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p53 Increases Experimental Metastatic Capacity of Murine Carcinoma Cells

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Transfection of a cloned p53 gene into a murine bladder carcinoma cell with a low metastatic capacity led to elevated levels of p53 protein in clonal transfectants. After intravenous inoculation into syngeneic mice, p53-transfected clones showed significantly increased metastatic potential in comparison with control transfectants. The observed change did not seem to be due to a change in growth potential per se since the cell lines showed similar growth properties in vitro.

A great majority of human cancers develop from epithelial cell layers of organs such as the lung, breast, and gastrointestinal and colorectal tracts. Major breakthroughs in the treatment of the respective carcinomas have been largely hampered by the generation of metastases which change a localized disease into a systemic disease with multiple-organ side effects. In awareness of our lack of knowledge of the biology of metastasis formation, major research efforts are directed toward genes and molecules which might play an important role in each of the successive steps that lead to the generation of metastases, i.e., detachment of tumor cells from the local primary tumor, invasion of intercellular matrices, penetration of basement membranes of blood vessels, circulation while undergoing homotypic aggregation, extravasation, immune escape, induction of angiogenesis, and growth in the afflicted target organ.

Most gene transfer studies in this field have been performed with oncogenes of the *ras* family which were used to induce metastatic competence in tumorigenic but nonmetastatic cells mostly of fibroblastic origin (1, 8, 24). In two independent studies (26, 27), activated *ras* genes were transfected together with a pSV2neoR plasmid into nonmetastatic murine mammary carcinoma cell lines. While the thus-generated transfectants showed significantly increased metastatic capacity, control transfectants also showed an increase, although to a lesser extent, of metastatic competence. Here we report on a different oncogene, the p53 gene, which belongs to the nuclear oncogenes (4). This oncogene was found to be overproduced in a wide range of various tumor cells (5, 18, 19). In conjunction with the *ras* oncogene, the p53 oncogene was shown to be capable of transforming primary embryonic rat cells (12, 16). Furthermore, the p53 protein was shown to play a role in the normal cell cycle. Total inhibition of p53 expression in transformed and nontransformed mouse cells led to their death (22).

Here we show that upon transfection of p53 into a murine bladder carcinoma cell line with a low metastatic capacity, these cells have a significantly increased capacity after intravenous inoculation to colonize and grow in the lungs of syngeneic recipients. Control transfectants showed no significant increase in this test for experimental metastatic capacity.

MATERIALS AND METHODS

Cell lines. The MB 63/T/36 (BL) bladder carcinoma cells originated from a chemically induced tumor (3) and were passaged in C57BL/6 mice (A. Radler-Pohl, J. Pohl, and V. Schirrmacher, Int. J. Cancer, in press). Cells were grown in tissue culture medium (Dulbecco modified Eagle medium; Flow Laboratories, Glasgow, Scotland) supplemented with 10% fetal calf serum and were passaged one to two times per week by careful trypsinization. Cell cultures were used within 12 passages from frozen stocks of low-passage cells to eliminate possible genotypic and phenotypic shift.

Experimental metastasis assay. Bladder carcinoma cell lines with or without the transfected p53 gene were mechanically removed from culture flasks where they had grown to subconfluency. After washing, the cells were adjusted to a suspension of 10^7 cells per ml and 50 μl (5×10^5 cell) was injected into the tail vein of normal male C57BL/6 mice. Animals were killed after 24 days and monitored for metastases in the lung, heart, kidney, liver, and spleen. The organs were fixed in Bouin solution and observed with a Binokular microscope. All metastases found were observed in the lungs. Significance level was determined by Kruskal-Wallace analysis and Dunn correction (21).

Antibodies. Monoclonal anti-p53 antibodies were obtained from the established hybridoma cell lines RA3-2C2 (2, 20) and PAb122 (10). Antibodies were purified and concentrated by binding to protein A-Sepharose columns (Sigma Chemical Co., St. Louis, Mo.). Monoclonal antibodies were obtained either from supernatants of the hybridoma cell lines or from the ascitic fluid of syngeneic mice injected intraperitoneally with the hybridoma cell lines.

