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Biochemical Characterization of α-Fetoprotein and Other Serum Proteins Produced by a Uterine Endometrial Adenocarcinoma

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A high serum α-fetoprotein (AFP) level was found in a patient with endometrial adenocarcinoma of the uterus, which appeared to be hepatoid on histological examination. The AFP of this unusual patient was purified by immunoaffinity chromatography and characterized. The electrophoretic profiles on sodium dodecyl sulfate-polyacrylamide gel electrophoresis both before and after glycopeptidase F treatment were indistinguishable from those of a hepatoma AFP. This indicates that the patient's AFP was also composed of a single polypeptide chain of Mr 67,000 and an N-linked sugar chain of Mr 3,000. Amino acid sequence analyses of this AFP, and of AFP from hepatoma and umbilical cord serum indicated that the N-terminal sequences were essentially the same. The sequence, Arg-Thr-Leu-His-Arg-Asn-Glu-Tyr-Gly-Ile, was slightly different from previous reports, but matched that deduced from the cDNA sequence. AFP isoforms due to microheterogeneity of the sugar chain were analyzed by lectin affinity electrophoresis using a series of lectins. The AFP isoform profiles were distinct from those of proteins derived from cord serum, hepatoma, yolk sac tumor and gastric cancer. The reverse-transcription of RNA from the tumor tissue followed by a polymerase chain reaction using primers with AFP-specific sequences gave a product of the size and nucleotide sequence expected for AFP. mRNAs possessing the requisite sequences for albumin and transferrin syntheses were also detected in the tumor. The expression of these hepatocyte-specific proteins supported the hepatoid nature of this tumor.

Key words: α-Fetoprotein — Endometrial adenocarcinoma — Isoform — Hepatoid — Lectin

α-Fetoprotein (AFP) is one of the major serum proteins in the developing embryo and is mainly synthesized by the liver and yolk sac.[1,2] It is well-established that it is a diagnostic marker for hepatoma or yolk sac tumor, since these tumors produce AFP and secrete it into the circulation. However, it is occasionally observed that some other cases of malignant tumors also have highly elevated serum AFP levels. Such malignancies reported to date include those of the gastrointestinal tract,[3–5] pancreas,[6] lung,[7] kidney,[8] urachal tract,[9] gallbladder[10–12] and uterus.[13,14] Endometrial adenocarcinoma of the uterus with elevated serum AFP is very rare and only two cases have been reported.[13,14] Recently, we experienced a third such case whose clinical and pathological details will be reported elsewhere.[15] Production of AFP by this type of cancer, including our case, was suggested by a highly elevated and variable level of serum AFP and by immunohistological localization of AFP in the tumors. Since tumors, as well as fetal cells, have been shown to take up AFP,[2] further evidence is needed to confirm its production by tumor cells. In this study, we partially sequenced the AFP mRNA of the tumor tissue together with those of albumin and transferrin, which are co-expressed in fetal liver, yolk sac and their malignant counterparts. AFP is composed of a single polypeptide chain, Mr 67,000, and an N-linked sugar chain of Mr approximately 3,000.[16,17] Isoforms of AFP relating to microheterogeneity of the sugar chains have been demonstrated by lectin affinity electrophoretic studies. AFP isoform profiles are distinct depending on the origins of the AFP.[18,19] We have isolated AFP from the serum of our endometrial cancer patient and found it to have the characteristic properties of AFP. We also determined its N-terminal amino acid sequence together with those of the proteins derived from hepatoma and fetal sources since conflicting results have been reported.[1,20,21]

MATERIALS AND METHODS

Purification and characterization of AFP AFP was purified from the serum of the patient by immunoaffinity chromatography as previously described,[22,23] proteins from a hepatoma patient (hepatoma AFP) and umbilical cord sera (fetal AFP) have been described previously.[24] The purified AFPs were deglycosylated by glycopeptidase F, which cleaves the linkage between Asn and carbohydrate chains.[25] The molecular sizes of the AFPs and the deglycosylated forms were analyzed by sodium

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dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For the N-terminal amino acid sequencing, AFPs were further purified by reversed-phase, high-performance liquid chromatography employing a phenyl 5PW-RP column (Toso, Tokyo) with an acetonitrile gradient (15–80%) containing 0.05% trifluoroacetic acid. They were then sequenced by automated Edman degradation using a gas-phase sequencer, model A470 (Applied Biosystems, Foster City, CA).

