate that the level of c-erbB-2/neu overexpression may correspond to the degree of malignancy in these ovarian carcinoma cells.

Inhibited Expression of c-erbB-2/neu-encoded p185 in EIA-expressing Ovarian Carcinoma Transfectants. We have previously shown that the adenovirus-5 EIA proteins can repress c-erbB-2/neu gene expression (31, 38) and suppress the tumorigenic and metastatic potential of neu-transformed mouse embryo fibroblast cells (29, 32). In the present study, we further questioned whether EIA can also repress c-erbB-2/neu overexpression in SKOV3.ip1 cells and function as a tumor suppressor gene in c-erbB-2/neu-overexpressing human cancer cells. Hence, we cotransfected into SKOV3.ip1 cells the EIA-expressing plasmid together with the pSV2-neo plasmid carrying the neomycin-resistance marker gene to generate the EIA-expressing ovarian carcinoma stable transfectants. The G418-resistant clones were selected and expanded into cell lines, which were designated ip1.E1A cell lines. The same approach was used to select control cell lines, in which the pElAdl343 plasmids containing a 2-base pair frameshift deletion in the EIA coding sequences and producing nonfunctional protein products were introduced into the SKOV3.ip1 cells to generate the ip1.Efs cell line. It is possible that some of the stable transfectants selected by such a cotransfection strategy may only harbor the neomycin resistance gene but not the EIA gene. Therefore, to identify those ip1.E1A transfectants that integrated the EIA gene and actually produced EIA proteins, immunoblot analysis with anti-EIA antibodies was performed (Fig. 5A). Two of the ip1.E1A transfectants expressed multiple species of EIA proteins as described previously (39), whereas the control ip1.Efs cell line, as expected, did not express EIA proteins. We thus established two kinds of stable transfectants: (a) ip1.E1A cells (i.e., SKOV3.ip1.E1A-expressing transfectants), which were used to test the tumor-suppressing function of EIA; and (b) ip1.Efs cells (i.e., SKOV3.ip1 transfectants containing EIA frameshift mutants), which were used as a control cell line to make sure that the

Fig. 4. A. increase in colony formation for the SKOV3.ip1 variant versus parental SK-OV-3 cells. Soft-agar assays were performed as described in "Materials and Methods." The numbers of soft agar colonies from each cell line were shown with SD. Experiments were repeated for each cell line at least twice. B. tumor formation by c-erbB-2/neu-overexpressing SK-OV-3 and SKOV3.ip1 ovarian cancer cells. Viable cells (3 x 10⁵) were injected s.c. into the right and left flanks of female homozygous nu/nu mice. For each cell line, six mice were given injections. Tumor formation was scored at indicated days, and tumor volumes were estimated as the product of three-dimensional caliper measurements.
changes in transformation phenotypes (if any) in ip1.E1A transfectants were not due to the selection process or to transfection of the plasmids and the pSv2-neo gene.

Our previous data indicate that EIA proteins can repress c-erbB-2/neu-encoded p185 expression in the c-erbB-2/neu oncogene-transformed NIH3T3 cells (32). In addition, we also demonstrated that EIA proteins can decrease the c-erbB-2/neu mRNA level as well as c-erbB-2/neu-encoded p185 in c-erbB-2/neu-overexpressing breast cancer cell lines (31, 38). To determine if the expression of EIA in ip1.E1A transfectants can inhibit p185 expression, immunoblot analysis of c-erbB-2/neu-encoded p185 protein was performed. p185 protein levels were dramatically decreased in both the ip1.E1A1 and ip1.E1A2 cell lines versus the control ip1.Efs cell line (Fig. 5B), which expressed an amount of c-erbB-2/neu-encoded p185 protein comparable to that of the parental SKOV3.ip1 cell line (data not shown). Since p185 proteins were dramatically reduced in ip1.E1A1 and ip1.E1A2 cells, the transformation potential of SKOV3.ip1 cells did not alter the c-erbB-2/neu gene at the DNA level. Furthermore, these results indicate that EIA can repress the c-erbB-2/neu-encoded p185 protein expression in ip1.E1A transfectants.