Southern blot analysis. DNA was prepared from individual BL-derived clones which had been transfected by a genomic p53 clone. Cells were washed twice with phosphate-buffered saline, suspended in lysis buffer (0.5% sodium dodecyl sulfate [SDS], 50 mM Tris hydrochloride, 5 mM EDTA [pH 7.5], 0.2 mg of proteinase K per ml [Boehringer Mannheim Biochemicals, Indianapolis, Ind.]), and incubated for 14 h at 37°C. The solution was extracted twice with an equal volume of redistilled phenol, followed by two extractions with equal volumes of chloroform-isoamyl alcohol in a ratio of 24:1.

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TABLE 1. Lung-colony-forming potential of clonal p53 transfectants of bladder carcinoma lines after intravenous inoculation[a]

Expt	Group	Cell line	No. of lung metastases per animal (total no. of metastases)	P value compared with control
1	A	BL (control)	0, 1, 0, 0, 1, 0, 2, 0, 0, 0 (4)	
	B	BLgpt	0, 2, 8, 1, 0, 0, 3, 0, 0, 0 (14)	NS[b]
	C	BLgpt CL8	7, 0, 4, 2, 3, 2, 11, 9, 2, 2 (42)	<0.0005
	D	BLgpt CL11	30, 4, 6, 18, 3, 8, 8, 3, 4, 1 (85)	<0.0005
2	A	BL (control)	0, 0, 0, 2, 0, 2, 6, 0, 0, 0 (10)	
	B	BLgpt	2, 0, 0, 8, 14, 0, 2, 0, 0, 0 (26)	NS
	C	BLgpt CL8	3, 2, 0, 21, 18, 11, 7, 1, 2, 4 (69)	<0.0005
	D	BLgpt CL11	3, 5, 0, 115, 7, 47, 15, 19, 1, 3 (215)	<0.0005

[a] A total of 5×10^5 cells were injected into the tail veins of 3-month-old C57BL/6 mice male recipients; animals were killed after 24 days and monitored for metastases in the lung, liver, kidney, spleen, and heart. Organs were fixed in Bouin solution and checked with a Binokular microscope under 10-fold magnification. All metastases found were localized in the lung. Results were confirmed by histology.

[b] NS, Not significant.

The DNA solution was adjusted to a final concentration of 0.3 M NaCl, and 2 volumes of cold ethanol were added. The DNA precipitate formed at room temperature was isolated, washed twice with 80% ethanol, and suspended in 10 mM Tris hydrochloride–1 mM EDTA (pH 7.5). DNA was digested and electrophoresed on a 0.8% agarose gel, blotted onto a nitrocellulose filter (23), and hybridized to a nick-translated (17) p53-specific insert probe (30).

DNA transfection. DNA transfection was performed by the calcium phosphate precipitation technique described by Graham and van der Eb (7). Briefly, 25 μg of the p53 genomic clone pULM8R6 (28, 29) and 5 μg of pSVgpt, the selective marker (15), were suspended in 1 ml of transfecting buffer, and 62.5 μl of 2 M $CaCl_2$ was added. After formation of a fine precipitate at room temperature, the mixture was added to a monolayer of BL cells. Cells were incubated for 10 min at room temperature and then transferred into 10 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. After 4 to 5 h of incubation at 37°C, cells were washed and plated in microtiter plates (Costar, Cambridge, Mass.), in nonselective medium. After 3 days, selective medium containing mycophenolic acid (2 μg/ml), xanthine (150 μg/ml), hypoxanthine (15 μg/ml), and L-glutamine (150 μg/ml) was added. Growing clones were detected 12 to 24 days after transfection and expanded.

Immunoprecipitation. Of each individual cell line, 10^7 cells were washed several times in phosphate-buffered saline and suspended in 1.5 ml of Dulbecco modified Eagle medium without methionine, enriched with 10% dialyzed, heat-inactivated fetal calf serum and 250 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.). Cells were incubated for 1 to 2 h at 37°C, washed in phosphate-buffered saline, and extracted into 2 ml of lysis buffer (10 mM Na_2HPO_4-NaH_2PO_4 [pH 7.5], 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) at 4°C. In vivo-labeled cell lysates were precleared by repeated absorption onto *Staphylococcus aureus* and nonimmune serum. Antigen-antibody complexes were collected with *S. aureus* (10), and SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (11).