AFP in the serum of the patient was analyzed for isoforms by lectin-affinity electrophoreses as described previously.[26] Briefly, 4 μl of serum sample diluted to contain 100 ng/ml of AFP was applied to an agarose gel plate containing concanavalin A (Con A), lentil lectin (LCA-A), erythroagglutinating phytohemagglutinin (E-PHA), or *Allomyrina dichotoma* lectin (allo A). Following electrophoresis, the AFP was transferred to a nitrocellulose membrane precoated with affinity-purified horse antibody to AFP. The membrane was reacted with $F(ab')_2$ fragments of rabbit immunoglobulins to AFP and then with affinity-purified goat anti-rabbit IgG conjugated with horseradish peroxidase. AFP bands were visualized by detecting the enzyme activity by the tetrazolium method. Samples analyzed in parallel experiments were from our frozen serum stocks[26] and included sera of a hepatoma patient, a gastric cancer patient, a yolk sac tumor patient and umbilical cord.

Detection and characterization of mRNA sequences Total cellular RNA was isolated from the endometrial adenocarcinoma and adjacent normal tissue by the acid guanidinium thiocyanate-phenol-chloroform method.[27] Detection of mRNA sequences was carried out by reverse transcription-polymerase chain reaction (RT-PCR) as described by Kawasaki and Wang.[28] Briefly, 10 μg of total RNA, 10 μg of random primers, 1 m*M* each of the 4 deoxyribonucleotides, and 50 U of RNase inhibitor (Takara Shuzo, Tokyo) were employed in a final volume of 25 μl. The cDNA synthesis reaction was initiated by the addition of 10 U of avian myeloblastosis virus reverse transcriptase (Seikagakukogyo, Tokyo) and run at 42°C for 60 min. The products were stored at −20°C until use. Primers used in PCR experiments are shown in Table I. PCR products generated after 20 or 50 cycles of denaturation (94°C for 30 s), primer annealing (37°C for 30 s), and DNA extension (72°C for 30 s) were analyzed by agarose gel electrophoresis. To ensure that the amplifications were specific, PCR products of double-stranded DNA were directly sequenced by the dideoxy method using Taq polymerase.[29] Primers used for the sequencing of AFP, albumin and transferrin were AFP789F,[16] ALB869F,[30,31] and TF338F,[32] respectively.

RESULTS

Biochemical characterization of AFP Approximately, 1 mg of uterine cancer AFP was purified from 1 ml of the patient's serum. It showed a single band on SDS-PAGE with Mr 70,000 (Fig. 1a). After glycopeptidase F treatment, the AFP was converted into a molecule of Mr 67,000 (Fig. 1c). Since the same results were obtained with hepatoma AFP (Figs. 1b and d), it was concluded that the polypeptide chain (Mr 67,000) of the uterine cancer AFP had a single N-linked oligosaccharide (Mr 3,000), as was found in AFPs from hepatoma, cord serum and yolk sac tumor.[2] The N-terminal amino acid sequences of the uterine cancer AFP and the fetal AFP were identical for 10 cycles and were: Arg-Thr-Leu-His-Arg-Asn-Glu-Tyr-Gly-Ile. This sequence was in good agreement with most previous reports of protein sequencing except for the N-terminal amino acid residue, where Ser instead of Arg has been found.[1,20,21] However, in hepatoma AFP, in addition to the above amino acids, other amino acids have been noted up to the 9th cycle, i.e., His, Arg, Asn, Glu, Tyr, Gly, Ile, Ala, and Ser, suggesting the presence of an additional N-terminus starting with His-4.

Table I. Primers Used in the PCR Experiments

Primer	Sequence	Product	(size, position)
AFP789F	5′-ACCAAAGTTAATTTTACTGAAAT-3′	AFP	(644 bp, 789–1452)
AFP1452R	5′-GTTTGTCCTCACTGAGTTGGCA-3′		
ALB869F	5′-TCCACACGGAATGCTGCCATGG-3′	albumin	(368 bp, 869–1236)
ALB1236R	5′-AGCGGCACAGCACTTCTCTAGA-3′		
TF338F	5′-TCTATGGGTCAAAAGAGGATCC-3′	transferrin	(612 bp, 338–949)
TF949R	5′-GAGAGCTGAATAGTTGGAATTC-3′		
β-P1	5′-CTGTCTGGCGGCACCACCAT-3′	β-actin	(254 bp, 936–1189)
β-P2	5′-GCAACTAAGTCATAGTCCGC-3′		

