Establishment and Characterization of a Pancreatic Carcinoma Cell Line Derived from Malignant Pleural Effusion

A.N. Starr a,b  A. Vexler b  S. Marmor c  D. Konik d  M. Ashkenasi-Voghera b
S. Lev-Ari b  Y. Greif a  R. Ben-Yosef b

a Lung and Allergy, b Oncology, c Pathology and d Genetic Institutes, Tel Aviv Sourasky Medical Center and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

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Abstract
Background/Aims: A novel cell line, designated p34, was developed from the malignant pleural effusion of a patient with carcinoma of pancreas. The objective of this work was to characterize this cell line. Method: The in vitro studies included karyotype analysis, immunohistochemistry, XTT cell proliferation assay, analysis of the cell cycle by FACS and cell sensitivity to chemotherapeutic drugs and irradiation. Subcutaneous and intraspleen inoculations into nude mice were carried out to study the tumorigenicity and the metastatic tendency of this cell line. Results: The p34 cell line showed typical morphological characteristics of epithelial pancreatic tumor cells. The cells were hyperdiploid with a modal number of 48, and had two markers, deletion in the short arm of chromosome 2 and duplication of the short arm of chromosome 8. The doubling time was 16 h. Subcutaneous inoculation of the cells into nude mice yielded 100% tumorigenicity, and intraspleen inoculation resulted in extensive intra-abdominal spread. The antiproliferative effect of chemotherapy (gemcitabine, cisplatin, taxol and vinorelbine), chemopreventive agents (celecoxib and curcumin) and radiotherapy showed dose-dependent cytotoxicity. Conclusions: This p34 cell line can be used as a new model for studying various aspects of the biology of human pancreatic cancer and potential treatment approaches for the disease.

Introduction

Adenocarcinoma of the pancreas is presently the fifth leading cause of cancer death in the western world [1]. The incidence appears to be increasing. At the time of diagnosis, patients usually have locally advanced or distant metastatic disease [1, 2]. Despite improvements in early diagnosis, surgical techniques, and chemoradiotherapy, the majority of patients succumb to their disease because of distant metastasis [3–5]. The average survival from diagnosis to death is about 4–6 months, and the overall 5-year survival rate is <10% [6, 7]. Only 10% of patients with pancreatic cancer undergo a curative resection. Effective treatment is inhibited in part by inherent tumor chemoresistance, the biological heterogeneity of pancreatic carcinoma cells and other, as yet unknown, epigenetic features [8].

Specific in vitro and animal models are required for further study of the pancreatic carcinoma mainly from a molecular and therapeutic standpoint. The most com-
The cell line, designated p34, was developed from a 53-year-old woman who had developed hemoptysis and a cough. Physical examination and X-ray studies revealed pleuropericardial effusion and mediastinal, hilar and axillary lymphadenopathy. Cytological investigation of the pleural effusion revealed adenocarcinomatous cells with multiple mitotic figures (fig. 1). Space-occupying lesions were found in the liver and both adrenals. The pathology findings of a liver biopsy were consistent with poorly differentiated carcinoma with a nested pattern and a focal desmoplastic reaction (fig. 2). Immunostaining showed a positive reaction for CK7 and pancreatic antigen (Santa Cruz, Calif., USA), a borderline reaction to CK20 and negative immunostaining for hepatocyte, α-fetoprotein, estrogen, progesterone and vimentin. A pathological diagnosis of pancreatic cancer was established.

Materials and Methods

Clinical History

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Human Cell Collection and Processing

An appropriate institutional review board approval was obtained before tissue collection. Malignant pleural effusion was obtained by thoracocentesis, and the carcinoma cells were sedimented by low-speed centrifugation. The supernatant was removed, and the cell pellet was resuspended in DMEM (Biological Industries, Beit HaEmeq, Israel) supplemented with 2 mM L-glutamine, 2% penicillin-streptomycin (10,000 U/ml) and 10% heat-inactivated FCS (Biological Industries, Beit HaEmeq, Israel). The cells were plated in tissue culture dishes and cultured in an atmosphere of 5% CO₂ at 37 °C. Fresh cultured medium was supplied twice a week, and confluent cells were dissociated with trypsin (0.25%) and plated in T25 flasks (Nunc, Roskilde, Denmark). The cells were routinely cultured in the same medium.

