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Expression of the pS2 gene in human gastric cancer cells derived from poorly differentiated adenocarcinoma

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NH_2-terminal amino acid sequence of the pS2 protein produced and secreted by human gastric cancer cells, MKN-45, was determined to be identical to that of MCF-7 cells. A clone encoding pS2 protein was isolated from the cDNA library constructed from MKN-45 cells. The nucleotide sequence was identical to that of pS2 cDNA previously isolated from human breast cancer cells, MCF-7, except for one nucleotide in the 3′ untranslated region. Thus, in this cell line, the pS2 gene product is translated and secreted as in MCF-7 cells. RNA blot hybridization analysis revealed that pS2 gene was expressed well in two (MKN-45 and KATO-III; derived from poorly differentiated adenocarcinoma) but not in three cell lines (MKN-1, MKN-28 and MKN-74; from well differentiated adenocarcinoma), suggesting that expression of the pS2 gene depends on the state of cell differentiation. These results suggest that pS2 is expressed in human gastric cancer cells in an estrogen-independent manner and is possibly associated with the malignant state of cells.

pS2; Human gastric cancer cell; Transcription; Processing

1. INTRODUCTION

The pS2 gene is originally isolated from human breast cancer cells, MCF-7, with its inducibility by estrogen [1]. This gene encodes a protein composed of 84 amino acids [2]. The protein is purified from the serum-free medium conditioned by MCF-7 cells and the amino acid sequence of this purified protein shows that it is secreted into the medium as a polypeptide of 60 amino acids after the signal polypeptide cleavage [3]. Activation of transcription of the pS2 gene is a primary response to estrogen in MCF-7 cells [4]. The pS2 protein was first deduced to be an autocrine growth factor for this cell line because expression and cell proliferation were likely to occur simultaneously. But experiments with MCF-7 variant cells [5] and time course experiments [6] suggest that this polypeptide is not an autocrine growth regulator. At present, the biological significance of this polypeptide remains to be uncovered.

pS2 is expressed well in estrogen receptor-positive breast cancers but not in normal breast cells [7]. Thus, in breast, the expression of pS2 is highly estrogen-dependent. However, pS2 is expressed well in stomach mucosa [8], suggesting the expression of this gene is not necessarily hormone-dependent in other cell types.

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In our current study, the expression of pS2 gene was investigated in gastric cancer cell lines derived from well or poorly differentiated and hormone-independent adenocarcinoma, at transcriptional and secreted levels in the conditioned medium.

2. MATERIALS AND METHODS

2.1. *Cell culture procedure*

Five human gastric cancer cell lines obtained from Immunobiological Laboratory (Japan) were cultured as described for MCF-7 cells but without insulin nor β-estradiol [6]. For preparation of RNA, cells were scraped from the dishes and stored at −80°C. Cells for purification of pS2 protein were cultured as described previously [3].

2.2. *Assay of pS2 protein*

Assay of pS2 samples was performed with the two site enzyme immunoassay as described previously [6].

2.3. *Purification of pS2 protein and amino acid sequence analysis*

Purification of pS2 protein and its sequence analysis were carried out as described previously [3].

2.4. *cDNA library construction*

Total RNA was extracted from MKN-45 cells and poly(A)$^+$ RNA was prepared as described previously [10]. The MKN-45 cell cDNA library was constructed using 5.2 μg of vector primer DNA (Pharmacia) and 30 μg of poly(A)$^+$ RNA according to the method of Okayama and Berg [9].

2.5. *Colony screening*

Escherichia coli HB101 was transformed with recombinant DNA and colony hybridization was carried out as described previously [10]. The DNA fragment used as a probe was a 358 base-pair (bp)

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*Nco*I–*Pvu*II fragment of pS2B1 cDNA obtained from MCF-7 cells [10], which was nick-translated with [α-^{32}P]dCTP (3000 Ci/mmol) to the specific activity of 2–3 × 10^8 cpm/μg according to the method of Weinstock et al. [11]. DNA sequencing was performed by the method of Sanger et al. [12].

2.6. *RNA blot hybridization analysis*

Poly(A)$^+$ RNAs were prepared (described above) from human breast cancer cells (MCF-7) cultured in the presence of estrogen (10^{-8} M) and from five gastric cancer cells. Poly(A)$^+$ RNAs (15 μg) were denatured in the presence of 50% formamide containing 2.2 M formaldehyde, 20 mM morpholinopropane sulfonic acid (pH 7.0), 5 mM sodium acetate and a nitrocellulose filter (Schleicher & Shuell), and then baked in a vacuum oven for 5 h at 75°C according to the standard method [13]. The filter was prehybridized, hybridized and then washed as described previously [10].

