Tumor Progression Studied by Analysis of Cellular Features of Serial Ascitic Ovarian Carcinoma Tumors

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ABSTRACT

Seven consecutive ascitic tumors were obtained over a 9-month period from a patient with serous adenocarcinoma of the ovary. The tumor cell populations were analyzed for cellular proliferation (labeling index, agar clonogenicity, and self-renewal capacity), for cell differentiation (cell surface expression of carcinoembryonic antigen and histochemical stain for fat accumulation), and for karyotypic changes. Evidence is presented of increased aggressiveness of proliferative features together with a decreasing proportion of cells with differentiated features. Parallel temporal changes were documented in density-volume characteristics of the tumor cell population, from small, high-density to large, low-density cells. The only karyotypic change identified over this period was the loss of one X-chromosome and the increased frequency of cells containing double minute bodies. The progressive characteristics described in this human tumor are not, therefore, associated with gross chromosomal changes. The accumulation of double minute chromosome bodies may be associated with a low-dose methotrexate exposure or with the tumor progression.

INTRODUCTION

The term "neoplastic progression" was first applied by Foulds (5) to describe temporal changes in biological characteristics of the tumor cells in a mouse mammary tumor system. It is now widely believed that progression has generated tumor cell diversity by the time of clinical diagnosis and thus has an impact on therapeutic effectiveness. It has been hypothesized by Nowell (17) that tumour progression is the basis for a stepwise selection of variant clones which have attained growth advantages through genetic lability. Information is available on a variety of cellular features associated with tumor progression in animal systems (6, 9--11). These include decreasing hormone dependence of endocrine-requiring tumors, increasing capacity for invasion and successful metastatic colonization, loss of differentiated cellular characteristics, and increased evidence of chromosomal abnormalities. In animal tumor systems, these changes have been described in the conversion of a solid tumor to ascites (11). Evidence of cellular changes due to progression in human cancers is restricted to documentation of changes of stem-line proportions through serial karyotypic studies particularly in hemopoietic neoplasms (19).

We have described recently procedures which allow the analysis of heterogeneity within human ovarian carcinoma cell populations. The diversity of tumor cell types present in the ascites was demonstrated through analysis of cell proliferation features (labeling index, clonogenicity in soft agar, and clonogenic cell self-renewal potential), cell differentiation features (histochemical differentiation and expression of ovarian differentiation antigens), and cell physical parameters (density-volume) (4, 14, 15).

In this paper, we apply such technology to the study of 7 consecutive ascites samples obtained from an untreated ovarian carcinoma patient over a period of 9 months to define the cellular characteristics relating to tumor progression in human ovarian carcinoma.

MATERIALS AND METHODS

Patient. The patient studied was diagnosed at exploratory laparotomy 2 years prior to the initiation of this study with a serum well-differentiated adenocarcinoma of the ovary with Stage 3 disease. Her treatment history was as follows. She attained partial remission subsequent to melphalan chemotherapy (1-year duration). On relapse, she received combination chemotherapy (Adriamycin, cis-platinum, and cyclophosphamide) (23) and had another partial remission lasting 5 months. For the 7 months leading up to this study and during the study, she was not treated. For the entire duration (2 years prior to and 9 months during this study), she was treated for psoriasis by the administration of low-dose methotrexate (2.5 mg, twice weekly).

Cells. Ascites were obtained by paracentesis into heparinized (10 units/ml) vacuum bottles. Cell counts were performed by hemocytometer, and then cells were harvested by centrifugation (600 × g, 10 min) and resuspended in McCoy's Medium 5A containing 10% HIFCS. Mononuclear cells were prepared by Ficoll-Hypaque (density, 1.077 g/ml) centrifugation (2000 × g, 20 min). In this patient, only erythrocytes were pelleted under these conditions. The tumor cell-rich layer was washed twice in McCoy's 10% HIFCS, and the resulting suspension was passed through needles of decreasing size to 23-gauge. The viability of all cell preparations was greater than 99%, as determined by trypan blue exclusion. Cellular differentials were performed by methods described previously (3). Briefly, positive identification of tumor cells was based on morphological criteria on Wright-Giemsa- and Papanicolaou-stained cytospin slides and negative identification through visualization of cells capable of rosette formation with sheep RBC and cells capable of latex phagocytosis.

