Loss of a $M_\text{}$, 78,000 Marker in Chemically Induced Transplantable Carcinomas and Primary Carcinoma of Human Pancreas

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ABSTRACT

Toward the identification of steps in the multiphasic process of human pancreas carcinogenesis we have developed a panel of monoclonal antibodies to normal and carcinogen-treated human pancreas cells. One of these, an IgG3 with strong affinity for a membrane-associated $M_\text{}$, 78,000 protein in fetal and adult parenchymal cells, was purified by high performance liquid chromatography, and used for the detection and characterization of tumorigenic stage in human pancreas carcinogenesis. This protein was present on the cell surface of human pancreas explants exposed to methylnitrosourea for up to 4 months and in nontumorigenic cell lines derived from these explants. It was absent in a morphologically transformed subpopulation of cells in explants treated with methylnitrosourea for longer than 4 months, in tumorigenic cell lines derived from these explants, and in primary carcinomas of human pancreas. The presence of this marker in normal pancreas adjacent to tumors, in hyperplastic cells induced by methylnitrosourea and in nontumorigenic cell lines suggests a correlation between the loss of this membrane-associated marker and cell tumorigenicity.

INTRODUCTION

Carcinogenesis in man is considered to be a multiphasic process and each step of progression is suggested to be determined by the activation, mutation, or deletion of specific genes. The identification of the steps in carcinogenesis, wherein new cell populations represent stages in cellular evolution from target, through preneoplastic, and premalignant cells to malignant tumor, has been difficult to achieve. There is on the other hand considerable evidence that certain genes function as suppressors of tumorigenicity (1-6), and that their deletion or absence of their product in preneoplastic cells may lead to malignancy and invasiveness. The recognition of any of these steps in the pancreas, an organ composed of several cell types, has been even more complex. An in vitro model of carcinogenesis of human pancreas was reported from this laboratory. In this model repeated MNU treatments of pancreas explants resulted in ductal hyperplasia within 8 weeks and the development of foci of anaplastic growth and transplantable carcinomas within 6 months (7, 8). Cell lines, nontumorigenic and tumorigenic, derived from clonal growths of explants at various stages of carcinogen treatment have been developed, defined by DNA fingerprinting, and verified as progenies of untreated human pancreas. In the search for markers to identify steps in pancreas carcinogenesis we have developed a panel of monoclonal antibodies to normal and MNU-treated human pancreas. The present report describes a protein marker of normal and nontumorigenic cells detectable by a monoclonal antibody and its absence in tumorigenic cells and primary carcinomas of human pancreas.

MATERIALS AND METHODS

General. BALB/c, athymic nude mice were obtained from Harlan Sprague Dawley, Indianapolis, IN. Acrylamide, agarose, amido black, SDS, standard low and high molecular weight proteins, 2-mercaptoethanol, and Coomassie blue were obtained from Bio-Rad Laboratories, Rockville Center, NY. 

I-Labeled rabbit anti-mouse IgG was purchased from New England Nuclear, Boston, MA, and Kodak X-Omat AR Film was purchased from Eastman Kodak Co., Rochester, NY.

Human Pancreas. Sections of Bouin’s-fixed adult pancreas from cadaveric donors 12-69 years old without pancreatic disease and pancreases from 3 prostaglandin-induced fetuses 12-14 weeks old were used as control.

Human Tissue Sections. Formaldehyde or Bouin’s-fixed and paraffin-embedded 2- to 4-µm-thick sections from fetal and adult human stomach, liver, gallbladder, large and small intestine, prostate, lung, bronchus, spleen, kidney, lymph nodes, and salivary glands were used. Sections from human carcinomas of stomach, liver, gallbladder, colon, and prostate were also used.

MNU-treated Explants. Human pancreas explants prepared from cadaveric donors 15-45 years old without pancreatic disease were cultured in a chemically defined medium and treated with MNU, 7 µg/ml, twice a week for up to 6 months as previously described (7). MNU-treated and untreated explants after 1, 2, 4, 6, 8, 12, 16, 20, and 26 weeks of culture were fixed in Bouin’s solution, embedded in paraffin, and sectioned at 2- to 4-µm thickness.

