Establishment of Different Clonal Strains from a Human Sarcoma of the Stomach: Tumorigenic Heterogeneity in Athymic Nude Mice

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ABSTRACT

Twenty five clonal strains have been isolated from a single human sarcoma of the stomach. Two different types of clones have been recognized by their morphology and behavior in vitro. Type I clones were characterized by criss-crossed arrays and multilayers with high terminal density. Type II clones grew in a well-organized monolayer with lower saturation density. Although both types of clones exhibited fibroblastic appearance, type I clones showed a more rounded, refractile shape. The cells of this type showed multiple regions of criss-crossed arrays and multilayers throughout the culture vessels. Saturation density of this type of clone was 2- to 3-fold (1.7 to 2.1 x 10^5 cells/sq cm) higher than that of type II clones. Chromosomal analysis revealed that type I clones were human aneuploid ones with modal chromosome numbers ranging from 51 to 61. With the exception of clones 11 and 19, type I clones were able to produce tumors in athymic nude mice when injected s.c. Type II clones exhibited a more flattened and elongated appearance. The cells grew in a well-organized monolayer resembling fingerprint whorls. They showed lower saturation density (0.7 to 0.9 x 10^5 cells/sq cm). Chromosomal examinations revealed the clones to be human aneuploid ones with modal numbers from 47 to 54. Tumor formation was not observed in nude mice given injections of this type of cell. Both types of clones did not bear antigens cross-reacting with the antiserum against mouse spleen cells but had surface antigens which were affected by the antibody against HL-60 cells and complement.

These results suggested that this human sarcoma was het- erologus and that cells with widely different tumorigenic potential preexisted in the parental cell population.

INTRODUCTION

The selection of cancer cell variants is a potentially powerful technique for aiding our understanding of tumor formation and metastasis. In animal tumors, correlation of in vitro growth properties and tumorigenicity has been described (3, 9, 10, 16). In human tumors, few such studies have been reported (7, 24, 30). Our approach has been to select cell lines for specific cancer-related properties and then attempt to correlate these variations with specific cellular properties. There has been increasing evidence that a single tumor consists of several clones which differ from each other in their cellular and biochemical properties (7-9). On the other hand, morphological changes and growth properties have been used to distinguish transformed cells from their progenitors (2, 3). It was recently suggested that cell transformation in culture, sufficient to confer oncogenic potential in vivo, is a multistep process (20), but definitive information about the number and the nature of discrete steps that result in neoplastic transformation is still needed. In this report, we describe 25 different clones derived from a human stomach sarcoma, only some of which were able to grow as tumors in athymic nude mice.

MATERIALS AND METHODS

A 27-year-old Japanese female was admitted to Tomohon Hospital because of bloody sputum and pain in the back in February 1975. Chest X-ray examinations revealed multiple abnormal shadows in both lung fields. A cervical mass was noted and biopsied for diagnosis. Histological examination of the tumor revealed a leiomyosarcoma. The tumor mass was transplanted into athymic nude mice at biopsy. The patient died of disseminated metastases of a tumor originating from a leiomyosarcoma of the stomach. The tumor has been maintained in nude mice through serial passages over a period of 2 years. In July 1977, a tumor of the seventh transfer generation was removed for use in attempts to establish a new human sarcoma cell line in vitro.

Culture Medium. The medium used for the primary culture was F-10 synthetic medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum (Flow Laboratories). Penicillin (100 units/ml) and streptomycin (100 µg/ml) were added to the medium. Cells were harvested with 0.25% trypsin (1:250; Difco Laboratories, Detroit, Mich.) in calcium- and magnesium-free balanced salt solution.

Primary Culture. The tumor tissues were rinsed twice with F-10 medium and minced into small pieces with surgical scissors. The pieces were then incubated in trypsin solution at 37° for 20 min. The treated pieces were then dispersed in F-10 medium with 10% fetal bovine serum, immediately dispensed into Falcon plastic flasks (Falcon No. 3013; Falcon Plastics, Oxnard, Calif.), and incubated at 37° in a humidified atmosphere of 5% CO2 in air. Spindle-like cells attached together and grew in groups within a week. Subcultures were performed at 1:3 dilution with trypsin solution.

Cloning Procedure. The original culture used for establishing clonal strains was the 17th passage culture. Single-cell-platings, without feeder layer, were made from the culture by the method described by Puck and Marcus (27). Stainless steel cylinders were used for the isolation of individual colonies. The isolated colonies were detached and dispersed into single cells with trypsin solution. The dispersed cells were serially diluted and distributed in fresh dishes. Original clonal strains were recloned twice.

Growth Curve. The cells of each clonal strain were studied to estimate the population-doubling time. The initial cell number was 1 x 10^4 cells per dish (Falcon No. 3001; 35 x 10 mm). The cells were grown in 2 ml of complete growth medium (F-10 plus 10% fetal bovine serum). A cell count was taken each day in 2 dishes, and the medium was changed every day.

