Primary Transfection as a Mechanism for Transformation of Host Cells by Human Tumor Cells Implanted in Nude Mice

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ABSTRACT

Establishment of cell lines in vitro from a human lung cancer xenograft in nude mice resulted in transformed mouse cell lines. The transformed mouse cell lines expressed both mouse-specific and human-specific histocompatibility antigens. Of 3 cell lines, 2 were tumorigenic in BALB/c nude mice but not in normal mice. Tumors formed by the transformed mouse cell lines were fibroblastoid and epithelial by histology. In addition, tumors exhibited neuroepithelial differentiation by ultrastructural and immunohistochemical analysis. Phenotypically they were similar to the original patient and human xenograft tumor. These data suggest that previous reports of host cell transformation and induction of fibrosarcomas may not be true fibrosarcomas. Human DNA sequences were present in the tumorigenic cell lines, indicating that spontaneous transfection of human tumor DNA into host cells had occurred. The implication of these findings is that human genetic information has been transferred to primary mouse host fibroblasts, which resulted in a transformed as well as a differentiated phenotype.

INTRODUCTION

Human tumors of varying histological types when xenografted in nude mice have been reported to result in the transformation of mouse fibroblast cells into fibrosarcomas (1-8). The transformed fibroblasts have been observed in vitro and in vivo (1-8). The mechanism by which this transformation of host fibroblasts occurs is unknown. We now report that tissue culture cell lines, initially established in serum-free conditioned media from a human small cell lung cancer, resulted in the transformation of mouse cells as evidenced by the formation of tumors when injected into nude mice. Immunocytochemical studies indicate that these tumor cells are similar to the original human lung cancer whereas cytogenetic analysis indicates that they may have a typical mouse karyotype. Because these mouse cell lines expressed human DNA sequences it is suggested that they have acquired human genetic information that resulted in the expression of the human gene products as well as induction of the lung cancer. On the basis of these findings we propose that the transformation of host cells can occur when foreign tumors are injected into nude mice and that this may be a potentially useful model for studies of spontaneous transfection and expression of differentiated phenotype.

MATERIALS AND METHODS

Xenograft History. A metastatic skin nodule from a patient with small cell carcinoma of the lung was established in male BALB/c nude mice (Life Sciences, St. Petersburg, FL) by s.c. implantation of 2- to 3-mm³ tumor fragments in 1982 and designated NES. The xenograft tumor line has been intermittently reestablished from frozen stock material as needed. This tumor has, in addition, retained its human karyotype and DNA content by flow cytometry through 10 serial passages. The NES tumor expresses 0.9 nmol 14CO₂/h/mg protein of dopa decarboxylase activity when compared to an activity of 71 nmol 14CO₂/h/mg protein for NCI line H69. The tumor has features which are consistent with small cell carcinoma of the lung (histology, neurosecretory granules, neuroendocrine markers such as neuron-specific enolase, and neurofilament by immunohistochemistry) and variant features (presence of tonofilaments, desmome-like cell attachment structures, and glandular differentiation on electron microscopy, low dopa decarboxylase activity, and epithelial differentiation markers such as cytokeratin and epithelial membrane antigen by immunohistochemistry) (9, 10). This tumor on repeated examinations through the 10th transplant generation has retained the above-mentioned features.

Establishment of Cell Lines. During the 4th transplant generation in November 1983, a tumor was excised, a portion processed for histology, and the rest of the tumor cut into 1- to 2-mm³ fragments and incubated in serum-free conditioned media for human small cell lung cancer in 75-cm² tissue culture flasks in order to suppress the growth of mouse fibroblasts. The serum-free media consisted of RPMI tissue culture media, selenium (30 nm), hydrocortisone (10 nm), bovine insulin (5 ug/ml), transferrin (100 µg/ml), and 17β-estradiol (10 nm) as previously described (11). Three permanent cell lines designated NES B, D, and E were thus established. NES B was changed after 2 months to RPMI media containing 15% fetal calf serum and 1% penicillin-streptomycin while the NES D and E were changed after 6 months of culture in serum-free media. The cell lines were routinely passaged 2-3 times weekly with the use of 0.25% trypsin.

The cell lines once permanently established were analyzed for karyotype, DNA content by flow cytometry, isoenzyme analysis, histology, electron microscopy, and immunohistochemistry.