Nontumorigenic Cell Lines. MNU-treated explants were minced into 0.2- to 0.5-mm-diameter fragments and cultured in 25-ml culture flasks in a chemically defined medium (8) for 1 week. Outgrowths from explants after 2, 4, 8, 12, and 16 weeks of treatment were trypsinized, propagated, and developed as cell lines. After 30 or more generations, cell lines were tested in nude mice for tumorigenicity. HP-DU-1 (9) and A-1 (10) antibodies were used to establish the expression of ductal or acinar determinants on these cells. Nude mice were inoculated s.c. with 106 cells and followed up to 16 weeks for tumor growth. Sections from cell blocks prepared from nontumorigenic cell lines NT-2, NT-8, and NT-16, derived from 2-, 8-, and 16-week-old explants, respectively, were used in this study.

Tumorigenic Cell Lines. Cell lines developed from MNU-treated human pancreas explants after 6 months of culture were tested for tumorigenicity in nude mice. Two of 11 tumorigenic lines, T-1 and T-2, were adapted to Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated bovine fetal serum and carried for 4 years. These cell lines were repeatedly tested in nude mice and were proven tumorigenic at 1-2 ¥ 106 cells per inoculum. Cell blocks prepared from tumorigenic cell lines NT-2, NT-8, and NT-16, derived from 2-, 8-, and 16-week-old explants, respectively, were used in this study.

Carcinoma. Formaldehyde-fixed paraffin sections from 3 autopsy cases of pancreatic carcinoma were included in this study.

DNA Fingerprinting. DNA was isolated from fresh or frozen tissue or cell pellets by incubation in 0.01 M Tris-HCl, 0.01 M EDTA, 0.1 M NaCl (pH 8.0) containing 2% SDS, 20 µg/ml proteinase K, and 0.04 M dithiothreitol, at 37°C for 6-24 h followed by two phenol-chloroform extractions and precipitation in 70% ethanol containing 0.07 M NaCl. The DNA was pelleted by centrifugation at 15,000 ¥ g for 10 min, washed in 70% ethanol, and repelleted. Samples of DNA, 10 µg, were digested with HaeIII at 37°C for 12 h. The DNA fragments were recovered after phenol extraction by ethanol precipitation and were electrophoresed through a 20-cm-long 0.6% agarose gel at 40 V overnight, and were transferred by blotting onto a nitrocellulose filter. The probe, 28-base pair M13 HaeII-ClaI fragment (11), was electroeouted from agarose gel after electrophoresis and 5'-end-labeled with [32P]ATP.
(New England Nuclear) to a specific activity of about $1 \times 10^9$ cpm/µg of DNA. The nitrocellulose filters were prebriedized for 2 h and then hybridized in 40% formamide, 6 × SSC, 5 mM EDTA, 0.25% casein (Difco), overnight at 42°C. The blots were washed in 2 × SSC containing 0.1% SDS twice at 20°C and twice at 65°C for 15 min, followed by twice 1 × SSC, 30 min each at 65°C, and then were exposed to Kodak XAR-5 films at ~70°C with intensifying screen. Frozen normal pancreas tissue and splenocytes from cadaveric donors were used to validate the fingerprinting technique. Frozen splenocytes or pancreatic tissues from donors were used to establish the authenticity of nontumorigenic and tumorigenic cell lines. Human bladder carcinoma cell line T-24 (American Type Culture Collection) was used as external reference.