3. RESULTS

The pS2 protein was purified from serum-free conditioned medium of MKN-45 cells by following essentially the same procedure as from human breast cancer cells (MCF-7) [3]. This pS2 protein synthesis in MKN-45 cells was not induced by estrogen and the content of pS2 in serum-free medium conditioned by this cell line was about 1/100 of that by MCF-7 cells supplemented with estrogen (10^{-8} M) (data not shown). Fig.1 shows the final step of purification of pS2 protein achieved by reverse-phase high-performance liquid chromatography with a μBondapak C_{18} column. pS2 was recovered at the same elution position as that from MCF-7 cell. Approximately 500 ng of pS2 protein was purified from 6 l of the conditioned medium containing 6 μg of pS2 protein. The NH_2-terminal amino acid sequence of the pS2 protein obtained was as follows: Glu-Ala-Gln-Thr-Glu-Thr-Cys-Thr-Val-Ala-Pro-. This sequence is completely identical to the NH_2-terminal sequence of pS2 protein purified from MCF-7 cells [3].

To determine the entire structure of pS2 protein pro-

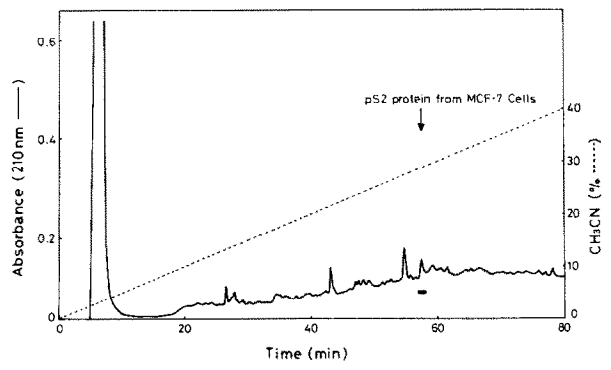

Fig.1. Final step of purification of pS2 protein from serum-free medium conditioned by MKN-45 cells. The pS2-containing fractions were separated by reverse-phase high-performance liquid chromatography on a μBondapak C_{18} column as described in section 2. The column eluate was monitored at 210 nm (solid line), and the broken line shows the percentages of acetonitrile in the elution medium. pS2 obtained is indicated by the black bar. The arrow denotes the elution position of pS2 protein purified from MCF-7 cells under the same chromatographic conditions.

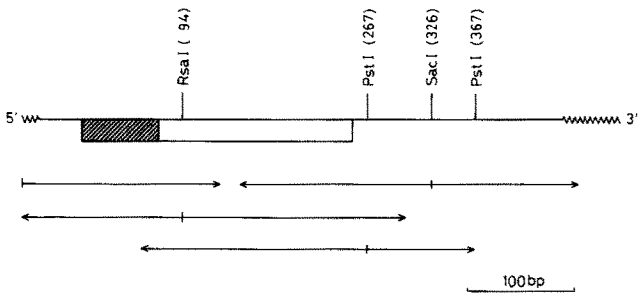

Fig.2. Restriction map and sequencing procedure of pS2GC. The open box and the hatched box denote the regions encoding mature pS2 protein and the signal peptide, respectively. Straight lines on both sides of these boxes indicate the 5′ and 3′ untranslated nucleotide sequences. 5′ and 3′ wavy lines are the poly(dG)-poly(dC) tail and the poly(dA)-poly(dT) tract, respectively. The relevant restriction sites are shown together with nucleotide numbers derived from fig.3 in the parentheses. The length and direction of the sequenced restriction fragments are illustrated by the arrows.

duced in this cell line, cDNA clones were isolated from the MKN-45 cell cDNA library as described in section 2. Seven were obtained from approximately 1 × 10^5 ampicillin-resistant colonies as hybridization-positive ones. Thus, about 0.007% of the total mRNA molecules prepared from the cells is thought to encode the pS2 protein. The content is about 1/100 of that in MCF-7 cells, which is reported to be about 0.8% [14]. Of these, three clones that seemed to contain a full-length cDNA were selected and analyzed for their nucleotide sequences as schematized in fig.2. All these clones contained an identical cDNA insert of 490 bp, excluding poly(dA)-poly(dT) tract and the poly(dG)-poly(dC) tail, hereafter referred to as pS2GC.

