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Tumor-associated Expression of a Serum Protein, Termed αX Protein (α_1-Inhibitor III), and Its mRNA in Rat Liver

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A cDNA clone bearing the mRNA sequence for rat αX protein (αX) was isolated from a cDNA library constructed from rat liver mRNA. The nucleotide sequence of αX protein cDNA showed 97% homology with that of the 3′-proximal domain of α_1-inhibitor III cDNA. The amino acid sequence deduced from that of αX cDNA also exhibited high homology with the primary sequences of α_1-inhibitor III and α_2-macroglobulin. K231 ascites hepatoma cells were transplanted into male ACI rats, and the level of αX mRNA in the liver of the tumor-bearing rats was determined by RNA blot hybridization with the cDNA probe. The serum concentration of αX decreased to about 30% of the control value with time after transplantation. The amount of αX mRNA in the liver of tumor-bearing rats was proportional to the serum concentration of αX. The serum concentrations of transferrin and albumin in the tumor-bearing rats also decreased to about 30 and 60% of the normal levels, respectively. However, the amounts of mRNAs for transferrin and albumin in the liver of tumor-bearing rats did not decrease. These findings indicate that the mechanisms of tumor-associated decrease in the concentrations of different serum proteins in tumor-bearing rats may differ.

Key words: Serum protein — αX protein — α_1-Inhibitor III — Expression — Tumor-bearing rats

Rat alpha-X protein, named in our laboratory,[1] is a major serum protein with a molecular weight of 200,000. Its serum concentration is about 10–15 mg/ml in normal rats, and is known to decrease in rats bearing various tumors.[1]

In tumor-bearing rats, the serum levels of some proteins, such as α_2-macroglobulin and α-fetoprotein, are known to increase, while those of some other proteins including αX protein, transferrin and albumin decrease. Possible reasons for these decreases are (1) decreased synthesis of these proteins in the liver, (2) increased degradation of proteins in general, (3) excretion of serum proteins from the body, and (4) consumption of these proteins by tumor or tissues of tumor-bearing rats. To determine the relationship between the serum level and hepatic synthesis of αX protein, we isolated a cDNA clone encoding αX protein mRNA, and measured the mRNA level in the liver of tumor-bearing rats. For comparison, we also measured the levels of transferrin and albumin mRNAs in the liver.

MATERIALS AND METHODS

Transplantation of tumor cells into rats Male ACI rats were purchased from CLEA Japan Co. and were inoculated with ascites hepatoma K231 cells (2×10^6 cells, ip or 4×10^6 cells, sc) at 5 weeks of age. Then they were maintained under conventional conditions for up to 15 days. Tumor-bearing rats were anesthetized with ether and their blood and liver were removed. The serum was separated by centrifugation and stored at −20°C, while the liver was quickly frozen in liquid nitrogen, and then stored at −70°C until use.

Determination of serum protein concentration The serum proteins were purified from normal Sprague-Dawley rats by published methods.[1-3] Antisera to the respective proteins were raised in rabbits as described previously.[4] The serum concentrations of these proteins were determined by the single radial immunodiffusion method[4] with the purified proteins as standards.

Cloning of cDNA for αX protein Recombinant phages carrying cDNA for the mRNA sequence of αX protein were isolated from a λgt11 expression library constructed from rat liver mRNA by immunological screening with anti-(αX protein) serum, as described previously.[5] A cDNA insert was isolated from one of the recombinant phages by *Eco*RI digestion of the phage DNA, and then was subcloned into pUC18 cloning vector by the method of Maniatis *et al.*[6] The resultant plasmid (prAX2) was used for nucleotide sequencing and as a hybridization probe.

Quantitation of serum protein mRNAs in the liver Total RNA was extracted from the livers of normal or tumor-bearing rats by the guanidine isothiocyanate/cesium chloride procedure of Chirgwin *et al.*[7]

Abbreviations used are: αX, αX protein; α_1I3, α_1-inhibitor III; Tf, transferrin; ALB, albumin; α_2M, α_2-macroglobulin; 1× SSC, 0.15 *M* NaCl and 15 m*M* sodium citrate.

