A M₅₈,000 Glycoprotein Specifically Expressed in Developing Hamster Pancreas and Reexpressed in Hamster and Human Pancreatic Carcinomas

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ABSTRACT

A glycoprotein with a molecular weight of 58,000 specifically expressed in exocrine hamster fetal pancreas was characterized using a monoclonal antibody (Mab B4).

By immunoperoxidase, Mab B4 stained pancreatic tissue from the 10th day of gestation (6 days before delivery) until the 10th day after birth. The maximal expression of the Mab B₄-specific protein called fetal pancreatic (FP) protein was reached between delivery and the 5th day of postnatal life. Endocrine pancreas was negative at any developmental stage. All adult pancreata examined were negative. Moreover, Mab B₄ was tested against a wide variety of fetal and adult tissues; only immature pancreata were stained. Chemically induced pancreatic carcinomas were strongly stained by this Mab. On the contrary, other tumors (liver and kidney) appearing simultaneously with pancreatic carcinomas were negative. Using a nitrocellulose immunofixation assay, FP protein was found in all sera from hamsters bearing pancreatic tumors (23 cases tested). This protein was not detected in normal sera.

Mab B₄ cross-reacted with a protein in human fetal pancreas extracts, that behaves similarly to the hamster FP protein: it is present exclusively in exocrine fetal pancreas and is reexpressed in pancreatic adenocarcinomas.

The high tissue specificity of this protein, its oncofetal character, and release into the blood circulation make the FP protein a potential tumor marker of pancreatic cancer.

INTRODUCTION

The presence in the pancreas of components specific for fetal acinar cells was first evidenced in Syrian golden hamster using a polyclonal antiserum raised against fetal pancreatic extracts (1, 2). Important features of these antigens are their association with development and reexpression in pancreatic carcinomas (3, 4). During experimental carcinogenesis in a BOP hamster model (5–7), these antigens were already present in preneoplastic lesions (8, 3, 4). They first appeared in altered acini and progressed through irregular hyperplasia to ductal carcinoma. This suggests an important role in transformation.

In Western blot, this antiserum recognized several antigens in fetal pancreatic extracts, in the relative molecular weight range of 27,000 to 94,000 (1). Two main components of M₅₈,000 and 80,000 were identified as being two antigenically unrelated glycoproteins (2).

Recently, a family of proteins presenting many analogies with hamster fetal pancreatic proteins has been described in human fetal pancreas. By Western blot, the main human components had a molecular weight of 110,000 and 60,000 (9). A monoclonal antibody exclusively recognizing the M₅₈,000 component has been obtained (10, 11). It is a concanavalin A-reactive protein associated with the development of the pancreas and located in fetal acinar cells. It has been referred to as FAP (for fetoacinar pancreatic) protein. The expression of FAP protein rose sharply in case of pancreatic carcinoma and was found to be present in the blood of most patients with this neoplasia (12, 13).

In the present study, we prepared a murine monoclonal antibody (Mab B₄) against the M₅₈,000 hamster fetal protein (14). This protein belongs to the family of fetal pancreas-specific proteins already defined by the polyclonal antiserum, and shares with FAP protein: pancreas specificity, association with development and reexpression in tumors. However the protein described here is located in acinar and ductular fetal cells whereas FAP-protein was described as acini-restricted (11). Therefore the M₅₈,000 component has been referred to as FP protein for fetal pancreas protein.

This work describes the biochemical characteristics and tissue distribution during development of the FP protein, its reexpression in chemically induced pancreatic tumors and its presence in sera from hamsters with pancreatic carcinoma. The cross-reaction of Mab B₄ with a human protein is also reported.

MATERIALS AND METHODS

Hamsters. Syrian golden hamsters maintained for many years in a syngeneic colony (15) were obtained from the IRSC facilities (Villejuif, France).

Normal Tissues. Whole embryos and fetuses of gestational ages 8, 9, 10, 11, and 12 days were removed. Gestational age was assessed by means of the size and number of somites in embryos and fetuses.

Pancreases were obtained from fetuses aged 13 and 15 days of gestation (the gestation period in hamsters is 16 days), from animals between 1 day and 15 days after delivery and also from adult hamsters 2–4 months old. Other fetal and adult organs, including lung, heart, kidney, spleen, liver, and the whole gastrointestinal tract were taken from the same animals.

Tumoral Tissues. Primary pancreatic carcinomas were obtained in 2-month-old hamsters by treatment with BOP (1, 4). Secondary tumors were raised by grafting pancreas from hamster treated with BOP into syngeneic recipients as described previously (1, 8). Kidney and liver tumors appearing simultaneously with pancreatic tumors after BOP injection were also collected.