Chromosomal Analysis. For chromosomal preparations, the cells in exponential growth phase were treated with Colcemid (0.2 µg/ml; Grand Island Biological Co.) for 2 hr at 37° and then hypotonic KCl solution...
(0.5%) for 20 min. The cells were then fixed with methanol/acetic acid (3:1). After 3 changes of fixative, the cells were dropped onto wet slides, air dried, and stained with conventional Giemsa dye. 

Transplantation into Nude Mice. The cells (1 × 10^6 to 1 × 10^8) were harvested in 0.5 ml of medium and inoculated into s.c. tissue on the flank of 3 nude mice (7-week-old female BALB/c-nu/nu mice, of congenitally athymic background; Clea Japan, Inc., Tokyo, Japan). 

Morphological Examination. All the extirpated tumors were fixed in isotonic buffered formalin (10%, v/v) and stained with hematoxylin and eosin. For staining reticulin fiber, the sections were stained with Gomori's silver stain. Cells in Petri dishes were photographed directly without stain by using a phase-contrast microscope. Cultured cells grown on coverslips were fixed with isotonic buffered formalin and stained with conventional Giemsa dye. 

For electron microscopy, the tumors were fixed with 2.5% glutaraldehyde and 2% formaldehyde in 0.1 m sodium cacodylate buffer (pH 7.4), postfixed in 1% OsO_4 in the same buffer, and embedded in Epon 812 after dehydration. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined with an electron microscope (Hitachi HU-12; Hitachi, Ltd., Tokyo, Japan). 

Immunological Examination. To determine the species from which the clones originated, antisera raised against human cells and nude mouse cells were prepared by immunizing rabbits with HL-60 cells (31) or nude mouse spleen cells as described previously (23). Briefly, a rabbit was given 3 i.v. injections at 2-week intervals with 1 × 10^6 viable cells. Two weeks after the third injection, blood was taken, and serum was separated and heated at 56° for 30 min to inactivate the complement. 

Cytotoxicity tests for cultured cells were carried out using rabbit serum as the complement source. Complement-dependent cytolysis was determined by trypan blue exclusion tests in which 50 μl of cell suspension (3 × 10^6 cells/ml) and 50 μl of antiserum in serial dilutions were mixed and 50 μl of a 10-fold diluted rabbit serum were added as the complement source. The mixtures were incubated at 37° for 45 min, and the surviving cells were counted following the addition of 150 μl of 0.5% trypan blue solution. Cultured cells in monolayers (1 × 10^6 cells/35- x 10-mm dish) were treated with 200 μl of antiserum in serial dilutions, 200 μl of a 10-fold diluted rabbit serum, and 200 μl of complete growth medium. The dishes were incubated at 37° in a 5% CO_2 chamber for 45 min. 

Type 1 clones grew to a final saturation density of 1.7 to 2.1 × 10^6 cells/sq cm, while that of type 2 clones was less than 1 × 10^6 cells/sq cm (Table 2). The plating efficiency of type 1 clones was 16.6 to 29.6%. Type 2 clones showed a somewhat lower plating efficiency of 10.5 to 23.4%. 

Immunological Identification of the Species of Origin of Clones. To see whether T3M-2 clones are derived from the human sarcoma cells, antigen analyses have been carried out using cytolytic tests. It has been shown that there is a difference in the specificity of the complement-fixing antibody for mouse and human cells (23). All the mouse cells were sensitive to immune cytolysis with the anti-mouse spleen cell antisera and complement. Human cell lines were not killed by the antiserum (Chart 1). None of the T3M-2 clones was sensitive to the immune cytolyis. Anti-human cell (HL-60) antisera specifically killed human cell lines (Chart 2). All the clones of T3M-2 were killed by the antiserum and complement. The mean titer for 50% cytolysis was 1:1000. 

Chromosomal Analysis. Determination of the modal number of the 25 clonal strains revealed significant differences in their karyotype (Table 2). Most of the type 1 clones showed modal numbers greater than 54. In contrast, type 2 clones showed modal chromosomal numbers less than 54. For determination of whether karyotypic heterogeneity preexisted in the parental culture from which these clones were originally derived, cells from T3M-2 parental culture were analyzed for their chromosomal numbers. Cells with karyotypes ranging from 34 to 64 were easily identified (Chart 3). Three major peaks, 47 to 49, 55 to 56, and 59, were found, which suggested that the genotypic heterogeneity observed between the clones has its origin in the neoplasm. 

Tumorigenicity of Clonal Strains. Cells were trypsinized and suspended in complete growth medium at varying concentrations (1 × 10^6 to 1 × 10^6), and 0.5 ml was injected s.c. into athymic nude mice. A minimum of 3 animals was used for each
Table 2
Characteristics of T3M-2 clones

<table>
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<tr>
<th>Clone</th>
<th>Type</th>
<th>Population-doubling time (hr)</th>
<th>Saturation density (x10^5/sq cm)</th>
<th>Plating efficiency (%)</th>
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* Numbers in parentheses, range.

