ABSTRACT

A rabbit antiserum raised against pancreatic extracts of newborn Syrian hamsters was used in a histological study of pancreas development. This antiserum, after being rendered specific by appropriate absorption, stained the cytoplasm of acinar cells in neonatal pancreas. The reaction was observed from the 13th day of gestation (3 days before delivery) until the 10th day after birth. This period was characterized by a progressive maturation of the endocrine pancreas. The disappearance of fetal pancreatic antigens coincided with the appearance of Langerhans islets. Adult pancreas was not stained with the antiserum, but a good reaction was observed in chemically induced pancreas adenocarcinomas. All reactions were confirmed by immunochemical studies. Polyacrylamide gel electrophoresis followed by immunodetection on nitrocellulose blots demonstrated the presence of two major fetal antigens.

Thus, this study demonstrates the existence of fetal pancreatic antigens associated with development which are reexpressed in pancreatic tumors.

INTRODUCTION

According to recent statistics, the incidence of cancer of the pancreas is increasing in Europe and the United States, so that this carcinoma is the second most frequent among gastrointestinal tumors, the fifth most frequent among all deaths caused by cancer, and the most frequent of the lowest 5-year survival rate (13). The therapy, and hence the prognosis, would probably be improved if the diagnosis could be achieved when the cancer is not too advanced. At the present time, in spite of modern diagnostic techniques, when symptoms appear, there is local or distant metastasis in most of the patients.

Pancreatic oncocytic and cancer-associated antigens have been proposed as potential tools for diagnosis (3, 6, 10, 19). However, the different antigens described so far exhibit different degrees of specificity and do not have the same physicochemical characteristics. It must be said that the difficulties inherent to all investigations dealing with human material are aggravated in this particular carcinoma which is only rarely resected. Furthermore, since the pancreas may easily undergo self-destruction, standard working conditions are not warranted. The same is true for a systematic study of fetal human pancreatic development.

In an attempt to define fetal pancreatic antigens in a standardizable animal model, we have undertaken an immunological study of the pancreas of the hamster. The Syrian golden hamster has been claimed to be the definitive model for human cancer studies (15). The bases for this statement are morphological and biological similarities between human cancer of the pancreas and pancreatic carcinoma induced in hamster by nitroso compounds (16). Our goal was first to decide whether fetal pancreatic antigens do exist before assigning them an eventual role as markers for malignant transformation. With the aid of a rabbit antiserum, we have obtained evidence of pancreatic fetal antigens that are relevant to development. This report describes the tissular localization, developmental features, and some physicochemical characteristics of such antigens. Examples of reexpression in pancreatic adenocarcinoma are also presented.

MATERIALS AND METHODS

Commercial Reagents. Aprotinin (TrasyloI) from bovine lung containing 15,300 Kallikrein-inhibitory units/ml, amino-n-caproic acid, EA3 crude powder, acrylamide, and N, N'-methylenebisacrylamide were obtained from Sigma Chemical Co. (St. Louis, MO). SDS was from BDH (Biochemicals Ltd., Poole, England). BOP was from Ash-Stevens (Detroit, MI). Peroxidase labeled goat anti-rabbit IgG was obtained from Institut Pasteur Production (Paris, France); peroxidase substrates, 3,3'-diaminobenzidine tetrachloride and chloronaphthol, were from Merck; and 3-amino-9-ethylcarbazole was from Sigma. Nitrocellulose membranes were obtained from Millipore (HAHY type) and Schleicher & Schüll (Dassel, Germany) (BA 85 type).

Hamsters. Syngeneic Syrian golden hamsters (Z strain) were from the I.R.S.C. animal colony (Villejuif, France) (22).

Pancreases. NB hamster pancreas was taken less than 24 h after delivery. The pancreas was clearly visible adjacent to stomach and spleen as a gland of about 2 mm and 0.1 to 0.2 mg. Fetal pancreas was taken from the 13th to 15th days of gestation (from 3 to 1 days before delivery). Before this date, the pancreas could not be visualized. Adult pancreas was obtained from animals more than 2 months old. Pancreas was taken also from animals between Days 1 and 15 after birth.

Homogenization. Immediately after excision, the pancreases were dipped in an antiprotease solution consisting of 0.4% amino-n-caproic acid (w/v) and aprotinin (40 units/ml) (10), and were homogenized twice in an ice-chilled bath with the aid of an Ultraturrax homogenizer (Janke & Kunkel, Staufen, Germany). The homogenates were mixed and centrifuged for 30 min at 20,000 rpm at 4°C. The supernatants were either frozen immediately at −60°C (fetal and NB extracts) or lyophilized (adult extracts).

Two hundred NB pancreases, 10 each day, from 12- to 15-day-old fetuses, and about 50 adult pancreases were needed for this study.