Human Material. Fetal pancreases were obtained after spontaneous or therapeutic abortion. Adult pancreases and other normal organs came from recently deceased donors of kidney grafts. Normal and pathological tissues were kindly donated by Dr. M. Gonzalez (Hôpital Saint Antoine, Paris, France) and Dr. M. Nap (Institute of Health Province of Friesland, Leeuwarden, The Netherlands).

Tissues for Histology. Immediately after excision, a piece of each organ was fixed in ethanol or formalin for 48 h and subjected to immunohistological procedures.

Tissue Extraction. Pancreases from fetuses 13 to 15 days-old and from newborns were taken for tissue extraction since previous results from our laboratory (1) showed that newborn and fetal hamster exhibit the same antigens. At birth individual pancreas weight is around 0.15 mg.
Immediately after removal, 100 pancreases were dipped into an antiproteinase solution consisting of 0.4% E-a-mannosidase a/w, aprotinin 40 units/ml and 10^{-5} M diisopropylfluorophosphate and homogenized. The homogenates were centrifuged (20,000 rpm for 30 min at 4°C) and the supernatants were immediately aliquoted and frozen at -80°C. Protein content in extracts evaluated by Lowry method (16) was on the average 5 mg/ml. Normal and tumor adult pancreases were processed similarly. Extracts of the other organs were prepared in the same manner, with the exception that antiprotease solution contained only aprotinin.

Con A-reactive Fraction. This was prepared from extract of hamster fetal pancreas as previously described (2). 5 ml of pancreatic extracts (5 mg protein/ml) were applied to a Sepharose-Con A column equilibrated with 0.02 M Tris buffer, pH 7.4, containing 1 mM each of MnCl2, MgCl2, and CaCl2. Elution was performed with 0.2 M methyl-a-D-manno-pyranoside. This Con A reactive (Con A⁺) fraction contained the two major fetal proteins of M, 58,000 and 80,000 (2), as well as minor components including adult pancreas and normal serum proteins as contaminants.

Production of Hybridomas. BALB/c mice, 8 weeks old, received 7 s.c. monthly injections of the fraction purified on Con A affinity chromatography from fetal pancreas extracts (15 µg total protein emulsified in complete Freund's adjuvant per injection). 3 days prior to fusion the animals were given one i.v. booster injection of crude fetal pancreas. Spleen cells were fused with murine myeloma cells SP2/0 according to the method of Buttin et al. (17). Fused cells were distributed in 24-well culture plates at a cell concentration of 10⁶ cells/ml. Hybridomas were selected in ER medium containing hypoxanthine, aprotinin 40 units/ml and 10⁻⁵ M diisopropylfluorophosphate and

Fetal pancreatic protein in cancer pancreas

RESULTS

Monoclonal Antibody Characterization

Three hybrid cells out of the 144 tested reacted in Western blot with M, 58,000 protein contained in fetal pancreas extracts. The hybridoma giving the strongest reaction was selected and cloned by limited dilution. After two cloning procedures, Mab B4 was produced in the form of ascites. Ascitic fluid showed the same specificity as culture supernatant.

After purification on protein A-Sepharose, antibody in ascitic fluid was typed as IgG1, by immunodiffusion analysis (20); this is in agreement with the fact that Mab B4 was fully eluted from a protein A column at pH 5.8 (24).

Antigen Characterization

In Western blot, Mab B4 in fetal pancreatic extract revealed a main component of M, 58,000 and a minor one of M, 54,000 (Fig. 1, lane D). According to the total protein level Coomassie-stained M, 58,000 protein appears at relatively high concentrations as compared to other proteins in fetal extract (Fig. 1, lane B). In IEF immunoblots, a rather heterogeneous pattern exhibiting several bands in the 5.5–6.1 region was observed (data not shown). Adult pancreas extracts were negative in both techniques.

The binding of Mab B4 to FP protein was not altered after antigen treatment with neuraminidase, periodic acid, and mercaptoethanol, whereas it was fully abolished after trypsin digestion.

Tissue Specificity

Mab B4 was tested by indirect immunoperoxidase in a large variety of normal fetal and adult tissues namely: pancreas, kidney, heart, spleen, liver, stomach, duodenum, small intestine, and colon. Only fetal pancreas was stained. Tissue specificity was confirmed by the negative reaction of extracts from

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FETAL PANCREATIC PROTEIN IN CANCER PANCREAS

B M Wicr

200

68

43

158

54

these tissues by dot blot nitrocellulose membrane assay. Controls performed using normal mouse serum were negative.