**DISCUSSION**

This study describes the establishment of different clonal strains from a single human leiomyosarcoma of the stomach. Although a number of human sarcoma cell lines have been isolated in vitro (13, 17, 24, 28, 29), few lines have been established from leiomyosarcomas (12, 15). This may be the first establishment of a cell line from human stomach leiomyosarcoma.
showed an infinite life span, human aneuploid karyotypes, and human antigenicity of the cell surface. Type 1 clones showed typical features of transformed mesenchymal cells, such as criss-crossed arrays, loss of contact inhibition, high terminal density, and tumorigenicity in nude mice. These clones may serve as a useful model of malignantly transformed human mesenchymal cells of the gastrointestinal tract. Type 2 clones did not show criss-crossed arrays, but they grew in well-organized monolayers resembling normal diploid fibroblasts. They showed rather lower saturation density and did not produce tumors in nude mice. These observations suggested that cells with different tumorigenic potential preexisted in the parental tumor, since the parental tumor from which these clones originated was tumorigenic and transplantable in nude mice. Type 2 clones showed morphological and behavioral features similar to those of nontransformed mesenchymal cells rather than to those of malignantly transformed cells. The role of such nontumorigenic clones in the parental tumor has not been understood. Recent studies on animal tumors suggested the ability of a tumor cell population to control the phenotypic properties of the other cell subpopulations in the parental tumor as seen in mammary carcinoma and B16 melanoma (18, 19). Further studies are required for elucidation of the role of type 2 clones in the parental tumor.

Type 1 clones may be further subclassified into 2 groups, tumorigenic and nontumorigenic. T3M-2 clones, therefore, may be subclassified into 3 groups: (a) morphologically transformed tumorigenic clones; (b) morphologically transformed nontumorigenic clones; and (c) morphologically nontransformed nontumorigenic clones (Type 2 clones). Since all 3 groups of clones showed an infinite life span, human aneuploid karyotypes, and human antigenicity, they are different from normal diploid mesenchymal cells which have a limited life span. There is increasing evidence that neoplastic transformation is not a one-step process, but definitive information about the number and nature of discrete steps that result in neoplastic transformation is still needed (20). Several investigators suggest that neoplastic cells display a morphologically transformed phenotype in vitro as an early change and that cells must be morphologically transformed in order to express tumorigenicity (4, 20, 33). This notion may be supported by our observations that type 1 clones which showed morphologically transformed phenotype were tumorigenic with the exception of 2 clones, but morphologically nontransformed type 2 clones did not produce tumors in nude mice. It is suggested that probably cells of this sarcoma must be morphologically transformed in order to express tumorigenicity. Recent studies suggest that the progression of neoplasms in host animals probably is the factor most responsible for their biological diversity (14, 26). Tumor populations are known to be less genetically stable than are normal cells, and then variants may arise spontaneously within the developing neoplasm. Those clones with increased survival capability are given a growth advantage and can dominate the tumor (14). In addition, in vitro mutagenesis experiments have resulted in the emergence of new clones (3, 6, 34), some of which have increased tumorigenic potential (5, 11). The most significant aspect of the tumor heterogeneity is considered to be the progression of noninvasive tumors from the benign to the malignant state. Type 2 clones were morphologically nontransformed and nontumorigenic. Clones 11 and 19 were morphologically transformed, but they did not produce tumors. The other type 1 clones showed mor-
phologically transformed phenotypes and were tumorigenic in nude mice. These clones with different tumorigenic potential might represent clones of different steps in the progression of a benign noninvasive tumor to malignancy.

In transformation experiments, the long-established permanent cell line of 3T3 cells is considered to contain unexpressed tumorigenic changes in many cells. Evidence supporting this possibility has been reported recently by Morris (20). Addition of a mutation conferring morphological transformation on such cells would simultaneously allow expression of previously cryptic changes that confer tumorigenicity. The accumulation of unexpressed changes is prominent in permanent cell lines. 3T3 cells were originally isolated from normal diploid fibroblasts of mouse tissue, but they exhibit chromosomal abnormalities and show an infinite life span in culture. Therefore, these cells are not considered to represent normal fibroblasts or malignant mesenchymal cells. Type 2 clones of T3M-2 cells may be similar to such cell lines in the sense that they would grow infinitely, but they did not show morphologically transformed phenotypes or tumorigenicity in nude mice (11). Some additional mutations conferring tumorigenicity may be required in order to express tumorigenicity in nude mice as described above (11, 13, 33).

T3M-2 clones may be useful for the study of each step of the malignant changes during the progression of malignant neoplasms of human mesenchymal origin as well as for investigators attempting to define those properties that contribute to tumorigenicity.

REFERENCES


Tumorigenic Heterogeneity of Human Sarcoma

Fig. 1. Morphological features of cultured cells. A, type 1 clone (clone 20); B, type 2 clone (clone 28) cells. Gomori's × 280.

Fig. 2. Histology of the tumor obtained by inoculating the cultured clone 20 cells. A, H & E; B, Gomori's silver. × 300.
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