Shipping cells to Tucson from Toronto for the cytogenetic analysis was done on dry ice after cell freezing in dimethyl sulfoxide (10% v/v)
and fetal calf serum (20% v/v). Cellular characteristics (analyzed in Toronto) were unaffected by freezing in this manner.

**Labeling Index.** Cell suspensions were washed twice in PBS, resuspended at $10^5$ cells/ml in a minimal Eagle's medium (without nucleosides) containing $\left[{^{3}H}\right]$thymidine (5 μCi/ml; 65 Ci/mmole), and incubated at 37°C for 1 hr. The cells were then washed 3 times in PBS, and cytotoxic preparations were made. Slides were fixed for 2 min in 95% ethyl alcohol. In the darkroom, the slides were dipped in Kodak NTB3 emulsion (diluted 1:1 with distilled water). Following exposure in the dark for 48 hr, the slides were developed with standard Kodak reagents and subsequently stained with Wright-Giemsa stain (0.3% in methyl alcohol for 4 min and 4% in water for 6 min, respectively). Positive cells were those with 10 or more grains over the nucleus.

**Quantitation of Clonogenic Cells.** Ovarian tumor colony-forming potential was assessed in agar culture with the enrichments described by Hamburger et al. (7), as modified by Buick and Fry (2). Layers (1 ml) of agar (0.5% w/v) in enriched McCoy's medium 5A containing 10% FIFCS were formed in 35-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.). Tumor cell populations were suspended in a plating layer of 0.3% (w/v) agar in enriched medium Connaught Medical Research Laboratories with 15% horse serum (1-ml volume plating layer). Cultures were incubated at 37°C in a 7.5% CO2 humidified atmosphere of air, and colonies (defined as aggregates of 40 or more cells) were scored with an inverted microscope at x100 after 10 to 14 days.

**Quantitation of Self-Renewal Capacity.** The method has been described (4). Briefly, primary colonies at 7 days of culture are harvested, pooled, disaggregated, and retested for further clonogenic capacity. The replating efficiency (PE2) is a measure of the frequency of secondary colony formation per primary colony.

**Histochemical Stain for Fat Accumulation.** The accumulation of fat-containing granules is a marker of cell differentiation in serous ovarian carcinoma (14). To visualize fat granules, the method of Lillie (5 x 10~) were prewashed with PBS and incubated for 30 min at 4°C with 0.05% (w/v) oil red O. Positive cells were those identified as containing a complete ring of red granules around the inner surface of the cytoplasmic membrane.

**Immunofluorescent Visualization of Cell Surface CEA.** Tumor cells (5 x 10^5) were prewashed with PBS and incubated for 30 min at 4°C with a 1:4 dilution of monoclonal mouse antibody to human CEA (Hybritech Incorporated). After 2 washes with PBS, the cells were incubated for 30 min at 4°C with a 1:10 dilution of fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories Dowingtown, Pa.). After 2 washes with PBS, the cells were incubated with fluorescein-conjugated goat anti-mouse IgG. Positive cells were enumerated under a microscope equipped with a light source with an excitation wavelength of 490 nm.

**Characterization of Cell Density-Volume.** Discontinuous density gradients of bovine serum albumin were used to fractionate cell populations by equilibrium density sedimentation as described previously (14). Briefly, 12- x 1-ml step gradients were constructed manually (covering the density range of 1.01 to 1.08 g/ml), and approximately 2 x 10^7 cells in 1 ml PBS were carefully layered on top. Density distributions were developed by spinning to equilibrium (30 min at 1000 x g), collecting cell fractions from the top by Pasteur pipet, and enumerating cell concentration by hemocytometer.

**Volume-density characterization was performed as described previously (15). Briefly, cell volume measurements for each density gradient fraction were made with an electronic cell sizer (Coulter Electronics, Hialeah, Fla.) connected to a pulse height analyzer (ND1200) and later transferred to a DPD 11/10 minicomputer (Digital Equipment Corporation, Maynard, Mass.) connected to a Tektronix Model 4662 plotter. Isometric and contour representations of the 2-parameter data were then computer generated (15).