RESULTS

Metastatic potential of p53-transfected BL variants. Cotransfection of the p53 gene with the *gpt* gene allowed us to select for positive bladder carcinoma transfectants. Two p53-transfected clones were picked and expanded for analysis. As controls we used untreated BL cells and BL cells transfected with the *gpt* gene only. All cell lines were clonally derived at the same time and showed comparable growth properties in vitro.

A total of 5×10^5 cells of the p53-transfected BL clones or of control BL cells were inoculated intravenously into syngeneic mice. Animals were killed after 24 days and monitored for metastases in lung, liver, kidney, spleen, and heart. It had been predetermined that 24 days were sufficient to develop visible metastases and that the number of nodules was comparable to that seen in respective moribund animals. Table 1 contains the results from two such experiments. Column 4 shows the distribution of metastases in 10 animals per group and the total number of metastases per group. All metastases found were localized in the lungs. Control transfected BLgpt cells showed no significant differences compared with the untreated BL cells. In contrast, both p53-

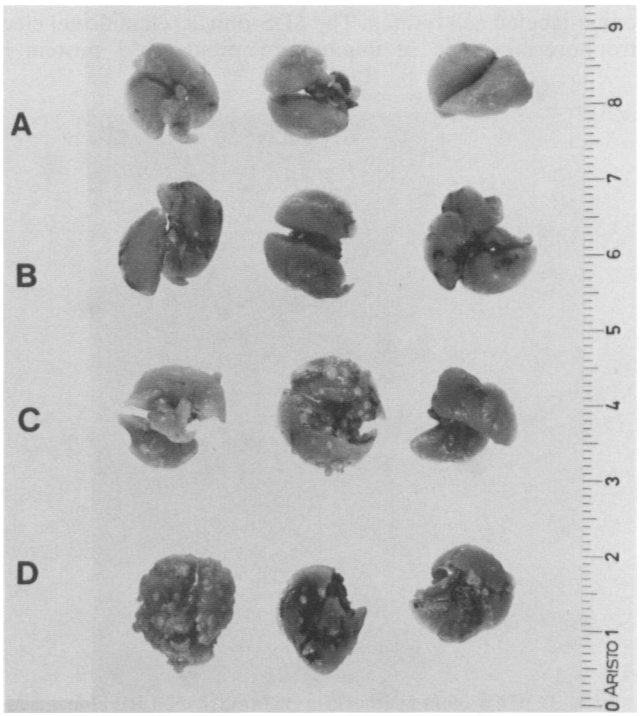

FIG. 1. Representative lung metastases from experiment 1, Table 1. (A) BL; (B) BLgpt; (C) BLgpt CL8; (D) BLgpt CL11. Lungs are fixed with Bouin solution.

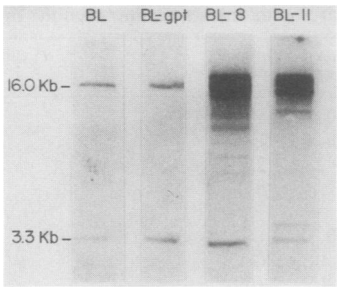

FIG. 2. Southern blot analysis of stably integrated genomic p53 sequences in BL clones. Samples of *Eco*RI-digested high-molecular-weight DNA (5 μg) were electrophoresed on a 0.8% agarose gel, blotted onto nitrocellulose filters, and hybridized to nick-translated p53-probing DNA. Kb, Kilobases.

transfected clones showed significantly increased metastatic potential ($P < 0.0005$). The factor of increase was approximately 10 for BLgpt CL8 and 20 for BLgpt CL11. Figure 1 shows representative lung metastases and organs from control animals. The results of Table 1 were corroborated by experimental data obtained with different clonal p53 transfectants.

Southern blot analysis of transfected clones. Southern blot analysis of *Eco*RI-digested genomic DNA from BL, BLgpt, BLgpt CL8, and BLgpt CL11 showed in addition to the endogenous p53 bands (Fig. 2, lane BL) new p53-specific bands (Fig. 2, lanes BL-8 and BL-11) in the transfected clones as a sign for new integration of the donor DNA. The results were similar to those obtained with the same probes in other transfected cell lines (21).