As shown in fig.3, pS2GC has a single open reading frame of 252 nucleotides. The sequence of pS2GC is identical to that of pS2 cDNA previously isolated from MCF-7 cells [2] except for one nucleotide (indicated by * in fig.3). The nucleotide 289 (C) is reported to be G in the sequence of pS2 cDNA [2] but to be C in the sequence of the pS2 gene [15]. pS2GC is thought to represent a full-length cDNA because its translational product was detected at the same migration position corresponding to about 600 nucleotides as pS2 mRNA from MCF-7 cells (fig.4). The sequence of the first 11 amino acids from NH_2-terminus of pS2 protein purified from the medium corresponds to that of the deduced protein extending from the amino acid 25, indicating that the preceding hydrophobic region consisting of 24 amino acids is a signal peptide (underlined in fig.3) and that the gene product of 84 amino acids is secreted as a mature protein of 60. These results clearly show that MKN-45 cells express the pS2 gene and process the gene product in the same manner as MCF-7 cells.

Levels of pS2 mRNA in five human gastric cancer cell lines were examined by RNA blot hybridization

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                -40       -30       -20       -10
                  ATCCCTGACTCGGGGTCGCCTTTGGAGCAGAGAGGAGGCA

1       10        20        30        40        50        60
ATGGCCACCATGGAGAACAAGGTGATCTGCGCCCTGGTCCTGGTGTCCATGCTGGCCCTC
MetAlaThrMetGluAsnLysValIleCysAlaLeuValLeuValSerMetLeuAlaLeu
  1                          10                          20

        70        80        90        100       110       120
GGCACCCTGGCCGAGGCCCAGACAGAGACGTGTACAGTGGCCCCCCGTGAAAGACAGAAT
GlyThrLeuAlaGluAlaGlnThrGluThrCysThrValAlaProArgGluArgGlnAsn
                           30                          40

       130       140       150       160       170       180
TGTGGTTTTCCTGGTGTCACGCCCTCCCAGTGTGCAAATAAGGGCTGCTGTTTCGACGAC
CysGlyPheProGlyValThrProSerGlnCysAlaAsnLysGlyCysCysPheAspAsp
                           50                          60

       190       200       210       220       230       240
ACCGTTCGTGGGGTCCCCTGGTGCTTCTATCCTAATACCATCGACGTCCCTCCAGAAGAG
ThrValArgGlyValProTrpCysPheTyrProAsnThrIleAspValProProGluGlu
                           70                          80

       250       260       270       280       290       300
GAGTGTGAATTTTAGACACTTCTGCAGGGATCTGCCTGCATCCTGACGCGGTGCCGTCCC
GluCysGluPhe                                   *