### RESULTS

**Cellular Features of the Malignant Ascites.** Table 1 presents the chronology and basic features of the consecutive malignant ascites. Paracentesis was performed on 7 occasions from February 29, 1980. The proportion of recovered cells which could be morphologically identified as tumor cells was consistent throughout (over 90%), and cell viability in all samples was >99%. The absolute viable tumor cell concentration in the ascites showed a trend to higher values with time.

**Tumor Cell Proliferation and Differentiation Characteristics.** The tumor cell populations were assessed for the proportions of cells within the proliferative compartment (labeling index), the clonogenic subpopulation (agar clonogenicity), and the renewing clonogenic subpopulation (secondary plating efficiency). The data for the 7 ascites populations are shown in Table 2. All 3 parameters of tumor cell proliferation indicated temporal changes. The proportion of cells identified as being...
in the process of DNA synthesis by labeling index increased from 1.7 (S.E.) ± 0.8 to 6.8 ± 1.2%. Clonogenicity in agar increased from 98 ± 32 to 650 ± 74 per 10^5 tumor cells, and replating efficiency increased from 1.5 × 10^{-2} (Sample 3) to 1.21 (Sample 7) colonies per primary colony.

We have described previously the cell surface expression of CEA and the accumulation of oil red O-staining fat granules as markers of undifferentiated and differentiated cells, respectively, in human serous adenocarcinoma cell populations (14). The temporal changes in these parameters are also shown in Table 2. The proportion of cells expressing CEA increased approximately 6-fold, while those cells demonstrating positive staining with oil red O decreased from 48.3 to 4.5% (Samples 2 to 7, respectively).

**Physical Properties of the Tumor Cells.** We have related previously cell differentiation heterogeneity within serous adenocarcinoma cell populations to differences in cell density and volume (14, 15). Chart 1 describes the cell density distributions of Samples 2 through 7 when fractionated in discontinuous gradients of bovine serum albumin. Sample 2 demonstrated essentially a unimodal cell population with respect to density with the majority of cells having a density >1.06 g/ml. With time, the tumor cell population showed a change in mean density to approximately 1.04 g/ml.

These changes were documented further by the application of a density-volume "fingerprinting" procedure. Chart 2 shows the 2-dimensional representation of density-volume heterogeneity of Samples 2 to 7. The fingerprints showed progressive changes in distribution from a predominantly small, high-density population to a large, low-density population. To further highlight these differences, Samples 2 and 7 are shown plotted in isometric form in Chart 3.

**Karyotypic Analysis.** Comparison of karyotypes was restricted to Samples 1 and 7. The modal chromosome number of Sample 1 tumor cells was 50 (n = 64). The stem-line karyotype is 50,XX,-3,+8,+12,+20,+2mar. The modal chromosome number of Sample 7 cells was 49 (n = 39). The stem-line karyotype is 49,X,-3,+8,+12,+20,+2mar. Q- and

**Chart 1.** Density distributions of tumor cells derived from Samples 2 through 7 sedimented in step gradients of bovine serum albumin.

**Chart 2.** Isocontour representation of density-volume parameters of Samples 2 through 7.

**Chart 3.** Isometric representation of density-volume distributions of Samples 2 (a) and 7 (b).
C-banded analysis demonstrated the origin of the marker chromosome [inv(3)(p13;q23)]. The breakpoints of this pericentric inversion are identical in Sample 1 and 7 cells. Clonal chromosomal alterations are shown in Fig. 1A. The only chromosome alteration unique to Sample 7 cells was the loss of one X-chromosome.

The karyotypes of Samples 1 and 7 cells did differ in one further cytological property; the percentage of cells displaying DMS (Fig. 1B). Sample 1 displayed DMS in <2% of cells, while Sample 7 contained DMS in 13% of cells. This increase in double minute-containing cells was statistically significant (n = 159, p = 0.03). The range of DMS per cell was similar for both samples (2 to 8 per cell).

**DISCUSSION**

In this communication, we have reported on the serial analysis of cell proliferation, differentiation, and karyotypic changes in an untreated patient with serous adenocarcinoma of the ovary. The consecutive ascitic tumors studied cannot be regarded as unmanipulated. By monthly paracentesis, there is clearly a challenge to the tumor to repopulate the peritoneal space. All the cell-proliferative parameters studied showed systematic temporal changes; the largest quantitative effects were seen in the replating efficiency of clonogenic cells. We have described previously data (4) to indicate that this parameter may quantitate the stem cell property of self-renewal. It is possible, therefore, that the increased PE2 represents a proportional increase in the stem cell population of the ascites tumor.