For preparation of labeled probes for RNA blot hybridization, plasmid DNAs, pRTfc1 for transferrin,[5] prAlb-1 (a kind gift from Dr. R. Makino, National Cancer Research Institute) for albumin[8] and prAX2 for αX protein, were labeled with [γ-^{32}P]dCTP by nick-translation with a nick-translation kit (Takara Shuzo Co.). For dot blotting, total RNA at defined concentrations was treated with 6% formaldehyde and 17×SSC at 70°C for 5 min, and then cooled in ice-water. RNA (3 μl) was spotted onto nylon membranes equilibrated with 20×SSC, and fixed to the membranes under UV illumination for 5 min. For Northern blotting, total RNA was denatured with dimethylsulfoxide and glyoxal, separated by 1% agarose gel electrophoresis in 10 m*M* NaH_2PO_4 (pH 7.0), and transferred to a nylon membrane (Hybond-N, Amersham).[9] The membrane was irradiated with UV light for 5 min, and then baked at 80°C for 1 h.

The irradiated membranes were prehybridized at 42°C for 4 h in prehybridization buffer consisting of 50% formamide, 5×SSC, 50 m*M* sodium phosphate buffer (pH 6.5), 0.25 mg/ml denatured salmon sperm DNA, 0.2% SDS, 5 m*M* EDTA, 0.2 mg/ml each of bovine serum albumin, Ficoll 400 and polyvinyl pyrrolidone. Hybridization was carried out in a 1:4 mixture of 50% dextran sulfate and the prehybridization buffer containing a nick-translated probe (4×10^6 cpm/80 ng/ml) at 55°C for 16–18 h.

DNA sequencing of αX cDNA clone The nucleotide sequence of the αX cDNA clone, prAX2, was determined by the dideoxy chain termination method[9] with a nucleotide sequencing kit (Takara Shuzo, Co.).

RESULTS

Nucleotide sequence of αX cDNA clone Three recombinant clones bearing the sequence for αX mRNA were isolated from a rat liver cDNA library (approximately 12,000 pfu) by immunological screening with a polyclonal antibody against rat αX protein. A cDNA insert of 294 base pairs in length was isolated from one of the recombinant phages, and subcloned into the pUC18 vector. The nucleotide sequence of the resultant recombinant plasmid, denoted as prAX2, was analyzed. The nucleotide sequence of the αX protein cDNA is shown in Fig. 1. A computer-assisted homology search revealed that the cloned cDNA bears 97% homology with the nucleotide sequence of the carboxyl proximal domain of rat α_1-inhibitor III (α_1I3).[10] The only mismatches between αX and α_1I3 cDNAs were single base-pair changes at ten locations. Most of these mismatches are G to A or A to G substitutions. Moreover, the amino

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αX:        1 CGGTACACCA AGACACTGAT GGCCTATGCT TTCGCTCTTG CGGGAAACCA GGAAAAGAGA
             ******** ** ******* ********** ******** * * ** ***** *** ******
αI3:  3437 GTGTACACCA AGGCACTGAT GGCCTATGCT TTCGCTCTGG CAGGGAACCA GGAGAAGAGA

αX:       61 AACGAAATCC TGAAATCCCT TGATAAGGAA GCTATAAGGG AAGACAACTC CATCCACTGG
             ** ******* ********** ********** ******* ** ********** **********
αI3:  3497 AATGAAATCC TGAAATCCCT TGATAAGGAA GCTATAAAGG AAGACAACTC CATCCACTGG

αX:      121 GAAAGACCTC AGAAACCCAC AAAATCAGAG GGTTATCTGT ACACACCCCA GGCTTCCTCT
             ********** ********** ********** ********** ********** **********
αI3:  3557 GAAAGACCTC AGAAACCCAC AAAATCAGAG GGTTATCTGT ACACACCCCA GGCTTCCTCT