The primers for AFP, albumin and transferrin were designed from the sequences reported by Morinaga *et al.*,[16] Lawn *et al.*,[30] Dugaiczyk *et al.*[31] and Yang *et al.*,[32] respectively. Those for β-actin were described by Nakajima-Iijima *et al.*[33]

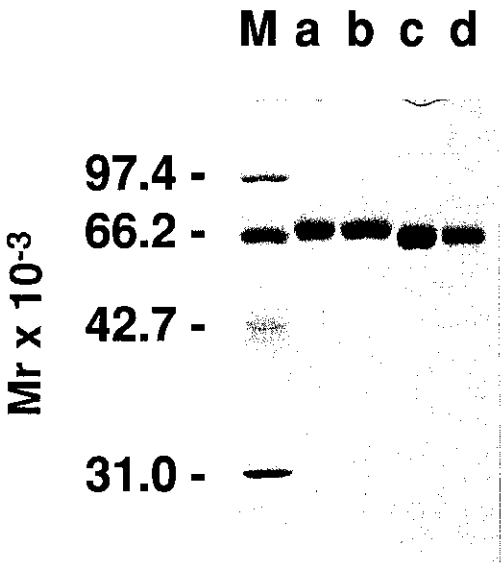

Fig. 1. SDS-PAGE analysis of AFPs. Uterine cancer AFP (a and c) and hepatoma AFP (b and d) were electrophoresed on an 8% gel before (a and b) and after (c and d) digestion with glycopeptidase F and stained with Coomassie Brilliant Blue. Molecular markers (M) were phosphorylase (97.4), bovine serum albumin (66.2), ovalbumin (42.7) and carbonic anhydrase (31.0).

AFP isoforms due to carbohydrate structural differences were analyzed by lectin affinity electrophoresis.[18,24,26] With Con A, AFP can be separated into 2 fractions, Con A-nonreactive C1 and Con A-reactive C2. As shown in Fig. 2, the 2 AFP isoforms were clearly detected in the serum of uterine cancer patient (Uterine ca.), gastric cancer patient (Gastric ca.) or yolk sac tumor patient (Yolk sac tumor), while the hepatoma patient's serum (Hepatoma) and cord serum (Umbilical cord) partially or completely lacked the C1 isoform. LCA-A separates AFP into 3 fractions, L1, L2 and L3. The uterine cancer AFP displayed L1 and a broad fused band of L2 and L3, as was seen with gastric cancer, but distinct from the patterns of cord serum, hepatoma and yolk sac tumor. Five isoforms, P1, P2, P3, P4 and P5, are demonstrable with E-PHA. The isoform profiles of AFP of 5 different origins were distinct from each other. With allo A, 3 major fractions, A1, A2 and A3 and one subpopulation, A1s, are demonstrable. Among the 4 isoforms, A1, A1s and A3 were detected in the uterine cancer AFP. This pattern was similar to that of yolk sac tumor, but distinct from those of AFP from other sources.

Analysis of mRNA by RT-PCR PCR amplification of cDNA generated from the total cellular RNA of normal uterus showed no detectable AFP-specific signal (Fig. 3c), whereas that of uterine cancer exhibited an intense