Monoclonal Antibody. Methods for the production of antibody were those of Kohler and Milstein (12). Briefly, BALB/c mice were immunized with dispersed cells derived from enzymatically dissociated human pancreas (13). Mice were given injections i.p. of 10³ cells every 2 weeks for up to 4 injections. After the last injection the presence of circulating antibody to pancreatic cells was established by an indirect fluorescence technique, using normal adult human pancreas sections, serum from tail veins of immunized mice, and fluorescein-conjugated rabbit anti-mouse immunoglobulin. Splenocytes from ice with antibody to pancreatic cells were fused with BALB/c myeloma cell line (SP2), 4 days after the mice received an i.v. injection of 10⁷ acinar cells. The immunoglobulin-producing hybrids were cloned and propagated and the affinity of their products for pancreas cells was tested by indirect microscopy. The IgG-secreting clones were isolated by limiting dilution and clones originating from single cells were propagated in vitro and inoculated i.p. into pristane-sensitized BALB/c mice. Ascitic fluid rich in specific IgG was collected and used for characterization of antibody in a double diffusion assay and for the characterization of the marker.

Purification of Antibody. The ascitic fluid was dialyzed against 0.02 M Tris-HCl, pH 8.5, and subjected to HPLC on a DEAE-anion exchange HPLC column (Bio-Rad). Samples were eluted with a linear gradient 0.0–0.5 M NaCl in 0.02 M Tris-HCl at a flow rate of 0.8 ml/min at room temperature. Peaks with 280 nm absorbance were collected and tested for their specificity by fluorescence microscopy.

Immunohistochemistry. Tissues were fixed in Bouin’s fluid, embedded in paraffin, and sectioned at 2- to 4-µm thickness. Deparaffinized sections were stained with hematoxylin and eosin. For fluorescent microscopy, sections were deparaffinized in xylene, hydrated gradually, and placed in PBS, pH 7.4. Sections were then incubated in 10% normal rabbit serum in PBS for 30 min, followed by 0.2% acetic fluid in PBS for 30 min, and 5% fluorescein-conjugated rabbit anti-mouse IgG in PBS for 30 min, and then were examined for fluorescence. All incubations were at room temperature and each incubation was followed by extensive rinsing with PBS. For peroxidase staining the sections were incubated in 3% hydrogen peroxide for 10 min, washed, and incubated in normal goat serum for 30 min at room temperature. The sections were then incubated for 30 min in 0.2% acetic fluid in PBS, washed, and incubated in goat anti-mouse immunoglobulin for 30 min, followed by reaction with 3,3’-diaminobenzidine tetrahydrochloride, and after reaction with 3,3’-diaminobenzidine tetrahydrochloride, were counterstained with methyl green.

Immunodiffusion. Agar plates, 1.5% Noble agar in barbital buffer, pH 8.6, were prepared for the Ouchterlony test by using double diffusion punches of 0.5 mm in diameter (Miles Laboratories, Inc., Elkart, IN). Ascitic fluid, 50 µl, 0.2% in PBS, was placed in the center well and 20 µl of rabbit anti-mouse IgG subclasses, 1, 2a, 2b, and 3 were placed in the peripheral wells and incubated at 37°C for 24 h. Gels were washed in PBS overnight and stained with 0.1% amido black.

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RESULTS

IgG. The HPLC-purified IgG obtained from ascites fluid as a single peak at 280 nm showed a single line of identity with anti-mouse IgG3.

Characterization of Antigen. Western blot analysis of homogenates prepared from normal pancreas electrophoresed in parallel with homogenates of tumorigenic cell lines and labeled with 125I-labeled anti-mouse immunoglobulin or PAP stained revealed a single immunoprecipitate band in normal pancreas homogenates. No detectable immunoprecipitates were noted in those of tumorigenic cell line homogenates. Western blot analysis of homogenates from nontumorigenic cell lines electrophoresed in parallel with homogenates of tumorigenic cell lines showed a single immunoprecipitate band in nontumorigenic cell homogenates and no immunoprecipitation in tumorigenic cell homogenates (Fig. 1). The molecular weight of this protein was 22669.