αX:      181 GCTGAAGTAG AGATGAGTGC CTATGTCGTC TTAGCTCGCC TCACTGCCCA GCCAGCCCCA
             ********** ********** ********** ********** ********** **********
αI3:  3617 GCTGAAGTAG AGATGAGTGC CTATGTCGTC TTAGCTCGCC TCACTGCCCA GCCAGCCCCA

αX:      241 TCCCCTGAGG ACCTGGCTTT GTCAATGGGC ACCATCAAGT GGCTCGCAAA GCAG
             ******** * ********** ********** ********** ***** **** ****
αI3:  3677 TCCCCTGAAG ACCTGGCTTT GTCAATGGGC ACCATCAAGT GGCTCACAAA GCAG
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Fig. 1. Comparison of nucleotide sequences of αX cDNA and α_1-inhibitor III cDNA. The nucleotide sequence of αX protein cDNA (prAX2), subcloned into pUC18 plasmid, was determined by the dideoxy-elongation procedure using a TaKaRa sequencing kit (Takara Shuzo Co., Japan). The sequence was compared with the sequence for α_1-inhibitor III (pRLA1I3/2J) reported by Braciak *et al.*[10] Identical residues are marked with asterisks.

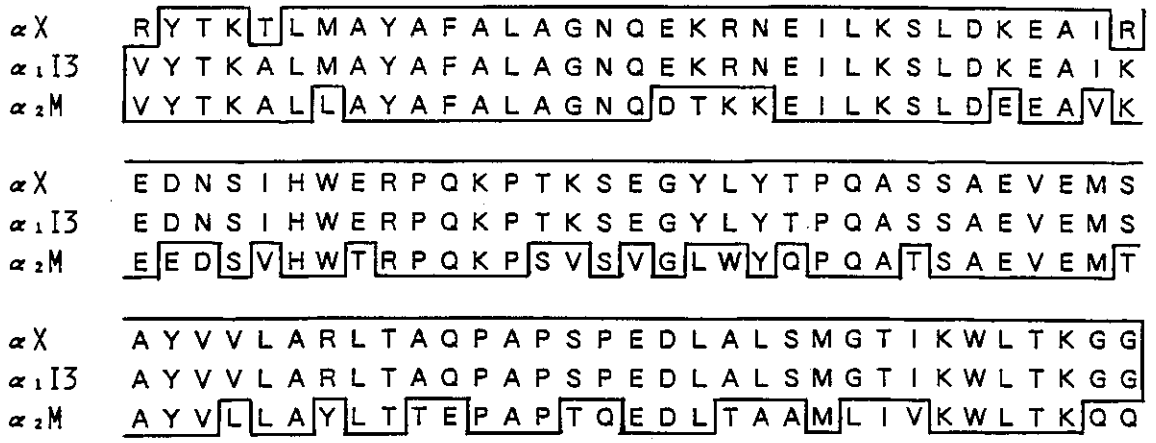

Fig. 2. Comparison of amino acid sequences of αX protein, α_1-inhibitor III and rat α_2-macroglobulin. The amino acid sequence of αX protein was deduced from the nucleotide sequence of prAX2 DNA shown in Fig. 1. The sequences of α_1I3 and α_2M are those reported by Braciak *et al.*,[10] and Gehring *et al.*,[11] respectively. Identical residues are boxed.

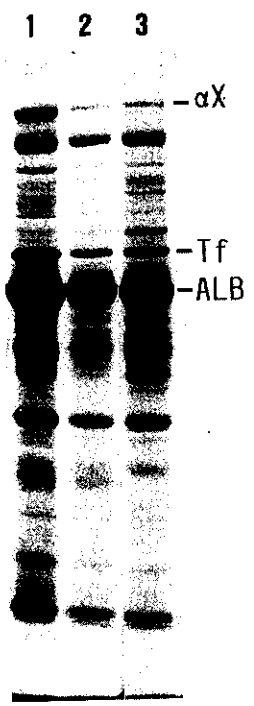

Fig. 3. SDS-polyacrylamide gel electrophoretic patterns of serum proteins of tumor-bearing rats. Ascites hepatoma K231 cells were inoculated into male ACI rats (2×10^6 cells, intraperitoneally and 4×10^6 cells, subcutaneously). Serum was obtained after 15 days, and samples equivalent to 0.5 μl of serum were subjected to 8% polyacrylamide gel electrophoresis by the method of Laemmli.[12] Proteins were stained with Coomassie brilliant blue R250. (1), serum of control rats; (2), serum of rats bearing ascites tumor; (3), serum of rats bearing subcutaneous tumor.