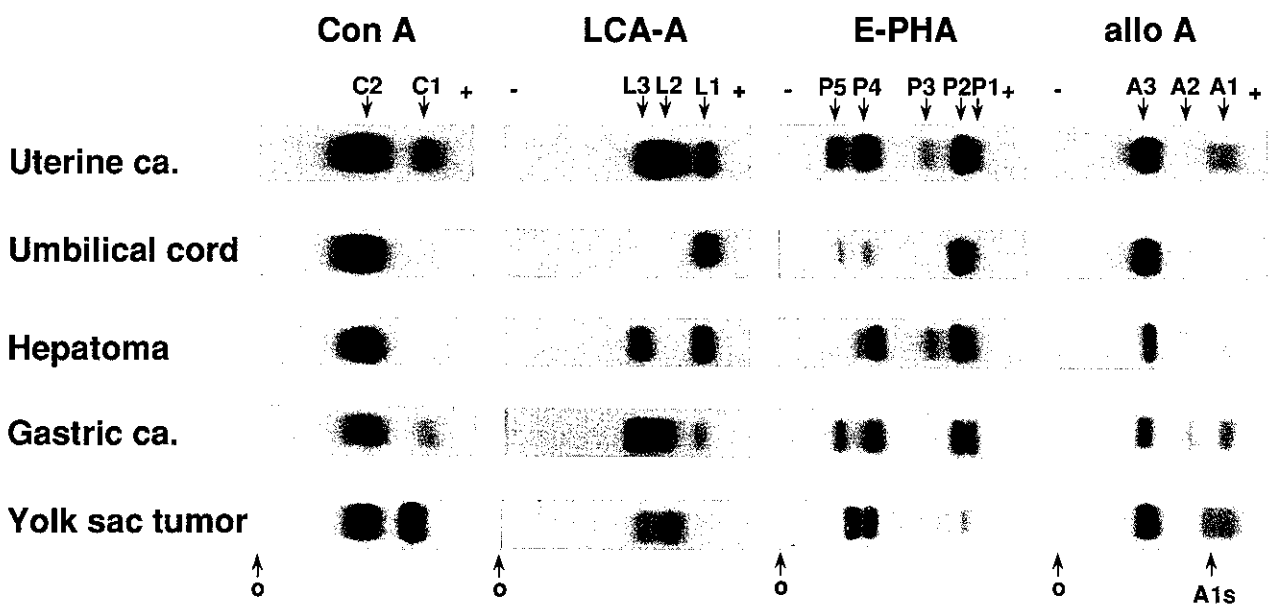

Fig. 2. Lectin affinity electrophoretic analysis of AFP isoforms. AFPs in the sera of the uterine cancer patient (Uterine ca.), umbilical cord (Umbilical cord), a hepatoma patient (Hepatoma), a gastric cancer patient (Gastric ca.) and a yolk sac tumor patient (Yolk sac tumor) were electrophoresed on agarose gel containing Con A, LCA-A, E-PHA or allo A. The letter "o" represents the origin of electrophoresis.

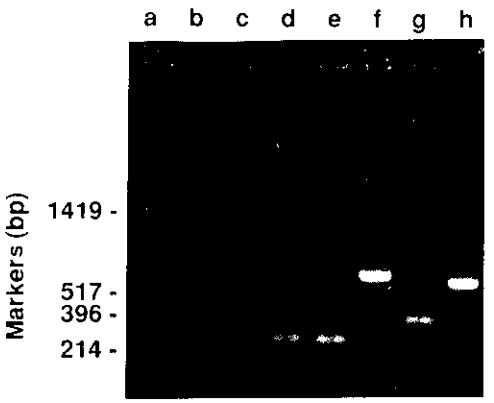

Fig. 3. Agarose gel electrophoresis of PCR products. The PCR reactions were done with template cDNA from normal tissue (a to d) and the cancer (e to h) employing primers for transferrin (a and h), albumin (b and g), AFP (c and f) and β-actin (d and e). Electrophoresis was done on a 1.2% gel and DNA was stained with ethidium bromide.

band of the expected size, which suggested the presence of a substantial amount of the AFP mRNA in the cancer tissue (Fig. 3f). In addition, the uterine cancer was found to express the mRNA of albumin and transferrin (Fig. 3, g and h), whereas normal uterus did not (Fig. 3, a and b). Since similar amounts of β-actin cDNA were generated with both templates (Fig. 3, d and e), the results indicated the specific expression of the mRNAs of these serum proteins in the tumor. The 3 mRNA sequences were detected with a 500-fold diluted template of tumor cDNA at 25 PCR cycles, while no such sequences were obtained with an undiluted template of normal tissue at 50 PCR cycles. For identification of the PCR products, direct nucleotide sequencing analyses were performed. Nucleotide sequences of AFP, albumin and transferrin determined corresponded to the reported cDNA regions of 836–948,[16] 920–985[30, 31] and 384–516,[32] respectively. Thus, the endometrial adenocarcinoma in this case expressed not only AFP, but also albumin and transferrin.

DISCUSSION

A patient with endometrial adenocarcinoma of the uterus displayed high AFP production; the AFP was isolated and its biochemical properties were studied.