Growth Rate Assay

Cells (10⁵ cells per flask) were plated in 25-cm flasks in growth medium. The culture medium was changed every 2 days. Triplicate flasks were trypsinized at various times, and the number of cells was counted. The doubling time of the cell line was calculated in the exponential growth phase.

Chromosomal Preparation and Banding

Exponentially dividing and freshly fed (24 h earlier) p34 cells were treated with colcemide (final concentration 0.02 μg/ml) for 45 min at 37 °C. The cells were then dislodged from the culture vessels by trypsin, exposed to hypotonic solution (0.06 M KCl), fixed in methanol and acetic acid mixture (3:1 by volume), and air dried on glass slides. G-banding of 5- to 6-day-old slides was performed following routine laboratory techniques as described previously [9, 10]. A total of 25 complete karyotypes were analyzed. We counted additional metaphase spreads to determine the modal chromosome number for the pancreatic carcinoma cell line.
Cell Cycle Analysis

p34 cells were seeded at a density of $5 \times 10^6$ in 100-mm culture plates and grown to 50% confluence. The cells were then treated with the drugs for 72 h in complete medium. They were harvested by trypsinization, centrifuged at 2,000 rpm for 5 min, washed in PBS, and resuspended in ice-cold 70% ethanol. The fixed cells ($10^6$ cells/ml) were stored at 4°C until cell cycle analysis. Before analysis, the cells were centrifuged and the pellet was resuspended in 810 μl PBS, after which RNase-A (10 mg/ml, 90 μl) was added. Following 30 min of incubation at 37°C, propidium iodide (PI, 0.5 mg/ml, 90 μl) was added and the samples were run for 1 h after PI had been added. FACScalibur (Becton Dickinson) was used for the flow cytometry. The percentages of cells in the different phases of the cell cycle were calculated using Cellquest software (Becton Dickinson).

Immunohistochemistry

For immunohistochemistry analysis, the cells were centrifuged into a cell pellet and embedded in paraffin for immunohistochemical staining. Antigen retrieval was performed at 95°C in citrate buffer pH 6.0, 6.4 M sodium citrate dehydrate, and 1.6 M citric acid monohydrate for 40 min. The slides were cooled at room temperature for 20 min and washed 3 x 3 min with Tris buffer pH 7.6, 0.15 M sodium chloride, 0.05 M Trizma HU. The slides were peroxidase blocked for 5 min and washed as above. They were incubated for 30 min with the primary antigen, then by the secondary antigen (Visualization Reagent), followed by the substrate-chromogen solution (3,3’-diaminobenzidine), and finally counterstained with hematoxylin. For a negative control, the primary antibodies were replaced with a nonspecific negative control antibody. A membrane’s staining was quantified from zero to 3+ according to the staining intensity and the percentage of stained cells.

Tumorigenicity Assay

Balb/c male athymic nude mice (Harlan, Jerusalem) were maintained in specific pathogen-free barrier animal facilities approved by the Israel Laboratory Animal Care Committee. They were used for experiments at age 6 weeks. To produce tumors, p34 cells were harvested from subconfluent cultures by treatment with 0.25% trypsin. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed once in serum-free medium and resuspended in HBSS. Only single-cell suspensions with >90% viability were used for injections. The cells ($5 \times 10^5$ cells in 0.2 ml PBS) were injected subcutaneously into the flank area of nude mice. The tumor size was measured twice weekly using digital calipers.

Intraspleen Implantation of p34 Cells

Intraspleen tumor cell injections were performed as described by Staroselsky et al. [12]. Mice were anesthetized, and their abdomens were sterilized with alcohol. An incision was made through the left upper abdominal pararectal line and peritoneum. The spleen was carefully exposed, and a tumor cell suspension of 5 x $10^5$ cells in 0.1 ml of PBS was inoculated into the spleen. The spleen was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed using 4 surgical sutures. All procedures were performed with standard surgical loupes. The mice were killed when they were moribund, and the size and weight of the primary tumors, the incidence of regional (celiac and paranomral) lymph node metastasis, and the number of liver nodules were determined. Histopathology confirmed the nature of the disease.