Developmental Pattern

In the hamster the gestation period is 16 days. Embryogenesis takes place during about 8 days (22) and the pancreas is morphologically mature at Day 10 after birth (1).

Embryonic and Fetal Tissues. Sections from whole embryos and fetuses ranging from 8 to 12 days of gestation (five to six specimens each at 8, 9, 10, 11, and 12 days) and pancreases obtained from fetuses aged 13 and 15 days of gestation were examined by immunohistology with Mab B4. The earliest staining was observed on the 10th day. This stage corresponds to the beginning of pancreas organogenesis. A pancreatic rudiment embedded in a loose mesenchyme was seen near the hepatic primordia, in all examined sections (Fig. 2). Moreover the undifferentiated cells forming this primitive structure were the only tissue Mab B4 immunostained in the whole fetus. During the following 24 h the epithelium of the developing pancreatic rudiment is arranged in branching tubules. Cells proliferate and become arranged into acinus-like structures with enlarged lumina. The stain with Mab B4 was located in the cytoplasm of these cells (Fig. 3A). The cytoplasmic compartment enlarged from this date and some morphologically distinguishable acini were seen at 13 days of gestation. Mab B4 immunostain regularly increased and was located in both still immature and newly formed acini. This persisted until delivery.

Neonates until 15 Days after Birth. Forty-four pancreases were tested (N = 20 for newborns and three cases each at 1, 3, 5, 7, 9, 10, 12, and 15 days). In newborns, the Mab B4 reaction was located in primitive ductular cells and acini (Fig. 3B). At this age staining intensity varied from one area to another, probably due to the different developmental stage of cells. Maximal expression of FP protein, as judged by the intensity of immunoreaction, was observed around birth and persisted until the 5th day of postnatal life. During this period intralobular ducts were patent and appeared stained (Fig. 3C), whereas interlobular ducts observed were negative. After the 5th day, staining quickly decreased, so that at the 7th day some acini remained faintly positive. Staining totally disappeared at the 10th postnatal day (Fig. 3D). Thus, all sections examined at Days 10, 12, and 15 were negative.

Hamsters 2-4 Months Old. No stain at all was observed in all examined pancreas at this age.

FP Reexpression in Pancreatic Carcinoma

In our experimental conditions, BOP-treated hamsters developed pancreatic adenocarcinomas from 6 to 7 months after the first carcinogen injection. However, morphological alterations were observed from the 2nd month. Evolution in time of pancreatic lesions were altered acinar cells [loss in cytoplasmic and zymogen contents and dilated lumina (26)], cystadenomas, and regular and irregular hyperplastic epithelium. Polyclonal anti-fetal pancreas serum showed immunoreactivity in preneoplastic lesions, mainly in distorted acini and ducts with marked atypical epithelium (3, 4). By contrast, the Mab B4 stain was only observed in pancreatic carcinomas, either primary (Fig. 4A) or produced by graft (Fig. 4B).

Reexpression of FP protein in pancreatic carcinomas was...
confirmed by the dot blot technique: Mab B4 positivity was seen in extracts from primary and grafted tumors.

Tumors of other organs which appeared simultaneously with pancreatic carcinomas after BOP treatment (liver, N = 10; kidney N = 4) were negative for Mab B4.

Serum Test

A qualitative test using the dot blot technique was performed in order to detect whether FP protein was present in the blood: 23 sera from hamsters with pancreatic tumors were analyzed. All cases were positive for Mab B4. On the contrary, no reaction was detected in normal sera. Its presence in other induced tumors could not be tested, since they appeared simultaneously with pancreatic carcinoma in our experimental model. Therefore, the control group was limited to healthy animals.

Cross-Reaction of Mab B4 with Human Tissues

Mab B4 cross-reacted in dot blot technique with human fetal pancreas extracts. In Western blot, this monoclonal antibody also reacted with a component which migrated at the M, 58,000 position (data not shown). Immunoperoxidase of all fetal pancreas sections analyzed (N = 10) showed a weak positive reaction in the cytoplasm of acinar cells. When Mab B4 was tested against other normal tissues summarized in Table 1, all cases were negative. We also examined the presence of FP protein in pathological tissues: pancreatic adenocarcinomas were positive (seven out of 10 tested) whereas other tumors were negative (Table 2). In pancreatic tumors, a positivity was observed in the malignant ductular epithelium (Fig. 4C). The immunoreaction was located in the cytoplasm of tumoral cells.