In Vitro Suppression of SKOV3.ip1 Cell Transformation by EIA Expression. Once the EIA-expressing ip1.E1A lines were established, we examined the effect of EIA expression on the c-erbB-2/neu-overexpressing ovarian cancer cells in vitro, assessing growth properties, DNA synthesis rate, and colony formation in soft agar. The growth curves of the EIA-expressing ip1.E1A1 and ip1.E1A2 cell lines and control ip1.Efs cell line indicated that EIA expression slightly reduced the growth rate of these ovarian cancer cells versus the control cells (Fig. 6A). Measurement of the DNA synthesis rate by [3H]thymidine incorporation assays revealed that the control ip1.Efs cells had a high level of [3H]thymidine incorporation that was similar to that of SKOV3.ip1 cells and significantly higher than the [3H]thymidine incorporation in the EIA-expressing ip1.E1A1 and ip1.E1A2 cell lines (Fig. 6B). Fig. 6C shows that the c-erbB-2/neu-overexpressing ip1.Efs cells exhibited high efficiency in forming soft agar colonies, whereas the colony-forming efficiencies of the two ip1.E1A transfectants were strikingly reduced. These data suggested that EIA proteins can suppress the effect of the c-erbB-2/neu overexpression in ovarian cancer cells and inhibit cell growth, DNA synthesis, and anchorage-independent growth.

EIA as a Tumor Suppressor Gene for c-erbB-2/neu-overexpressing Human Ovarian Carcinoma SKOV3.ip1 Cells. A critical test for EIA-mediated transformation suppression function in ovarian cancer cells is the ability of EIA to suppress tumor formation in vivo. Therefore, tumorigenicity assays were performed in mice that were injected s.c. with 3 × 10⁶ cells from either the EIA-expressing ip1.E1A1 and ip1.E1A2 cell lines or the control ip1.Efs cell line (Fig. 7A). Like mice given injections of the parental SKOV3.ip1 cells, mice given injections of the control ip1.Efs cells formed tumors 7 days after injection and had huge tumor burdens of 3280 ± 1310 mm³ by 80 days postinjection. However, nu/nu mice given injections of the same number of ip1.E1A1 transfectants did not form tumors until 21–30 days after injection, and their tumor burdens were only 460 ± 170 mm³ by 80 days postinjection. The tumor-suppressing function of EIA was more dramatic in mice given injections of the ip1.E1A2 transfectants, which did not induce tumors until 40–50 days postinjection, and 2 of 6 mice did not develop any tumor, even at 160 days postinjection. The tumor sizes in the four mice given injections of ip1.E1A2 were 290 ± 220 mm³ at 160 days postinjection. Therefore, these results clearly demonstrated that EIA can suppress the tumorigenic potential of the ovarian carcinoma SKOV3.ip1 cells.

We have shown in the previous section that SKOV3.ip1 cells, when compared to SK-OV-3 cells, induced a higher mortality rate and shorter survival following i.p. injection into nu/nu mice. To determine whether EIA expression in SKOV3.ip1 cells could counteract the effect of c-erbB-2/neu overexpression and reduce the mortality rate, we gave mice i.p. injections of the EIA-expressing ip1.E1A1 and ip1.E1A2 cell lines and the control ip1.Efs cell line. Mice given injections of 1 × 10⁶ ip1.Efs cells developed tumor symptoms similar to those described in the previous section; one of the mice died of tumor as early as 19 days postinjection, and all of the other mice died within 75 days postinjection (Fig. 7B). However, there was a significant increase in survival for mice given injections of the EIA-transfected lines versus the parent SKOV3.ip1 frame-shift-transfectant ip1.Efs cell line ($P < 0.01$) (Fig. 7B). The results indicated that EIA expression can reduce the mortality of mice given injections of c-erbB-2/neu-overexpressing human ovarian carcinoma cells.
EIA SUPPRESSION OF OVARIAN CANCER MALIGNANCY

Fig. 6. A, reduced growth rate of the ip1.EIA transfectants versus control ip1.Efs cells. MTT assay was performed as described in "Materials and Methods" at the indicated days after plating. Results were analyzed by regression analysis. Experiments were repeated for each cell line at least twice. B, decreased \[^{[H]}\]thymidine incorporation by the ip1.EIA transfectants versus control ip1.Efs cells (P < 0.01). Soft-agar assays were performed as described in "Materials and Methods." The numbers of soft-agar colony were shown. Experiments were repeated four times for each cell line.