Lung, liver, spleen, and the whole gastrointestinal tract from 10 NB animals were homogenized in a similar manner and kept at −60°C.

Protein Quantitation. The protein content in extracts was evaluated by Lowry’s technique (14) by reference to a standard curve made with commercial bovine serum albumin (Sigma). Liquid extracts from fetal and NB pancreas contained approximately 5 mg/ml of protein. Relative to the pancreas weight, the protein content was about 30%. Lyophilized

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3 The abbreviations used are: EA, egg albumin; NIF, nitrocellulose immunofixation; SDS, sodium dodecyl sulfate (sodium lauryl sulfate); PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered 0.15 M NaCl, pH 7.4; NB, newborn; BOP, 2,2'-dioxido-4-propynitrinosamine.
adult pancreas extracts contained 10 to 30% proteins relative to the dry powder.

Antiserum. One Flemish Giant rabbit was immunized with the NB pancreas homogenate as follows. One ml (5 mg protein) emulsified in complete Freund’s adjuvant was injected s.c. in the back. Three and 6 weeks later, second and third injections were done in the same conditions but of 1.5 and 1 mg of protein. Six weeks after the third injection, the rabbit was boosted with 0.6 mg of protein i.v., and the animal was bled 3 days later. The serum thus obtained was used in all experiments.

Absorption. This antiserum was decomplemented (1 hr at 56°C), absorbed 3 times on glutaraldehyde-polymerized (2) adult hamster serum, and stored at -60°C. Before use, the serum was further adsorbed by mixing it with fresh adult hamster serum (v/v) and adding adult pancreas extract (100 mg powder/ml of antiserum). After incubation for 1 hr at room temperature, the serum was centrifuged for 30 min at 40,000 rpm. This extensively absorbed serum will be quoted as anti-NB pancreas in this report.

NIF. This technique permits detection of minute amounts of both antigen and antibody (5) (sensitivity threshold less than 0.01 μg/ml). In brief, 2 to 5 μl of organ homogenates or serum were dotted on nitrocellulose membranes and were then allowed to dry at room temperature. The membranes were successively incubated for 1 hr at 4°C in: (a) a 2% solution of EA in PBS in order to block all binding sites in the membrane; (b) the anti-NB pancreas antiserum diluted 1/50 to 1/500 in 2% EA-PBS; and (c) peroxidase-labeled anti-rabbit antibodies diluted (1/100) in PBS just before use.

Histology and Immunohistology. The organs were fixed in 95% ethanol and embedded in paraffin according to the technique of Sainte-Marie (17). Sections 2 to 3 μm thick were cut with an autocut (R. Jung, Heidelberg, Germany). After deparaffinization, the sections were incubated for 30 min in 0.15% H2O2 in methanol in order to inhibit endogeneous peroxidase. They were then hydrated and incubated for 30 min at room temperature with anti-NB pancreas serum diluted 1/5 in 2% EA-PBS, then with peroxidase-labeled antirabbit antibodies also diluted (1/100) in EA-PBS. Addition of EA considerably reduced nonspecific absorption of antiserum by pancreatic tissues. Immunoreaction was revealed with 0.01% H2O2 and aminoethylcarbazole (8). Sections were counterstained with 1% hematoxylin and mounted in PBS containing 50% glycerol. For histological studies, they were stained with hematoxylin-eosin.

SDS-PAGE Electrophoresis and Immunodetection on Nitrocellulose Blots. PAGE was performed in 12- x 12-cm vertical glass plates using 10% acrylamide and 0.1% SDS (12). The samples (5 mg/ml) were heated at 50°C for 5 min in presence of or without 2.5% β-mercaptoethanol, and 50 μl were electrophoresed under 30 mA/plate in a cool room (4°C) until the marker dye (Coomassie blue) had reached the edge of the plate. Electrophoreses of freshly prepared homogenates were also performed as follows. Pancreas from newborn or adult animals were quickly removed, homogenized in antiprotease solution, centrifuged, and immediately electrophoresed. The whole operation from sacrifice until the start of electrophoresis took 45 min.

For electrophoretic transfer from the gel to nitrocellulose sheets, the technique proposed by Towbin et al. (20) was used. After transfer, the membranes were incubated with EA and the antiserum, both specific and peroxidase labeled. The experimental conditions, i.e., incubation, time, temperature, dilution of antiserum, and visualization were the same as these used in the NIF technique, except that incubation in anti-NB pancreas serum was prolonged for 14 hr (overnight) instead of 1 to 2 hr in NIF.