acid sequences deduced from the nucleotide sequences of the two cDNAs were almost identical, only three of the 99 amino acids in αX and α_1I3 being different (Fig. 2). The amino acid sequence of αX cDNA also showed 63% homology with that of rat α_2-macroglobulin (Fig. 2, ref. 11).

Serum levels of αX protein in tumor-bearing rats The levels of serum proteins in the tumor-bearing rats were compared with those in normal rats. As shown in Fig. 3, the concentrations of total serum proteins of tumor-bearing rats decreased a few days before death to about 70% of the normal level. Of the serum proteins, αX protein decreased the most, its level in tumor-bearing animals on day 15 after implantation of tumor cells being 30% of that of normal rats (Table I).

The serum concentration of transferrin decreased to nearly the same extent as that of αX, but the serum level of albumin in tumor-bearing rats was about 60% of that in normal rats (Table I).

The relationship between the serum protein levels and the weights of tumors was studied in rats after sc implantation of K231 cells. The results showed that marked decreases in αX protein and transferrin concentrations were followed by tumor growth (Fig. 4).

Amounts of αX protein mRNA in the liver of tumor-bearing rats The levels of αX mRNA in the livers of normal and tumor-bearing rats were compared. Total RNA was isolated from the liver and subjected to Northern blot hybridization analysis with cloned αX protein cDNA (prAX2) as a probe. Almost the same amounts of total RNA per unit weight of liver were obtained from normal and tumor-bearing rats. As depicted in Fig. 5A, αX protein cDNA hybridized to mRNA with a molecular

Table I. Serum and mRNA Levels of αX Protein, Transferrin and Albumin

Rats[a] (n=4)	Serum concentration (mg±SD/ml)[b]			mRNA amounts (cpm±SD/μg RNA)[c]		
	αX protein	Transferrin	Albumin	αX protein	Transferrin	Albumin
Untreated	13.1±0.1	3.7±0.1	34.1±0.2	520±89	3240±269	5393±99
sc tumor	4.3±0.1	1.0±0.1	20.7±0.1	156±17	3295±66	5236±13
Ascites tumor	2.5±0.0	1.2±0.1	22.1±2.1	200±29	3157±61	6085±160

a) Experimental conditions were as for Fig. 3.
b) Serum levels of serum proteins were determined by the single radial immunodiffusion method with specific antisera for each protein.[4]
c) Amounts of mRNA in total liver RNA were determined by dot blot hybridization as in Fig. 6.