Tumorigenicity. Cells (1 x 10⁶) from NES B, D, and E cell lines in 0.1 ml volume were injected s.c., bilaterally, in normal and nude male BALB/c mice. Mice were monitored weekly for tumor formation for 4 months. Tumor formation was usually evident within 4 weeks. Nude mice were housed in the Nude Mouse Facility, University of Texas Medical Branch Cancer Center.

Anchorage Independence. Cells (1 x 10⁶) from NES B, D, and E cell lines were plated in 0.3% agar (Difco Laboratories, Inc., Detroit, MI) and 0.3% agarose (Sea Plaque, FMC Corp., Rockland, ME) containing RPMI tissue culture media and 15% fetal calf serum in 35-mm Petri dishes and incubated in an atmosphere of air and 5% CO₂. After 21 days of growth colonies greater than 50 cells were counted at x40 with an inverted microscope.

Isoenzyme Analysis. Extracts of 1 x 10⁷ cells were prepared by freezing and thawing 6 times in saline. The supernatant fluid was analyzed for origin of species and for evidence of hybrid formation. The following enzymes were examined: lactate dehydrogenase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, and purine nucleoside phosphorylase. Karyotype studies, Giemsa G and pH 11 banding, and isoenzyme analysis (12, 13) were performed by Dr. Ward D. Peterson, Jr. (Children's Hospital of Michigan, Detroit, MI) and Dr. Ward D. Peterson, Jr. (Children's Hospital of Michigan, Detroit, MI) under contract NCI NO1-CP-21017.

Flow Cytometry. Cells (1 x 10⁶) were suspended in propidium iodide- and thymidine-hypotonic citrate (14) and analyzed on a TPS-1 cell sorter ( Coulter Electronics, Inc., Hialeah, FL). Data are expressed as the DNA index (ratio of model G1 DNA content of tumor cells versus diploid reference standard) that is used as a measure of ploidy (15). Human lymphocytes and mouse bone marrow cells were used as diploid controls for human and mouse cells, respectively.
Immunohistochemistry. Cultured cell lines NES B, D, and E and cell suspensions enzymatically dissociated from nude mouse xenografts NES D and E were studied. The cell suspensions were digested in 0.3 M sodium citrate, 65°C for 12 h. The high molecular weight DNA was isolated by sequential washes in phosphate-buffered saline the cells were incubated with fluorescein-conjugated goat anti-mouse IgG (Cooper Biomedical) (1:40) for 40 min and washed. A drop of cell suspension was placed on a glass slide, coveredslipped, and examined by a Leitz orthoplan fluorescence microscope. Controls included omission of primary antibodies and substitution of the primary antibodies with nonimmune mouse ascites fluid.

Fresh frozen tissue samples and formalin-fixed, paraffin-embedded tissue samples were studied by a 3-layer immunohistochemical method. The primary antibodies used were murine monoclonal antibodies to cytokeratin—AE1/3 (Hybritech, San Diego, CA), neurofilament (Lab Systems, Chicago, IL), epithelial membrane antigen (Dako Corporation, Santa Barbara, CA)—and monoclonal antibodies to calcitonin, neuron-specific enolase, and neurotensin (Dako Corporation). The optimal dilutions were 1:10 for monoclonal antibodies and 1:100–1:1,000 for polyclonal antibodies. The tissue sections were sequentially incubated with biotinylated sheep anti-mouse IgG F(ab’)2 or biotinylated goat anti-rabbit IgG F(ab’)2 (1:40) followed by avidin-fluorescein isothiocyanate (1:250) (Vector Labs, Burlingame, CA) for immunofluorescence and streptavidin biotinylated peroxidase complex (1:500) (Amersham Corporation, Arlington Heights, IL) for immunoperoxidase technique. The peroxidase reaction product was visualized by incubating with diaminobenzidine (0.05%) and hydrogen peroxide (0.01%). Controls included omission of primary antibodies and substitution of primary antibodies with nonimmune sera from the same species.

Light Microscopy. Tissue samples (0.4 cm3) were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Sections (4 µm) stained with hematoxylin and eosin were examined.

Electron Microscope. Tissue samples (1 mm3) were fixed in half-strength Karnovsky’s solution, postfixed in osmium tetroxide, and embedded in Epon. Sections (70 nm) were examined with a Philips 410 Electron Microscope.

Preparation and Source of DNA. High molecular weight DNA from solid tumors was prepared as follows. Tumors (0.5 gm of frozen tissue) were ground by using a pestle in a mortar containing liquid nitrogen. The resulting powder was added to 20 ml of Tris-HCl buffer, pH 7.5, and were ground by using a pestle in a mortar containing liquid nitrogen.