Consistent with the analysis of proliferative features, the proportion of cells expressing CEA showed a 6-fold increase and the proportion demonstrating terminal differentiation (oil red O positive) decreased markedly. These population changes occur concurrently with the changes in physical properties of tumor cells. The cell population displays markedly increased heterogeneity with respect to density and volume (Charts 1 to 3). We have described previously evidence that cell differentiation in serous ovarian tumor cells is associated with an increase in cell density and a decrease in cell volume (14, 15). The presently described data are consistent, therefore, with the trend towards loss of differentiative features with time. This is similar to features of progression described for animal tumors (5, 6, 9–11).

Human hemopoietic cancers often demonstrate the appearance of additional or secondary clonal chromosomal changes onto an initial chromosomal alteration with progression of the disease (19). The relationship between tumor progression and karyotypic alteration in human solid tumors is largely unstudied (19). Although a few previous studies of malignant effusions have been performed, these have been complicated by therapeutic intervention and importantly have not utilized chromosome-banding techniques (20, 21).

Our results demonstrate the maintenance of a substantial degree of karyotypic stability over time, despite large changes in the cellular organization of the tumor cell population. In examination of karyotypes from Sample 1, 20 of 21 cells demonstrated maintenance of both normal X chromosomes. However, in Sample 7 karyotypes, 11 of 11 cells demonstrated loss of one X chromosome. It is possible that the loss of an X chromosome from Sample 7 may represent a chromosome alteration involved in progression. However, the loss of sex chromosomes occurs often in a wide variety of cancers, as well as occurring increasingly in normal tissue as an apparent consequence of advanced age (for a review, see Ref. 19).

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Perhaps of more importance was the finding of an increased percentage of DMS-containing cells over time. DMS are small paired chromatin bodies which have been found in a variety of human and animal tumors (12), including ovarian adenocarci-

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**Fig. 1.** Clonal chromosomal alterations from tumor cells obtained on April 29, 1980, and December 16, 1980. A, comparison of karyotypic alterations demonstrated by C-banding (April 29, 1980) and G-banding (December 16, 1980) techniques. Breakpoints on Marker 1 chromosome are identical [inv(3); (p13q23)]. Monosomy X is present in cells from the December sample only. B, double minute bodies (arrows) from tumor cells. Metaphase chromosomes have been photographically overexposed to enhance identification of DMS.
nom (26, 28). It is interesting to speculate that a relationship may exist between tumor progression and double minutes. This viewpoint can be supported by recent studies relating DMS to poor prognosis in neuroblastoma (1).

Although a low number (<10) of DMS per cell was observed in Samples 1 and 7 cells, the genetic content of minute bodies may not have been found to be extremely large. In recent work by Hubbell (8), the DNA content of DMS from a human colon cell line averaged 3000 kilobases. Thus, “amplification” of double minute-DNA may represent a significant addition to the genetic material per cell, despite their presence in low numbers.

In addition to their association with a variety of cancers, DMS are often observed in experimental systems apparently acting as mediators of drug resistance through amplification of specific genes (22). The clinical history of this patient does include a prolonged exposure to low doses of methotrexate. The appearance of DMS in this case may therefore represent a situation similar to the amplification of dihydrofolate reductase genes in murine systems. We are investigating currently this possibility.

Cellular features of tumor progression have been difficult to study in human carcinomas partly due to a paucity of techniques applicable to the primary human cells and partly to the rarity of an untreated patient with accessible tumor tissue. We describe temporal changes in the cellular features of a human ovarian carcinoma, unmanipulated by cytotoxic therapy. Our data lend support to the use of the described cell proliferation and differentiation characteristics as markers of biological function in human ovarian carcinoma. The changes seen indicate a progressive loss of cell differentiation features and are consistent with the predictions of Nowell (17) and with reports of tumor progression in animal systems (5, 6, 9–11).

In addition to providing substantial evidence in support of the view that tumor progression can occur in human ovarian carcinoma, the data also provide support for a model of tumor progression in which the proportion of primitive (? stem) cells in the tumor cell population increases with a concomitant decrease in the proportion of (?) end cells with differentiation characteristics.

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