Comparative Study of FP and FAP Proteins

In order to confirm whether FP and FAP (10, 11) are different proteins, a comparative study by Western blot and immunohistochemistry was made. Thus Mab B4 and Mab J28 (specific for FAP protein) (10, 11) were tested by Western blot against hamster and human fetal pancreas extracts. Mab B4 recognized the M, 58,000 protein in both, but Mab J28 reacted only with the M, 110,000 FAP protein in human fetal pancreas extracts. By immunohistochemistry, we tested the same pancreatic tumors with both monoclonal antibodies (B4 and J28) and observed a very different localization, i.e., Mab B4 reacted with the tumoral structures themselves whereas as previously reported the stain-
Table 1 Reactivity of monoclonal antibody B4 with human normal tissues

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Number tested</th>
<th>Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal pancreas</td>
<td>10</td>
<td>+*</td>
</tr>
<tr>
<td>Adult pancreas</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Adult Wirsung duct</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Adult duodenum</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Adult stomach</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Adult liver</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>Adult colon</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Adult oesophagus</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Adult lung</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Adult kidney</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

* Indirect immunoperoxidase was applied in all instances. +, positive staining; -, no reaction.

Table 2 Reactivity of monoclonal antibody B4 with human pathological tissues

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Number tested</th>
<th>Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatites</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Pancreas CA*</td>
<td>10</td>
<td>+*</td>
</tr>
<tr>
<td>Duodenum CA</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Gastric CA</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Breast CA</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Colonic adenomas</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Colonic CA</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Ovarian tumors</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>

* CA, carcinomas.

Immunoreaction was performed by indirect immunoperoxidase procedure. +, positive staining in 7 of 10 specimens; -, no reaction.

DISCUSSION

The FP protein reported in this study behaves, in SDS-PAGE immunoblots, identically to the major antigen evidenced by Benedi et al. in hamster fetal pancreas extracts with the aid of a polyclonal antiserum (1). The bands immunostained by Mab B4 at the M, 58,000–54,000 position were comparable (size and shape) to those recognized by the polyclonal antibody. The M, 54,000 component appears as a molecular variant of the M, 58,000 protein. This antigen has been characterized as a glycoprotein which binds to concanavalin A. Changes in glycosylation can easily explain the double band pattern recognized by Mab B4. In this regard, FP protein is similar to human FAP protein (11) since in Western blot, Mab J28 (specific for FAP protein) stained two bands at positions M, 110,000 and 100,000. Results obtained after treatment of fetal pancreas extracts with enzymes or chemical reagents suggest that the epitope recognized by Mab B4 may be expressed on the peptide moiety.

By immunohistology, we demonstrated that FP protein expression is development related: it is present from the earliest stage of pancreatic ontogenesis (10 days of gestation) when the only pancreatic structure consists of a primitive duct-like structure embedded in a loose mesenchymal stroma. Later, this protein is expressed in exocrine fetal pancreas (cytoplasm of acinar and ductular cells), but never in endocrine fetal pancreas. The synthesis of this protein as well as FAP protein starts at a very early stage of exocrine pancreas differentiation, and therefore these proteins can be regarded as organogenesis-specific proteins. Expression of FP protein slowly increased, reaching the highest level between birth and 5 days after delivery. Thereafter, FP protein quickly decreased and totally disappeared when the definitive morphology of adult pancreas was acquired (on the 10th day of postnatal life).

As for the fetus, endocrine cells remained negative for Mab B4 in neonates and adults in agreement with previous results obtained with polyclonal antiserum in both hamster (3, 4) and...
humans (9), suggesting the existence of independent pathways of differentiation for exocrine and endocrine pancreas.

The FP protein is not expressed in adult pancreas, but was reexpressed in pancreatic carcinomas. FP protein was found in neoplastic stages and not in benign lesions observed during carcinogenesis, this suggests that not all oncofetal pancreatic antigens revealed using the polyclonal antiserum are simultaneously expressed (4).

The absence of FP protein in a wide variety of normal and pathological tissues, apart from immature pancreas and pancreatic tumours, demonstrates its high tissue specificity.

The serum test performed in order to verify the presence of FP protein in the circulation showed clear positivity in 100% of the sera from hamsters with pancreatic tumors. This finding indicates that FP protein is released into the blood in this malignancy.

Mab B4 cross-reacted with human tissues: in immunohistology, fetal pancreas and pancreatic carcinomas were stained with the M, 58,000 FP protein. The fact that Mab B4 recognizes all tissues. They also wish to thank Dr. B. Loridon-Rosa for the histolog

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