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RESULTS

For purposes of clarification and in following the text the origin of the various xenografts and cell lines is depicted in Fig. 1.

The cell lines NES B, D, and E always grew as an adherent monolayer with fibro-epithelialoid morphology. Chromosome analysis of the cell lines was performed after 6 and 18 months in culture while the NES xenograft was examined in the 5th and 10th transplant generation. One hundred metaphases of each cell line were examined for karyotype morphology and were exclusively of mouse origin while the NES xenograft retained human karyotype. Representative Giemsa-banded kar-

Fig. 1. Schematic graph depicting the life history and origin of various xenografts and cell lines referred to in the text.
Fig. 2. Representative Giemsa-banded karyotype from cell lines NES E indicating mouse origin. Probable origin of marker M1, Rob. t(2:2); M2, unknown. The number of unassignable chromosomes varied from 1 to 10 in different karyotypes.

Fig. 3. Representative Giemsa-banded karyotype from cells of the NES xenograft from the 10th transplant generation indicating the presence of the human karyotype. Marker M1 is probably chromosome 14 with extra material; other chromosomes were unassignable.

on frozen sections from the human xenograft tumor and the transformed mouse tumors NES D and E in the implant stage expressed human HLA antigens Class I and II. However, this expression of human HLA Class I and II was lost in the cell lines with passage of time (Fig. 5C) but was retained in some of the transformed mouse tumors NES E but not NES D on serial passage.

The patient's tumor and the human NES xenograft (Fig. 6,
A and B) were composed of round to ovoid cells with high nuclear:cytoplasmic ratio, arranged as masses in a mononuclear fashion, with frequent mitoses and large areas of necrosis. The histology was consistent with an undifferentiated small cell carcinoma of the lung, intermediate type. The tumor formed by cell lines NES D (Fig. 6C) was composed of spindle-shaped, fibroblastic cells admixed with large, plump, epithelialoid cells. The latter cells predominated in the tumor formed by the cell lines NES E (Fig. 6D). Ultrastructural examination of the NES xenograft revealed cells with definite tonofilaments and membrane-bound neurosecretory granules (Fig. 7, A and B) as well as desmosome-like cell attachment structures (data not shown). Membrane-bound neurosecretory granules and desmosome-like cell attachment plaques were also identifiable in tumors formed by the cell lines NES D and NES E (Fig. 7C). Viral particles were not detected in the xenografted tumors by electron microscopy.

Immunohistochemical analysis revealed that the tumors formed by cell lines NES D and E exhibited cytokeatin, epithelial membrane antigen, neuron-specific enolase, and neurofilament antigens similar to the patient's tumor and the human NES xenograft. Some representative data are shown in Fig. 8.

DNA from NES lines D and E and their secondary tumors were prepared and analyzed by Southern blotting for the presence of human repetitive sequences (Fig. 9). This method allows the comparison of putative transforming genes before their actual isolation. Tumors formed by NES D and E contain a common set of human repetitive sequences when the DNA was analyzed with an alu sequence probe (Fig. 9A). Although only one discrete band is observed in lanes 3 and 4, the intensity of staining would suggest that alu sequences are dispersed throughout the gels when compared to mouse DNA as a control, lane 2. When DNA from cell lines NES D and E and tumor formed by line E are analyzed by Southern blot using human lymphocyte DNA as the probe, human repetitive sequences were present in large amount (Fig. 9B).

DISCUSSION

Human tumor xenografts growing in nude mouse or immunosuppressed animals are composed of a mixed population of cells. While the malignant cells that grow are human, the stromal cells are that of host origin. It has been previously reported that host fibroblasts may acquire the transformed phenotype in vitro or in vivo resulting in the formation of fibrosarcomas (1-8). Results from 2 studies suggest that the frequency with which host cell transformation occurs is low (5, 8). Although cell fusion (18, 19), activation or presence of host viruses (5, 20), and transforming growth factors (21, 22) may be implicated in the process of host cell transformation, the mechanism by which nude mouse host fibrosarcomatous transformation occurs is unknown. The data presented help us to define this process at the molecular level.