Statistics. The results for each variant in the different experimental designs were represented as an average of 2–4 experiments; each was typically performed in triplicate. Mean values and standard errors were calculated for each point from the pooled normalized data. Statistical analyses of the significance of paired data points were performed by the Mann-Whitney unpaired test with two-tailed p value, assuming equal and unequal variance as determined by the ANOVA test and the nature of the experiment. The mean value and the SE were calculated for each variant of treatment tested.
Results

Cell Morphology, Growth Rate and Cell Cycle
The cells grew readily in the standard culture conditions and had been stable for over 20 passages at the time when this report was prepared. They were polygonal and grew in close proximity to each other, forming a monolayer of epithelial cells. Immunostaining showed that they had a positive reaction for CK7 and pancreatic antigen while CK20 was positive in only few cells. Negative immunostaining was found for hepatocyte, α-fetoprotein, estrogen, progesterone, vimentin and EGFR family proteins, ErbB-2 and ErbB-4. The absence of the last two receptors in p34 cells was confirmed by Western blot analysis. The growth curve which resulted from 3 experiments with prolonged cell cultivation showed that the doubling time for p34 cells in the logarithmic stage of growth was 16 h. FACS analysis demonstrated aneuploidy of p34 cells. The distribution of cells in the cell cycle was as follows: G1 phase 30%, S phase 10%, and G2-M phase 50%, while the remaining 10% of the cells were apoptotic.

Ploidy Study and Karyotype Analysis
The p34 cell line was aneuploid with a DNA index of 1.25. The chromosome number was determined by counting chromosomes in 50 metaphase cells that were trypsin-Giemsa banded. The cell line consisted of hyperdiploid cells with a modal number of 48. Numerical chromosomal changes included trisomy of chromosome 2, 5 and 7 and monosomy of chromosome 20. These numerical changes were observed in each cell and represented one clone (fig. 3). Cytogenetic analysis of 25 metaphases identified two marker chromosomes (a marker chromosome is defined as a structurally abnormal chromosome observed in two or more karyotypes): the first one was a deletion of the short arm of chromosome 2 and the second one was a large metacentric chromosome that was com-
posed of a normal chromosome 8 with extrachromosomal material on its short arm. Both markers were present in each studied cell.

**Tumorigenicity and Metastasis**

Tumorigenicity of the p34 cells was determined by subcutaneous injection into nude mice. All inoculated mice developed a subcutaneous tumor that reached a size of 7 mm in diameter on day 23 (fig. 4). The growth of p34 cells in the subcutaneous environment resulted in extensive necrosis of tumor tissue. Examination of the internal organs after sacrifice of the animals did fail to reveal any metastatic spread. To study their metastatic capacity, we injected p34 cells into the spleen of nude mice, and palpable tumors were detected in all mice within 30 days. The animals were killed on day 46 after inoculation at the time that one of the mice developed massive bloody ascites. All the mice had large, nodular masses in the spleen with multiple liver metastases and peritoneal dissemination, which proved to be metastatic adenocarcinomas by histology.

**In vitro Cytotoxicity**

All four chemotherapeutic medications and the two chemopreventive agents inhibited the growth of the p34 cells in a dose-dependent manner. Of the four chemotherapeutic medications, gemcitabine was the most potent inhibitor with an IC50 of 7 nM, followed by vinorelbine with an average IC50 of 20 nM, taxol with an IC50 of 150 nM, and cisplatin, the least potent agent, with an IC50 of 2 μM (fig. 5). In addition to cell growth inhibition, we observed morphological changes in the treated cells, i.e., blebbing and cell detachment. These changes were especially prominent 72 h after incubation, while the untreated control cells showed no morphological changes at that time. These morphological changes suggested that the
chemotherapeutic agents induced apoptotic cell death as well. The chemopreventive agents, celecoxib and curcumin, inhibited the cell growth in a dose-dependent manner (IC$_{50}$ of 40 and 5 $\mu$M, respectively) (fig. 5). Irradiation of these cells using a single dose of 2–8 Gy significantly decreased the cell survival with an LD$_{50}$ of 4.5 Gy (fig. 6).