Immunoprecipitation of p53-specific proteins. Next we investigated whether the transfected p53 gene was also expressed in the carcinoma lines. By using two different anti-p53 antibodies, we immunoprecipitated [^{35}S]methionine-labeled cell lysates. The SDS-polyacrylamide gel electrophoresis profile of immunoprecipitated p53 protein is shown in Fig. 3. Group 1 (2M3) represents a positive control (27), and group 2 (BL) shows the immunoprecipitation of the endogenous p53 protein of untreated BL cells. Transfection of the *gpt* gene alone did not result in changes of p53 protein expression (BL-gpt). Both p53-transfected BL variants, however, showed elevated levels of p53 protein (BL-8 and BL-11). We thus demonstrated that murine carcinoma cells transfected with a genomic p53 oncogene contained integrated transfected p53 and showed elevated levels of the oncogene product. It seems possible that the elevated level of p53 protein is responsible for the increase in metastatic capacity.

DISCUSSION

The p53 nuclear protein seems to possess a function in regulation of cellular growth (13, 14). Many transformed cells in culture have been found to express elevated levels of p53, thus pointing to an association of p53 and malignancy. Furthermore, recent studies on transfection of plasmids encoding p53 antisense demonstrated the importance of the expression for the maintenance of rapid proliferation of tumor cells (22).

Here we investigated a possible function of p53 in an established murine bladder carcinoma cell line. This tumorigenic but low-metastatic-capacity cell line showed low-level expression of its endogenous p53 gene. Upon transfection with genomic p53, new p53 genes became integrated and expressed. Two independently derived clones with elevated p53 expression showed upon intravenous inoculation into syngeneic mice a 10- to 20-fold-increased capacity to metastasize in the lungs. This observed change was highly significant and suggests that a single oncogene by transfection can lead to progression from low to high metastatic capacity in a carcinoma model system. In contrast to many other model systems, control transfectants retained a low metastatic capacity and did not progress. Since transfection of the p53 did not change the metastatic behavior of NIH 3T3 fibroblast cells (6) but did so in the carcinoma lines of this study, it is

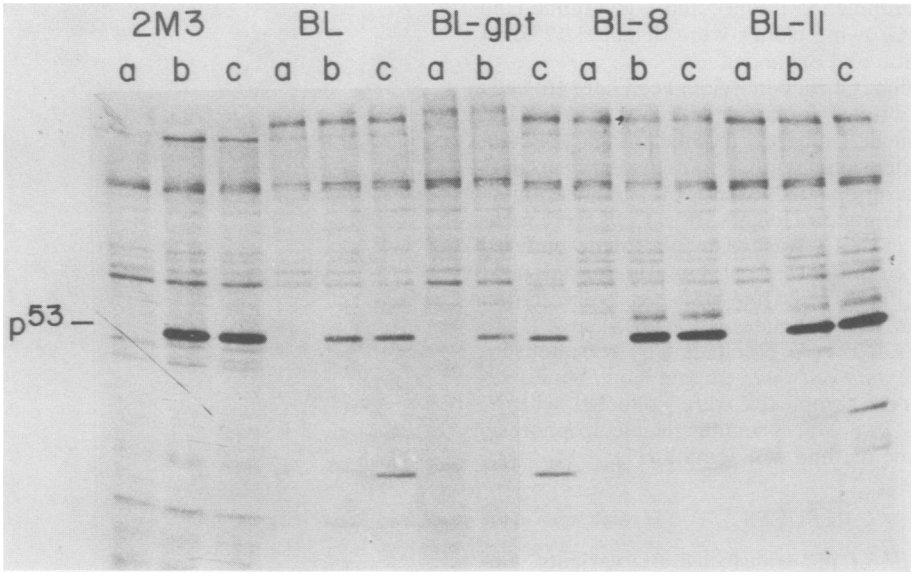

FIG. 3. p53 protein synthesis in p53-transfected BL clones and controls. Equal amounts of radioactive protein were immunoprecipitated with specific antibodies: lanes a, nonimmune serum; lanes b, PAb122; lanes c, RA3-2C2. Antigen-antibody complexes were collected with *S. aureus* (9). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (11). 2M3 cells are Abelson murine leukemia virus-transformed bone marrow cells with a high expression of p53 (27).

possible that the effect of the p53 on the metastatic capacity depends on the differentiation state of the recipient cell and on interaction with other cellular genes. It is likely from our results that the increased expression of the p53 protein observed in the transfected clones was the reason for the increased capacity of the cells to colonize and grow in the lungs. Further experiments are required to find out why and how an increased expression of p53 in these cells leads to increased malignancy.

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