       310       320       330       340       350       360
CAGCACGGTGATTAGTCCCAGAGCTCGGCTGCCACCTCCACCGGACACCTCAGACACGCT

       370       380       390       400       410       420
TCTGCAGCTGTGCCTCGGCTCACAACACAGATTGACTGCTCTGACTTTGACTACTCAAAA

       430       440       450
TTGGCCTAAAAATTAAAAGAGATCGATATT
```

Fig.3. Nucleotide sequence of pS2GC and the deduced amino acid sequence. Nucleotides are numbered in the 5′- to 3′-direction, beginning with the first adenine of the initiating methionine and ending with the last nucleotide just before the poly(dA) tract. Nucleotides in the 5′ untranslated region have negative numbers. The deduced amino acid sequence is shown below the nucleotide sequence with numbering starting from the initiating methionine. The region of the signal peptide is underlined. One nucleotide, 289, different from the sequence of pS2 cDNA previously isolated from MCF-7 cells [10] is indicated by *.

analysis. As shown in fig.4A, a single band of RNA was detected at the same migration position as that from MCF-7 cells (lane 1) in two cell lines (MKN-45 and KATO-III) derived from poorly differentiated adenocarcinoma (lanes 2 and 3, respectively). The amount of pS2 mRNA in MKN-45 cells was much less than that in MCF-7 cells cultured in the presence of estrogen as described above. As shown in fig.4B, the two cell lines synthesized and secreted pS2 protein into the conditioned medium with comparable levels. At present, discrepancies on the mRNA levels and pS2 protein between MKN-45 and KATO-III cells are unclear. No bands were detected in another cll line (MKN-74) derived from well-differentiated adenocarcinoma (lane 4), although the cells did synthesize and secrete pS2 protein. This is probably due to the presence of a level of pS2 mRNA in the cells lower than that detectable in the assay because the amount of pS2 protein secreted by the cells was 1/10 of that by MKN-45 or KATO-III cells. Thus, the levels of pS2 mRNA in five human gastric cancer cells correlate well with those of pS2 protein secreted by these cells. These results seem to indicate correlation between expression of pS2 gene and malignant level of gastric cancer cells.

4. DISCUSSION

The five human gastric cancer cell lines used in the present study were established from metastatic carcinoma of patients with various types of gastric cancer [16,17]. Microscopically, MKN-1, MKN-28 and MKN-74 cells were classified in the category of well differentiated adenocarcinomas whereas MKN-45 and KATO-III cells were considered to be poorly differentiated ones. Production of pS2 protein by these cells appeared to depend on the state of differentiation of the cells in the case of cells originated from adenocarcinomas because poorly differentiated cells synthesized and secreted this polypeptide but well differentiated ones did not and because the level of pS2 protein in the medium was reduced significantly by treatment of MKN-45 cells with retinoic acid, which is known to induce differentiation of some tumor cells [18–20].

In human breast cancer cells (MCF-7), expression of

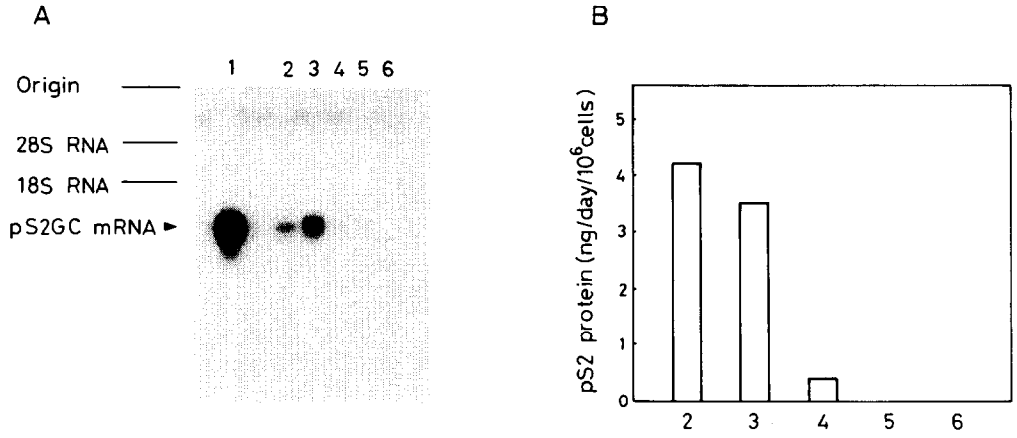

Fig.4. Levels of pS2 mRNA and pS2 protein produced by five human gastric cancer cells. (A) RNA blot hybridization analysis for pS2 mRNA was carried out on poly(A)$^+$ RNAs separated from MCF-7 cells cultured in the presence of estrogen (10^{-8} M) (1), MKN-45 (2), KATO-III (3), MKN-74 (4), MKN-28 (5), and MKN-1 cells (6) as described in section 2. Human rRNA was used as a molecular size marker. (B) The amount of pS2 protein secreted into the medium by five human gastric cancer cells was determined as described in section 2 and expressed as ng per day per 1×10^6 cells. Numbers on the abscissa correspond to the lane numbers in (A).

the pS2 gene is under strict control of estrogen at the transcriptional level [4]. Recent extensive investigation in human tissue specimens indicates that expression of the pS2 gene could be classified into two categories depending on tissue type. One is estrogen-dependent, as in MCF-7 cells; and the other is estrogen-independent. Of 180 breast cancer specimens, 129 (72%) are estrogen receptor-positive; and 87 (67%) of these are pS2 mRNA and/or pS2 protein-negative, whereas only 2 (4%) of 51 estrogen receptor-negative specimens are pS2 mRNA and/or pS2 protein-positive [7]. From those findings, expression of the pS2 gene was concluded to be limited to a subclass of human breast cancer cells containing estrogen receptor as represented by MCF-7 cells.

As to the second category of expression of the pS2 gene, normal stomach mucosa is believed to synthesize pS2 mRNA and secrete pS2 protein [7], both of which seem identical to those in MCF-7 cells [8]. The level of pS2 mRNA in the stomach was comparable to that in MCF-7 cells, but expression of the pS2 gene in the stomach cells was not under the control of estrogen because estrogen receptors were not detected in the stomach and because there was no significant difference in the amount of pS2 mRNA and pS2 protein between males and females.

MKN-45 cells do not respond to estrogen with regard to cell growth and induction of pS2 protein synthesis (data not shown). Thus, MKN-45 cells are thought to represent the stomach-type expression of the gene, which is not regulated by estrogen. As regards the unresponsiveness of pS2 gene to estrogen in MKN-45 cells, further research is warranted.

The results obtained showed that the pS2 gene is expressed in gastric cancer cell lines as in MCF-7 cells and implicated the association with the malignant level of the cells.

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