**Discussion**

We describe the establishment and characterization of a novel p34 cell line derived from a human pancreatic carcinoma involving the pleura. There are number of reports of human pancreatic carcinoma cell lines originating from primary pancreatic tumors and/or abdominal metastases of pancreatic cancer [13–17]. To the best of our knowledge, however, the p34 cell line is among the few pancreatic carcinoma cell lines developed from a pleural effusion and is a true distant metastatic cell line [18]. Evidence that this cell line originated from human pancreatic carcinoma includes the characteristic phenotype and specific immunohistochemical markers (i.e., general pancreatic antigen and cytokeratin 7) positive for pancreatic cancer. p34 cells grew readily in culture and formed a tumor model in nude mouse. The cell line displayed an aneuploid karyotype with a median of 48 chromosomes, but had a low number of chromosomes with structural abnormalities. In fact, aneuploidy is frequently observed in pancreatic cancer, but not in normal or inflamed pancreatic cells and also has been reported as a marker of poor prognosis. The neoplastic nature of p34 cells is well demonstrated by their ploidy status, the high modal number of chromosomes and the frequency of multinucleated cells. A comparison with G-banding of early passages revealed p34 cells to be remarkably stable after 12 months of cultivation because both clonal markers and numerical changes were present in early passages. Several consistent numerical and structural chromosomal aberrations were found in this cell line, including trisomy in chromosomes 2, 5 and 7, a deleted chromosome 2 and a translocated chromosome 8. The most prominent among these abnormalities is the aberrant chromosome 8. Alterations in this chromosome are frequently detected in surgically resected pancreatic cancer specimens as well as in established cell lines [19, 20]. Furthermore, numerical imbalances for chromosome 8 are found more frequently in metastatic pancreatic carcinomas than in primary tumors [21]. Similar results were reported by Sato et al. [22] who found that the majority of pancreatic cancer cell lines show numerical aberrations in chromosome 8 and these abnormalities are associated with malignant tumor growth. These findings suggest a possible involvement of gene(s) on chromosome 8 in the acquisition of aggressive phenotypes.

Our investigation revealed an in vitro antiproliferative effect of chemotherapeutic agents (gemcitabine, vinorelbine, taxol and cisplatin) on p34 cells in a dose-dependent manner. The growth inhibition ranged from 10 nM to 50 $\mu$M over a time course of 24–72 h. Cells treated with the drugs became rounded, detached from adjacent cells, and showed membrane blebbing, a typical feature seen prior to the initiation of apoptotic processes. The most potent ones were gemcitabine followed by vinorelbine. Irradiation of this cell line revealed that it was a radiosensitive cell line with an LD$_{50}$ of 4.5 Gy. We have shown that the chemopreventive agent celecoxib inhibits cell proliferation in a dose-dependent manner. This is consistent with previous studies showing that COX-2 inhibitors play a role in the growth and metastasis of established tumors [23]. In addition, we found that curcumin may inhibit pancreatic cancer cell growth and this is similar to its effect on other neoplastic cell lines [24]. Further in vitro and in vivo studies are necessary to investigate its effectiveness in controlling pancreatic cancer growth and metastasis.

In conclusion, the development of the p34 cell line is a step forward in elucidating the biology of metastatic
pancreatic carcinoma. The p34 cell line is tumorigenic and has metastatic features that reliably represent a clinical situation and thereby provide a basis for the evaluation of the efficacy of various therapeutic interventions. The cells expressed different levels of sensitivity in vitro to chemotherapeutic and chemopreventive drugs and to irradiation. This model can facilitate in vivo studies of systemic cellular and molecular mechanisms of tumorigenicity, growth, and metastasis in pancreatic cancer and may lead to improvements in the treatment of what is generally considered an unresponsive disease.

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