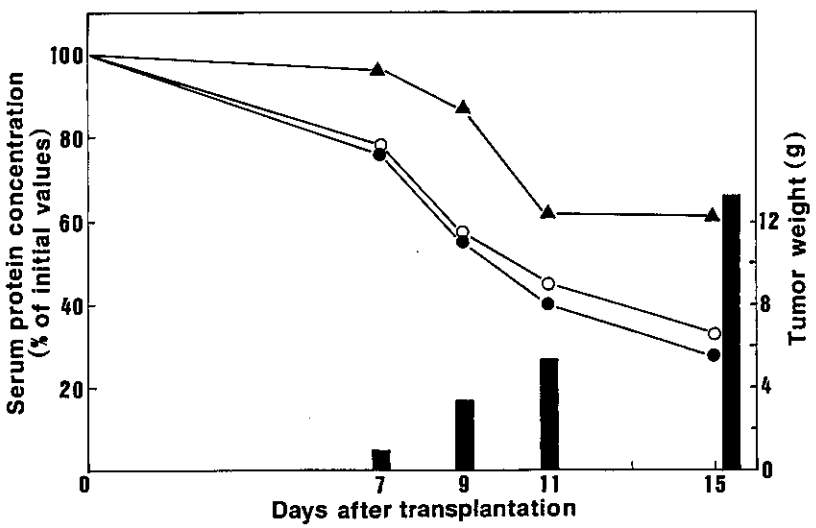

Fig. 4. Relationship of serum protein levels with tumor development. Ascites hepatoma K231 cells (4×10^6 cells) were inoculated subcutaneously into male ACI rats. At the indicated times, groups of three rats were killed, and their blood and subcutaneous tumors were collected. The serum concentrations of αX, transferrin and albumin were determined by the single radial immunodiffusion method[4] with specific antiserum for each protein and purified proteins as standards. ○, αX protein; ●, transferrin; ▲, albumin; ■, tumor weight.

size of about 4700 bases. No difference was found in the sizes of αX mRNAs of normal and tumor-bearing rats.

The amounts of αX mRNA in total RNA of the livers were determined by RNA dot blot hybridization. In the rats bearing subcutaneous tumors, the level of αX mRNA in the liver decreased progressively after transplantation of tumor cells. The profile of decrease in αX mRNA in the liver during tumor development was very similar to that of decrease in the serum αX concentration in these rats (Fig. 6). On day 15, the amount of the mRNA was about 30% of that in normal rats (Table I).

In contrast to αX protein mRNA, Northern blot hybridization analysis showed that the mRNA levels of transferrin and albumin in the liver of tumor-bearing rats were similar to those in the liver of normal rats (Fig. 5B, C). RNA dot blot hybridization also confirmed that the amounts of transferrin and albumin mRNAs in tumor-bearing rats were similar to those in normal rats (Table I).

DISCUSSION

Alpha-X protein was first found by us as a serum protein whose concentration decreases in the early stage of tumor progression in the rat.[1] In this paper, we showed that the nucleotide sequence of cDNA for αX protein bears 97% homology with that of cDNA for the carboxyl proximal domain of α_1-inhibitor III (pRLA1I3/2J) reported by Braciak *et al.*[10] Braciak *et al.* also reported the heterogeneity of α_1I3 mRNA. The sequence of cDNA for α_1I3 in a clone isolated by Aigello *et al.*[13] showed 96% identity with that of clone pRLA1I3/2J. In the present study, Northern blot hybridization of rat liver mRNA indicated that mRNA complementary to a clone containing cDNA for αX protein is identical in molecular size to α_1-inhibitor III mRNA. The molecular weight of αX protein (about 200,000 daltons) is also similar to that of α_1I3. Therefore, αX protein is probably identical to α_1-inhibitor III.