DISCUSSION

We have isolated a derivative cell line termed SKOV3.ip1 from the ascites that developed in mice given injections of human ovarian carcinoma SK-OV-3 cells. Compared with parental SK-OV-3 cells, the SKOV3.ip1 cell line expresses higher levels of c-erbB-2/neu-encoded p185 protein and correspondingly exhibits more malignant phenotypes determined by in vitro and in vivo assays. This association between enhanced c-erbB-2/neu expression and more severe malignancy is very consistent with previous studies in which c-erbB-2/neu overexpression was shown to correlate with poor prognosis in ovarian cancer patients (13). Our data provided experimental evidence to support those clinical studies that c-erbB-2/neu overexpression may play an important role in the pathogenesis of certain human malignancies such as ovarian cancer. It is important to further study the molecular mechanisms and biochemical pathways involved in c-erbB-2/neu overexpression and the associated malignant phenotype. The recent identification and molecular cloning of the ligands for c-erbB-2/neu-encoded p185, which can increase the tyrosine phosphorylation of p185, will enable future direct examination of the molecular mechanisms and the biological effects of c-erbB-2/neu gene overexpression in human cancer and cancer metastasis (40-45).

The adenovirus EIA gene was originally defined as a transforming oncogene that can substitute for the myc oncogene and simian virus 40 large tumor antigen gene in the ras cotransformation assay of primary embryo fibroblasts (46-48). Recently, we reported that EIA products can act as transformation and metastasis suppressors in the mutation-activated rat neu-transformed mouse 3T3 cells (29, 32). In the present study, we further demonstrated that the EIA gene products effectively repressed c-erbB-2/neu gene expression in SKOV3.ip1 ovarian carcinoma cells, suppressed transformation phenotypes in vitro, and

Fig. 7. A, EIA suppressed tumor formation by c-erbB-2/neu-overexpressing ovarian cancer cells. Tumor volumes from mice injected with the indicated cell lines at the indicated days are shown. Experiments were performed as described in "Materials and Methods" and as in Fig. 4B. For each cell line, five to six mice were given injections. B, longer survival of mice given injections of EIA-expressing ip1.EIA cells versus mice given injections of ip1.Efs human ovarian cancer cells (P < 0.001). Mice were given i.p. injections of 1 X 10^6 viable cells from the indicated cell lines. In two experiments, totals of nine mice for the ip1.Efs line, eight mice for the ip1.EIA1 line, and nine mice for the ip1.EIA2 line were given injections. Mice were examined for tumor symptoms and were killed when they appeared moribund (see "Materials and Methods", Fig. 1). Similar results were obtained from the two experiments, and results were combined for analysis.
EIA SUPPRESSION OF OVARIAN CANCER MALIGNANCY

reduced tumorigenicity and mortality rate in vivo. These results indicate that the adenovirus EIA gene can function as a tumor suppressor gene for c-erbB-2/neu-overexpressing human cancer cells as well as inhibit transformation induced by mutation-activated new oncogene in rodent cells. Since we have previously demonstrated that EIA products can dramatically inhibit the c-erbB-2/neu mRNA level and c-erbB-2/neu-encoded p185 expression in human breast cancer cell lines (31, 38) and we have shown that the EIA gene products can repress new gene expression at the transcriptional level by targeting at a specific DNA element in the new gene promoter (31), it is therefore likely that the reduced p185 expression in the ipl.EIA cell lines is due to transcriptional repression of the overexpressed c-erbB-2/newe gene, which may be one of the diverse molecular mechanisms that account for the tumor suppressor function of EIA in SKOV3.ip1 ovarian cancer cells. Interestingly, it has been shown that adenovirus EIA can render hamster cell lines more susceptible to lysis by natural killer cells and macrophages (49, 50). It has also been reported that EIA can induce an increased sensitivity to cytotoxicity by tumor necrosis factor in transfected NIH3T3 cells (51). Therefore, it is conceivable that the tumor-suppressing function of EIA may be partly due to an increased susceptibility to cytolytic lymphoid cells and molecules. Recently, EIA protein was shown to induce a cytotoxic response that resembles programmed cell death (apoptosis) (52), which may also contribute to the tumor-suppressing function of EIA. In addition, EIA has been reported to convert three unrelated types of human cancer cells into a nontransformed state (53). This suggests that EIA may also function as a tumor suppressor gene for certain human cancer cells in which c-erbB-2/newe is not overexpressed. It is not yet clear whether growth signals associated with the c-erbB-2/neu-encoded p185 protein might be activated in these human cancer cells and whether EIA might repress transforming phenotypes of these human cancer cells by blocking the signal transduction pathway associated with p185 protein via repressing c-erbB-2/newe expression; or EIA might suppress tumor formation through other mechanisms in certain human cancer cells. Despite the potential involvement of different molecular mechanisms, our results have established EIA as a tumor suppressor gene for c-erbB-2/newe-overexpressing human ovarian cancer cells; therefore EIA may be useful in developing potential therapeutic reagents for the treatment of these human cancers.