Induction of Carcinogenesis. Fifty hamsters 2 months old weighing 100 g were given monthly injections in the right flank of 2 mg/kg body weight of BOP in 0.9% NaCl solution (saline). None of the animals manifested pathological symptoms up to 6 months later. Twenty animals were then sacrificed. Tumor in liver was evident in some, but the pancreas looked macroscopically normal in all animals. In the hope that some tumoral cells were nevertheless present, each pancreas was transferred by trocar in the flank of 20-day-old hamsters. About 50% of animals developed tumors at the point of inoculation. Two to 4 months after implantations, most tumors were relatively large (mean diameter, 2 cm) and were excised. Sections were cut for histology, and the remainder was homogenized in the same conditions as for pancreas.

Primary pancreatic adenocarcinoma started from the eighth month following the first BOP injection. They were analyzed by immunohistology.

RESULTS

Specificity of the Anti-NB Pancreas Serum. This antiserum did not display precipitin reactions with the immunogen when tested in various gel diffusion techniques. In the more sensitive NIF technique (Fig. 1), a clear reaction was observed. The serum did not react with normal hamster serum nor with adult pancreas extract. In the same technique, the serum was negative with NB serum and the following NB extracts: spleen; lung; liver; and the gastrointestinal tract.

Histology and Immunohistology. At birth, the pancreas showed significant differences from the adult organ (Fig. 2). While the latter had the typical endocrine and exocrine organization, distinct islets of Langerhans were absent in the former. The acinar cells were visible in the NB pancreas, although they were smaller than in the adult organ. The connective tissue was more abundant at birth. We also noted the presence in the NB pancreas of atypical cellular grouping, quoted by Sak et al. (18) as “areas resembling islets.”

These areas disappeared progressively from the first to the seventh days after birth and, by the 10th day, distinct Langerhans islets were already formed.

Fetal pancreas (13 and 15 days of gestation) were morphologically quite similar to NB pancreas.

Immunoperoxidase detection with anti-NB pancreas serum gave the following results: (a) In NB pancreas, reaction was seen in the cytoplasm of acinar cells (Fig. 3). All acini were positive. Connective tissue, vessels, ducts, and “areas resembling islets” were never stained. (b) Fetal pancreas stained like NB pancreas in that the reaction was restricted to acini. (c) The intensity of the immunoperoxidase staining progressively decreased during the first week after birth and definitively disappeared at the 10th day.

The specificity of the histological immune reaction was ascertained by 3 controls: (a) normal rabbit serum was negative; (b) adult pancreas was not stained with the antiserum; and (c) absorption with NB pancreas extract (1 mg/0.1 ml antiserum) completely abolished the reaction in NB and fetal pancreas sections.

Immune Characterization after PAGE Electrophoresis. The electrophoretic patterns of NB and adult pancreas extracts are depicted in Fig. 4. In the nitrocellulose blot, only the NB pancreas extract was stained. Two main antigens were seen with apparent molecular weights of approximately 58,000 and 54,000. Both appeared also in the Coomassie-stained counterpart, suggesting that they are proteins. Minor components with molecular weights of 77,000, 44,000, and 27,000 were also visible. The electrophoretic profiles of freshly prepared or stored samples were quite similar in both adult and fetal extracts. Mercaptoethanol-treated or untreated samples exhibited analogous banding patterns. It
may be observed in the Coomassie-stained lanes that the M, 77 fetal component is lacking in the adult homogenate and that the M, 58 moves slightly behind a quantitatively comparable component in the adult organ.

**Reexpression of Fetal Pancreatic Antigens in Pancreatic Adenocarcinoma.** Tumors obtained after transfer of BOP-treated pancreas were well-differentiated ductal adenocarcinoma. Morphologically, they were analogous to primary pancreatic tumors obtained from the eighth month following the initiation of BOP treatment. In primary tumors, Langerhans islets were often conserved, whereas exocrine tissue was, in general, absent. On the contrary, no normal tissue was seen in transplanted tumors. Anti-NB pancreas serum stained the glandular (ductal)-like structures in both primary or implanted tumors. The reaction was seen mainly in the cytoplasm of malignant duct cells (Fig. 5). Isolated tumoral cells were also stained in their cytoplasm (data not shown). Reexpression of fetal antigens in tumors was confirmed by the fact that all homogenates from tumors obtained by graft gave a positive reaction with anti-NB pancreas serum in the NIF technique.