Fig. 1. Western blot. Immunoblots of mouse IgG, 1 µg, showing immunoprecipitation of heavy and light chain (A); tumorigenic cell (T-1) homogenate, 10 µg (B); human pancreas homogenate, 5 µg (C); tumorigenic cell (T-2) homogenate 10 µg (D); and nontumorigenic cell (NT-16) homogenates, 10 µg (E); showing negatively stained protein profiles except for Lanes C and E with an immunoprecipitate band of about M, 78,000.
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Fig. 2. Micrograph from a paraffin section of adult human pancreas, incubated in myeloma medium and PAP, showing no peroxidase reaction of the luminal surface (arrow). × 400.

Fig. 3. Human pancreas section stained with p78 and PAP showing peroxidase reaction of the luminal surface of the acini (arrows). × 1000.

Fig. 4. Human pancreas section stained with p78 and PAP, showing peroxidase staining of the intralobular duct and acinar lumen (arrow). × 1000.

Fig. 5. Human pancreas section stained with p78 and PAP, showing peroxidase reaction on larger duct and acinar lumen (arrows). × 1000.

A band was calculated, based on standard [35S]methionine-labeled markers to be approximately 78,000. This protein appeared to have approximately the same apparent molecular weight in normal human pancreas lysate treated with (M, 78,000) or without (M, 77,600) 2'-mercaptoethanol. The Rf value of the nonreduced protein was the same whether the nonreduced homogenates were electrophoresed on the same gel adjacent to the mercaptoethanol-treated homogenates or on a separate gel. The slight decrease in Rf value of this protein in mercaptoethanol-treated homogenates suggests the presence of internal S-S bonds. The comparison of the total protein profiles of normal human pancreas and tumorigenic and nontumorigenic cell lines, stained with Coomassie blue, revealed the absence of several major normal proteins (acinar cell zymogens) in tumorigenic and nontumorigenic cells. No difference was noted in the 78,000 molecular weight range in the Coomassie blue-stained
profiles of normal pancreas, tumorigenic, and nontumorigenic cell homogenates.

Tissue Specificity and Characterization of Antibody. Sections from normal adult pancreas incubated in myeloma medium, followed by incubation in fluorescein-conjugated anti-mouse IgG, or processed for peroxidase staining, showed no fluorescence or peroxidase reaction product, respectively (Fig. 2). Fluorescence microscopy examination of sections from adult human pancreases stained with this antibody and fluorescein-conjugated rabbit anti-mouse IgG revealed diffuse fluorescence of luminal aspects of parenchymal cells. The peroxidase method was more sensitive than the fluorescence technique and showed staining of small and large lumens. The peroxidase reaction was present on basal and lateral aspects of some cells but...
Fig. 10. Micrograph from a section of 4-week MNU-treated adult human pancreas explant incubated in p78 and fluorescein-conjugated anti-mouse IgG, showing intense luminal fluorescence of ductules (arrow) and acini (arrowhead). X 400.

Fig. 12. Micrograph from a section of 24-week MNU-treated adult human pancreas explant, incubated in p78 antibody and fluorescein-conjugated anti-mouse IgG, showing atypical cells lacking fluorescence (arrowheads). X 400.

Fig. 11. Micrograph from a section of 20-week MNU-treated explant incubated in p78 antibody and fluorescein-conjugated anti-mouse IgG, showing strong fluorescence of some glandular structures (arrows) and the lack of immunofluorescence in others (arrowheads). X 400.

Fig. 13. Hypervariable polymorphism in HaeIII fragments of human pancreatic cell lines probed by 5' end-labeled ([γ-32P]ATP) 282-base pair Clal-HaeIII fragments of M13 DNA; T-24 (human bladder carcinoma cell line) (A); adult human pancreas and spleen (B and C); tumorigenic cell lines (T-1), (T-2), and E (T-11); splenocytes from their respective cadaveric donors (F and G); and I (NT-16), and J and K; and HindIII fragments of λ-DNA (L).

appeared as sharp lines on the luminal aspect of acini (Fig. 3), in interlobular ducts (Fig. 4), and in the larger and main pancreatic ducts (Fig. 5). The connective tissue and blood vessels were negative. Sections from fetal pancreases showed a similar pattern of peroxidase staining of the luminal cell surface (Fig. 6). The fetal and adult pancreases were the only tissue showing affinity for this antibody. Sections from other organs and tissues were negative.