The SDS-PAGE of the AFPs from this case and from a hepatoma indicated that the sizes of polypeptide chain and N-linked oligosaccharide chain were the same (Fig. 1). Amino acid sequence analyses of the above 2 AFP preparations, as well as fetal AFP, showed two new significant findings on the amino terminus of human AFP. First, Arg was identified as the N-terminal amino acid in all samples. Previously, Ser, but not Arg, has been reported as the N-terminus by amino acid sequencing from several laboratories.[1, 20, 21] Human AFP cDNA sequence analysis has indicated that the precursor form of AFP, preAFP, is composed of 609 amino acid residues.[16] The present result showing that the N-terminal amino acid is primarily Arg strongly suggests that preAFP is processed to a mature AFP of 591 amino acids. The present proposal for the cleavage site, between the 18th (Ser) and 19th residue (Arg), is different from the previous assignment[16] between the 19th and 20th residue (Thr), but seems more reasonable, since it is more firmly based on the experimental results described above. Furthermore, the observation of posttranslational modification of human albumin, whose processing is well-understood,[34] supports the above assignment. The albumin, which has marked structural similarities to AFP, is synthesized as preproalbumin of 609 amino acids in which an extra signal sequence of 24 residues is attached to the mature albumin of 585 residues. After or during translation, a signal peptidase, which recognizes the Ser (-7)-Arg (-6) dipeptidyl sequence, cleaves off the presequence of 18 amino acid residues to form proalbumin (albumin with an extra hexapeptide) with an N-terminal Arg. The N-terminal portion of preAFP is highly homologous to the albumin presequence, 9 out of 18 residues being identical, and contains Ser-Arg at the 18th and 19th residue. Therefore, it is highly likely that similar processing takes place, resulting in mature AFP having an Arg residue at its N-terminus. In the case of albumin, a second processing occurs in the Golgi by a convertase which cleaves off the hexapeptide.[34] The recognition sequence of the convertase, Arg-Arg, is absent in AFP.

Second, upon Edman degradation of hepatoma AFP, additional amino acids were also noted that could match a sequence starting with His-4. Such results may suggest that hepatoma AFP has 2 N-terminals, one being identical to that of fetal AFP or uterine cancer AFP, and the other lacking the N-terminal Arg-Thr-Leu and beginning with His-4. This type of variant AFP has also been reported for AFP of hepatoma origin.[2, 20] It was suggested that peptidases in the ascites fluid or in the serum could have generated this heterogeneity.[20]

We analyzed AFP isoforms of the uterine cancer AFP by means of lectin affinity electrophoresis, and the isoforms were distinct from those compared in this study (Fig. 2). This result suggested that a set of glycosyl transferases and/or modifying enzymes which were different from those in other types of cells were operating in the uterine cancer cells to generate the unique carbohydrate structure.

Our previous report, which showed positive immunostaining of the tumor with anti-AFP antibody, suggested AFP production by the tumor.[15] In the present study, a

high level of AFP mRNA was detected by RT-PCR, indicating that the AFP was expressed in the tumor rather than incorporated by the tumor. Furthermore, the mRNAs of hepatocyte-specific molecules, albumin and transferrin, were also detected in the tumor. Ishikura *et al.* studied 7 cases of hepatoid adenocarcinomas of stomach which produced AFP, and they detected production of albumin in 6 out of 7 cases and transferrin with less frequency by immunohistological analyses.[4] The detection of mRNAs of albumin, transferrin and AFP in this study provides further evidence that hepatoid tumor exhibits a hepatocyte-like phenotype. It is known that the 5′ upstream regions of the genes of these proteins, as well as several others which are specifically produced in liver, contain similar promoter/enhancer elements required for hepatocyte-specific transcription.[35–38] It is suggested that the uterine cancer in the present studies expressed hepatocyte-specific transcription factor(s), resulting in gene activations which lead to hepatoid histology and expression of albumin, transferrin and AFP. Malignancies other than hepatoma and yolk sac tumor sometimes express AFP.[3–14] Many of them are categorized as hepatoid adenocarcinomas, exhibiting the histological appearance of hepatoma, that could result from similar mechanisms to those described above.

In conclusion, the AFP produced by uterine cancer demonstrated distinct carbohydrate isoforms, while other biochemical properties were similar to those from other sources. The uterine cancer in this case, histopathologically characterized as hepatoid, was also confirmed to be hepatoid at the molecular level by RT-PCR, which detected hepatocyte-specific gene products, AFP, albumin and transferrin. This approach for detection of tissue-specific molecules will aid the pathologist in establishing and correlating morphological features with specific biological properties.

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