It has been proposed that there are cellular "EIA-like" factors that may mimic the function of EIA in certain cell types (54). Many common features between EIA and c-myc suggest that the c-myc gene product may be one of the cellular homologue of the EIA protein. These common features include the following: EIA and c-myc share a similar structural motif (55, 56); both EIA and c-myc can transform primary embryo fibroblasts in cooperation with the ras oncogene (46, 47); both can bind specifically to the human Rb gene product, the RB protein (57, 58); both can induce apoptosis in certain cell types (52, 59); and both have been shown to block transformation of certain transformed cell lines (53, 60). In addition, we have found that, similar to the EIA proteins, the c-myc gene product can repress c-erbB-2/newe gene expression at the transcription level, resulting in reversal of the new-induced transformed morphology in NIH3T3 cells (61). Whether c-myc can suppress the malignancy of c-erbB-2/newe-overexpressing human cancer cells is an interesting issue that needs to be further examined.

EIA can inactivate the RB tumor suppressor gene by complexing with the RB gene product, RB protein, and by inducing RB protein phosphorylation (57, 61). Therefore, we have recently examined whether RB might also regulate c-erbB-2/newe expression. Similar to EIA, RB can also repress c-erbB-2/newe gene expression at the transcriptional level (62). The cis-acting elements responding to EIA and RB are different but only a few base pairs away from each other. It would certainly be interesting to study further the possibility that EIA and RB might interact with each other to regulate c-erbB-2/newe transcription.

The EIA gene of adenovirus 2, a close sera type of adenovirus 5, was shown to reduce the metastatic potential of ras-transformed rat embryo cells (63). It was hypothesized that the Ad-2 EIA gene may regulate the expression of one or more cellular genes that contribute to the metastatic phenotype and expression of nm23, a gene associated with low metastatic potential in certain cell types that was subsequently shown to be elevated in EIA-expressing ras-transformed rat embryo cells (64). Although we have found that EIA can repress c-erbB-2/newe gene expression and suppress the metastatic potential of c-erbB-2/newe-transformed 3T3 cell (29), the c-erbB-2/newe gene expression levels in the parental ras-transformed rat embryo cells and EIA-expressing ras-transformed rat embryo cells is not known. Therefore, it is not clear at this moment whether repression of c-erbB-2/newe gene expression contributes to the metastasis suppression function of EIA in ras-transformed rat embryo cells.

One of the interesting issues on the correlation between c-erbB-2/newe overexpression and poor clinical outcome in human breast and ovarian cancers is whether c-erbB-2/newe overexpression is the result of an aggressive tumor or has a causative role for aggressive tumors. The data presented here support, although they do not prove, a direct role for c-erbB-2/newe overexpression in the pathogenesis of aggressive tumors. First, comparison of the SK-OV-3 cell line and the derivative SKOV3.ip1 cell line revealed a direct relationship between an increased c-erbB-2/newe expression level and an enhanced malignant phenotype measured by in vitro and in vivo assays. Second, c-erbB-2/newe expression in the EIA-expressing ipl.EIA cells was dramatically repressed, and, accordingly, the malignant potential of these cells was diminished. Taken together, these observations argue for a causative role of c-erbB-2/newe overexpression in the more malignant tumor pattern. Since c-erbB-2/newe-overexpressing ovarian tumors may be more malignant, more aggressive therapy might be beneficial to those ovarian cancer patients whose tumors overexpress c-erbB-2/newe-encoded p185.

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