**DISCUSSION**

It is known that, in hamster, endocrine pancreas develops postnatally. A study of insulin secretion demonstrated that β-cell differentiation begins at 4 to 6 hr after birth (11). In the pancreas of the NB hamster, an acinar-to-islet continuity was observed (1, 18). β-Cell clusters appeared progressively during the first postnatal week, and well-formed islets of Langerhans were seen from the 10th day after birth. This seems peculiar to hamster since, in other species such as rabbit, mouse, and rat, morphological and biological development of endocrine pancreas occurs during fetal life (4, 7, 9). Histological results in the study presented here are in agreement with the preceding reports, while our immunohistological observations demonstrate another interesting feature of pancreas development. Fetal pancreatic antigens defined with the aid of the antisera raised against homogenates of NB hamster pancreas were seen from the 13th day of fetal life (earliest time when the pancreas was macroscopically evident) until the 10th day after birth. Moreover, the gradual decrease in intensity of the immunoperoxidase stain in tissue sections suggested a time-dependent loss of fetal antigens. Apparently, a disappearance of fetal antigens parallels the maturation of endocrine pancreas. Whether this is merely circumstantial or both phenomena are functionally linked requires further study. At the present time, the only conclusion that can be drawn is that exocrine pancreas in hamster matures postnatally. This conclusion could not be deduced from simple histology since, apart from their dissimilar size (1), fetal acinar cells are morphologically indistinguishable from adult pancreas cells.

According to PAGE experiments, there probably are several pancreatic oncofetal antigens in hamster. None of them is clearly related to α-fetoprotein, which has been demonstrated to be present in rat fetal pancreas (21). In effect, anti-NB pancreas serum reacted with neither fetal liver nor fetal serum, and the molecular weights of the fetal components are not compatible with α-fetoprotein.

The possibility of autodigestion in extracts was considered. For this reason, electrophoresis of freshly prepared samples was compared to that of stored lyophilized (adult) or frozen (neonatal) pancreas homogenates. No significant differences in the profiles were observed, and the migration of fetal antigens remained unchanged. Taking into account that, in fresh samples, only 45 min elapsed between extraction (made in antiprotease solution) and the electrophoretic run and that all operations including electrophoresis were performed at 4°, autolysis seems improbable, although it cannot be completely ruled out.

The pancreatic oncofetal antigens described in humans have molecular weights of 40,000 (10) and approximately N, 1,000,000 (6). It is difficult at the moment to establish a further comparison between hamster and human antigens. In experiments performed to detect cross-reactions, the anti-NB pancreas serum did not stain sections of fetal human pancreas. However, only fetuses of 5 months or older were examined and since, at this age, human pancreas is functionally mature, the possibility that fetal pancreatic antigens had already disappeared cannot be ruled out.

The presence of pancreatic fetal antigens in pancreatic adenocarcinoma illustrates again the reexpression of fetal or embryonic components in cancer and makes these antigens suitable for further studies concerning pancreatic carcinogenesis. In particular, it would be of interest to look for the earliest appearance of these antigens in the course of experimental carcinogenesis and to search for pancreatic oncofetal antigens in blood for possible markers in diagnosis.

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Fig. 1. NIF technique showing the specificity of the antiserum against NB pancreas extract before (top) and after (bottom) absorption with serum and adult pancreas. Five μl of NB and adult pancreas (AP) homogenates (1 mg/ml) and normal adult serum (S) (diluted 1/10) were dotted on a Millipore membrane. Anti-NB pancreas serum was used at 1/50 dilution, and peroxidase-labeled antiserum was used at 1/1000 dilution. Substrate, chloronaphthol.

Fig. 2. A, NB pancreas sections showing acini ducts and "areas resembling islets" (arrows); B, adult pancreas section showing vessels, ducts, acini, and islets. H & E, × 400.

Fig. 3. A, section of NB hamster pancreas stained with anti-NB pancreas serum and the indirect immunoperoxidase technique. For experimental conditions, see "Materials and Methods." × 400. B, control of negative NB pancreas section made with anti-NB pancreas serum absorbed with fetal pancreatic extract (10 mg/ml antiserum), × 400.

Fig. 4. SDS-polyacrylamide slab electrophoresis of adult (AP) and NB pancreas homogenates. All samples including standards were heated for 5 min in presence of 2.5% β-mercaptoethanol. The molecular weight standards were bovine serum albumin (M, 67,000), catalase subunit (M, 60,000), and lactate dehydrogenase subunit (M, 36,000) from Pharmacia Fine Chemical Co. (Uppsala, Sweden). Left, Coomassie stain. Right, reaction with anti-NB-pancreas serum (dilution 1/100) after transfer to a Schleicher & Schuell membrane. Peroxidase-labeled antiserum was diluted 1/2000. Substrate, diaminobenzidine tetrachloride.

Fig. 5. Immunoperoxidase staining of areas of implanted ductal pancreatic adenocarcinoma sections. (a) The immunoreaction is seen in the glandular-like structures (× 100). At greater magnification (× 250) (c, d), the reaction can be observed in the cytoplasm of malignant cells. Note the absence of reaction in the control section (b) (× 100) treated with normal rabbit serum instead of anti-NB pancreas serum.
Oncofetal Pancreatic Antigens in Hamster
Fetal Pancreatic Antigens in the Syrian Golden Hamster and Their Relationship to Development and Carcinogenesis

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