Control Explants. Explants cultured in the absence of MNU revealed increasing central necrosis after the first 4 weeks in...
Fig. 14. Micrograph from a H&E-stained paraffin section of nontumorigenic cells NT-16, showing cells of similar size and shape with regular nuclei. × 280.

Fig. 15. Micrograph from a section of nontumorigenic cells NT-16, incubated in p78 and PAP, showing an abundance of peroxidase reaction in particular along the slit-like lumens formed by two or more cells (arrows). × 400.

Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Acinar marker</th>
<th>Ductal marker</th>
<th>Tumor in nude mice</th>
<th>p78 cell surface</th>
<th>M, 78,000 in Western blot</th>
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<tr>
<td>NT-2</td>
<td>−</td>
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Fig. 16. Micrograph from a H&E-stained section of tumorigenic cells T-1, showing cells of irregular size and shape with abnormal nuclei. × 280.

Fig. 17. Micrograph from a section of tumorigenic cells, incubated in p78 and PAP, showing a total absence of peroxidase reaction. × 400.

culture (16). The living parenchymal cells in explants cultured for up to 6 months showed the presence of the p78 marker detected by fluorescence and peroxidase techniques.

MNU-treated Explants. Parenchymal cells in MNU-treated explants cultured for up to 16 weeks, including cells in hyperplastic glands, revealed the presence of the marker detected by this antibody. The distribution of this marker was similar to that of normal pancreas during the first 4 weeks of culture. Sections from explants treated with MNU for 4 to 6 weeks showed irregular and intense peroxidase staining of the apical
Fig. 18. Micrograph from a section of a poorly differentiated primary carcinoma of human pancreas, incubated in p78 and fluorescein-conjugated anti-mouse IgG, showing a total absence of fluorescence. × 400.

Fig. 19. Micrograph from a section of a well-differentiated primary carcinoma of human pancreas, incubated in p78 and fluorescein-conjugated anti-mouse IgG, showing a total absence of fluorescence in tumor cells (arrowhead) and strong fluorescence in the adjacent normal pancreas (arrows). × 400.

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The staining irregularity of luminal surface increased with duration of treatment, and the absence of staining in a number of atypical cells and glands was apparent in explants exposed to MNU for more than 16 weeks (Figs. 8 and 9). The normal distribution of this marker in explants treated with MNU for 4 weeks was confirmed by immunofluorescence (Fig. 10). Bright fluorescence was present on the apical cytoplasm of most cells in explants treated with MNU for more than 16 weeks (Fig. 11). Atypical glandular structures, formed by cells, some of which were completely devoid of fluorescence, were also present (Fig. 12).

DNA Fingerprinting. Fingerprints of DNA from splenocytes and pancreatic tissues of each cadaveric donor revealed identical profiles. Each cell line showed DNA fingerprints (Fig. 13), identical with those of splenocytes or pancreatic tissues from the cadaveric donor of its origin.

Nontumorigenic Cell Lines. Only one of the 24 clones derived from the 2-week MNU-treated explants developed into a cell line and propagated beyond 3 years. The remaining isolates underwent senescence between the 2nd and 15th generations. Two and four cell lines were developed from six clonal growths of the 8- and 16-week-old MNU-treated explants, respectively. Only three cell lines NT-2, NT-8, and NT-16, were adapted to Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Morphologically the nontumorigenic cell lines showed cells of regular size and shape with abundant cytoplasm and relatively small regular nuclei (Fig. 14). All nontumorigenic cell lines derived from MNU-treated explants expressed cell surface ductal determinants detected by HP-DU-1 antibody and were negative for acinar cell surface markers. The presence of the p78 marker on the cell surface and in particular along the slit-like lumens formed by two or more cells was detectable by immunofluorescence. The peroxidase staining was also pronounced along the lumens formed by these cells. The cytoplasmic and nuclear staining was weak, however, but some cells appeared to contain more peroxidase reaction than others (Fig. 15). While the intensity of fluorescence and peroxidase reactions varied greatly from cell to cell in each cell line, there seemed to be no differences in overall presence of this marker among cell lines derived from explants after 2, 8, and 16 weeks of treatment with MNU (Table 1).