In order to establish cell lines from a small cell lung cancer growing as a xenograft in nude mice we had initiated cell cultures under serum-free conditions for culturing small cell carcinoma of the lung with the purpose of excluding the growth of host fibroblasts. We were, therefore, surprised by the analysis of the cell lines which indicated that they were of murine origin. While the malignant cells that grow are human, the stromal cells are that of host origin. It has been previously reported that host fibroblasts may acquire the transformed phenotype in vitro or in vivo resulting in the formation of fibrosarcomas (1-8). Results from 2 studies suggest that the frequency with which host cell transformation occurs is low (5, 8). Although cell fusion (18, 19), activation or presence of host viruses (5, 20), and transforming growth factors (21, 22) may be implicated in the process of host cell transformation, the mechanism by which nude mouse host fibrosarcomatous transformation occurs is unknown. The data presented help us to define this process at the molecular level.
cross-react with control mouse cells. Although human small cell lung cancer cells, in general, have decreased HLA expression, they may express it strongly in some cases (23). We, therefore, were fortunate that the mouse tumor cells expressed human HLA antigens and provided evidence of human gene product expression. The latter suggested to us that human genetic information must be present in the mouse transformed cell lines and explained why these cell lines failed to form tumors when injected into normal BALB/c mice.

The mouse cell lines expressed a transformed as well as a differentiated phenotype. The most likely mechanism to explain this would be a process such as cell fusion or transfection (24). However, by cytogenetic or isoenzyme analysis we were unable to demonstrate evidence for cell fusion. Human DNA sequences present in the transformed mouse cell lines are evidence for natural or spontaneous transfection. This finding does not totally exclude cell fusion as a mechanism because cell fusion may have been the original step with subsequent loss of human chromosomal material such that it could not be detected by cytogenetic analysis. Because the NES xenograft showed a typical human karyotype in the 5th and 10th transplant generations and because no evidence of change in histology of the

Fig. 5. Photomicrographs of cell suspensions of cell lines NES D stained by indirect immunofluorescence technique for A, HLA-ABC, and B, HLA-DR antigens; C, same as A except late passage cells; D, blank.

Fig. 6. Photomicrographs of sections of A, patient tumor; B, NES xenograft, 4th transplant; C and D, tumors formed by cell lines NES D and E, respectively. Stained with hematoxylin and eosin, x 575.
xenograft was present through the 10th transplant generation, it is suggested that the transfection of mouse cells may have occurred in vitro. However, the possibility that a small focus of transfection in vivo was passaged in vitro cannot be totally excluded. Another possibility is that growth conditions in vivo may favor the transplanted human tumor cells while keeping the transformed and transfected fibroblasts under control, whereas those in vitro favor the growth of the transformed mouse fibroblasts.

Transfection mediated by human tumor DNA or cloned oncogenes of the mouse NIH 3T3 cell line has been reported to result in malignant transformation of NIH 3T3 cells (25). However, it has been demonstrated that transfection with 2 different oncogenes may be necessary for malignant transformation of primary cells (26, 27). Injection of transfected rodent cells into nude mice results in the formation of undifferentiated fibrosarcomas (28). The ability to demonstrate a differentiated phenotype in transfected cells has usually required that the recipient cell already possess the differentiated phenotype (29–31). In our example of spontaneous transfection it is important to note that the neuroepithelial phenotype similar to that of the original patient and xenografted small cell lung cancer were
present as well as the ability of these cells to be cultured in serum-free media designed for small cell lung cancer. This suggests that either the human regulatory genes for this lung cancer may have been passed onto the transfected cells and one is now activating mouse genes for the differentiated phenotype or that the human genes responsible for the differentiated phenotype are present in the mouse host cells.

Our example of host cell transformation of primary cells would appear to be also one of transfection. How could this have come about? Transforming growth factors are liberated in vitro by a number of human lung cancer cell lines and in vivo by a variety of human tumor xenografts in nude mice (21, 22). In addition, the isolation of transformed human fibroblasts has been reported from patients with lung cancers (32). It is, therefore, possible that primary fibroblasts coexisting with human tumor cells in vitro or in vivo become transformed with serial passage because of elaboration of growth factors resulting in increased susceptibility to transfection.

Although the nude mouse system has been reported to be a useful method for the study and isolation of oncogenes of cells previously transfected in vitro (33, 34), the data presented here suggest the use of the nude mouse system to study the oncogenic process as it relates to spontaneous or natural transfection. The importance of this system for studies of oncogenesis may be that it represents spontaneous transfection of primary cells and can result in a differentiated phenotype. The usefulness of this method will ultimately be determined by the frequency with which this process occurs and requires prospective evaluation. We would also suggest that previous reports of nude mouse host fibrosarcoma induction may actually represent cases of transfection with potentially important human tumor genetic information.

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