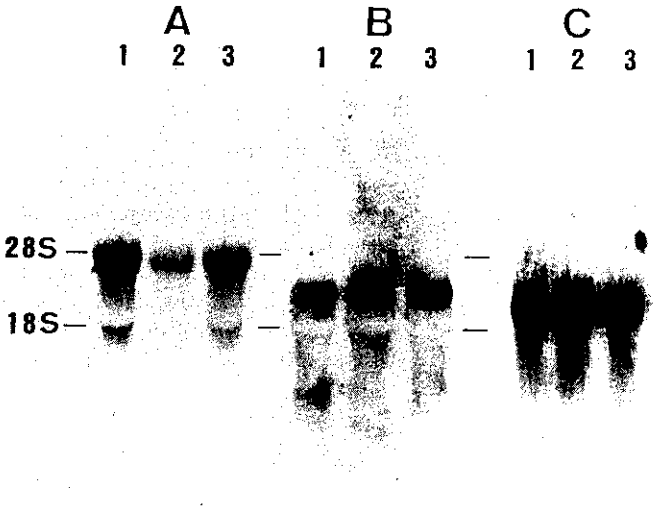

Fig. 5. Northern blot hybridization analysis. Total RNA was isolated by the method Chirgwin *et al.*[7] from pooled livers of groups of four normal and four tumor-bearing rats on day 15, as for Fig. 3. Samples of 20 μg of total RNA were denatured by treatment with glyoxal and dimethyl sulfoxide, separated by 1% agarose gel electrophoresis in the presence of 10 m*M* NaH_2PO_4 (pH 7.0), and transferred to Nylon membranes (Hybond-N, Amersham). Hybridization was performed as described in the "Materials and Methods." (A), αX protein mRNA; (B), transferrin mRNA; (C), albumin mRNA; (1), control rats; (2), rats bearing subcutaneous tumors; (3), rats bearing ascites tumors.

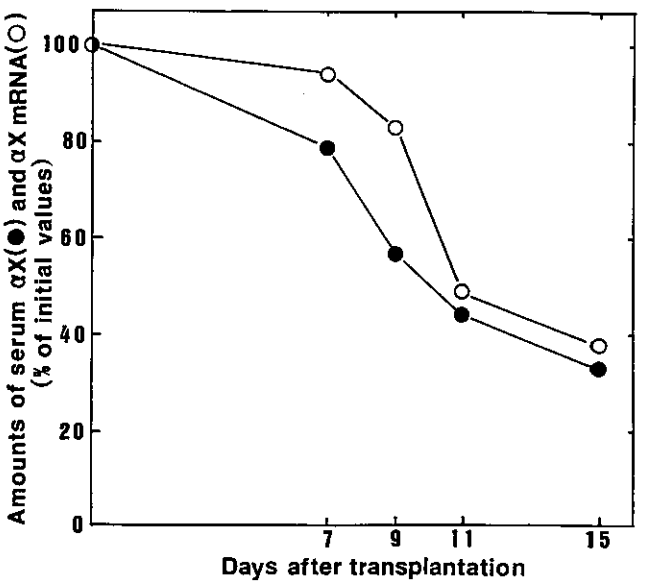

Fig. 6. Correlation between serum levels and amounts of mRNA of αX protein in rats bearing subcutaneous tumors. Serum and total RNA were obtained from rats on the indicated days after sc inoculation of ascites hepatoma K231 cells. Serum αX levels were determined by the single radial immunodiffusion method, and amounts of αX mRNA in total liver RNA were calculated from the radioactivities of nick-translated prAX2 DNA hybridized on dot blot hybridization.

The α_1-inhibitor III is known to be a negative acute-phase protein, and we found that its level decreases with tumor growth. This decrease in the serum concentration of αX is parallel with the decrease in the amount of αX mRNA in the liver of tumor-bearing rats (Fig. 6). Thus, the serum level of αX protein appears to be controlled mainly by the amount of hepatic αX mRNA.

The extent of this tumor-associated decrease in the serum αX level was almost the same (to about 30% of the normal level) as that observed in the acute-phase response induced by injection of complete Freund's adjuvant or turpentine.[10] In acute-phase responses, this reduction also results from a decrease in the α_1I3 mRNA level.[13] The serum levels of transferrin and albumin also decrease in tumor-bearing rats, but the amounts of their mRNAs in the liver remain unchanged (Table I). These findings indicate that the mechanisms of the tumor-associated reductions in serum levels of major protein components in rats may differ for different serum proteins. Albumin also decreases transiently during the acute-phase response, but there are conflicting reports that in this response the amount of albumin mRNA decreases[14, 15] or remains constant.[13] As it is unknown whether the mRNAs of transferrin and albumin are translationally functional in tumor-bearing rats, studies on the biosyntheses of these proteins during tumor progression are needed.

No loss in body weight of rats bearing subcutaneous tumor was found, while serum triglycerides in these rats increased to about 4 times the normal level (unpublished results). Hypertriglyceridemia in tumor-bearing animals or animals treated with lipopolysaccharide is known to be caused by tumor necrosis factor (cachectin).[16] Thus, it would be of interest to know whether lymphokines, including tumor necrosis factor, affect the expression of serum proteins during tumor development.

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