Tumorigenic Cell Lines. Tumor nodules were developed in nude mice within 8 weeks following s.c. injection of 1–2 × 10⁶ cells from tumorigenic cell lines. Morphologically the cells appeared irregular in size, shape, and contained relatively large eccentric nuclei (Fig. 16). The sections from tumorigenic cell lines showed the presence of ductal determinants on cell surface detected by HP-DU-1 antibody. The intensity of cell surface fluorescence due to HP-DU-1 antibody varied greatly from cell to cell but most cells revealed bright fluorescence. Both cell lines, PT-1 and PT-2, showed a total absence of p78 marker (Table 1) by immunofluorescence and peroxidase staining (Fig. 17).

Pancreas Carcinoma. One poorly differentiated and two well-differentiated adenocarcinomas were studied. Histologically, the degree of tissue preservation varied from case to case and from section to section in each case. The p78, marker was undetectable in tumor cells by peroxidase or fluorescent method (Fig. 18). The marker was however, present in normal pancreas adjacent to carcinoma cells in all three cases (Fig. 19).

DISCUSSION

A monoclonal antibody was produced by using adult human pancreas parenchymal cell suspension as immunogen. The antibody reacted exclusively with a membrane-associated marker of normal human pancreas epithelium. This IgG3 antibody detected an immunoprecipitable protein with an approximate molecular weight of 78,000. The marker protein detected by immunohistochemistry was membrane associated in paraffin-
embedded and fixed tissue sections from fetal and adult pancreas, nontumorigenic cell lines and normal parenchyma in three cases of primary carcinoma of pancreas. It was not detected in primary carcinomas of pancreas, in anaplastic cells in human pancreas explants treated with MNU, or in tumorigenic cell lines derived from these explants. The presence of this marker in fetal pancreas, in the absence of mature acinar or ductal structures, and in nontumorigenic cell lines with no morphological signs of differentiation, suggests it to be other than a differentiation or a postdifferentiation marker. Also, its abundance in nondividing adult parenchymal cells, seems to negate its direct relationship with cell proliferation. Its loss in tumorigenic cell lines suggests a relationship between its loss and tumorigenicity.

All cell lines derived from MNU-treated human pancreas explants express cell surface HP-DU-1 determinants which indicate lineage identity. The tumorigenic cells, however, differ from nontumorigenic cell lines by the absence of a p78 marker. This, together with the absence of this marker in primary carcinoma of human pancreas and its presence in the normal pancreatic parenchyma adjacent to cancer cells, suggests a possible role for the loss of this marker in tumorigenesis.

It is recognized that most carcinomas are monoclonal and therefore the loss of a number of differentiation markers, belonging to other parenchymal cell types, from the total protein profile of tumor cells is expected. Indeed, this was the case when the total protein profiles of normal human pancreas and tumorigenic and nontumorigenic cell lines were compared. There were qualitative differences, loss of bands, in the Coomassie blue-stained total protein profiles of tumorigenic and nontumorigenic cells when compared with those of normal pancreas homogenates. The loss of the p78 marker, however, is only demonstrable by Western analysis.

The control of tumorigenicity in human cells is complex and appears to involve both genetic and epigenetic mechanisms. The absence of some normal chromosomes, including chromosomes 13 and 21, in tumorigenic cell lines was recently reported from this laboratory (17). The localization of the gene for p78 marker on any of these chromosomes may provide additional support for the role of this marker in tumorigenicity.

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Loss of a $M_r$ 78,000 Marker in Chemically Induced Transplantable Carcinomas and Primary Carcinoma of Human Pancreas

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