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ARTICLE

Effective Production of Carcinoembryonic Antigen by Conversion of the Membrane-bound Into a Recombinant Secretory Protein by Site-specific Mutagenesis

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Carcinoembryonic antigen (CEA), the most widely used human tumor marker, is a heavily glycosylated protein over-expressed by a wide range of tumors. It has been indicated that CEA might be a useful target for human anti-tumor immunotherapy. CEA assay for research as well as clinical trials demands a continuous source of CEA protein preparations. In a multi-purpose research program to provide a reliable source for large production of CEA, we converted the membrane-bound carcinoembryonic antigen into a secretory protein by site-specific mutagenesis. We made the secretory CEA protein by introducing a new stop codon at 99 bp upstream of the original stop codon in CEA cDNA by PCR-based mutagenesis. The glycosylation of recombinant CEA proteins, especially those destined for administration to human trials is crucially important. To produce CEA with the same glycosylation pattern and immunogenicity as the native CEA expressed by human tumors *in vivo*, the truncated CEA cDNA which does not encode the last C-terminal 33-amino acid hydrophobic domain was transfected into HT29, a human colon carcinoma cell line by the calcium phosphate method. Stable transfectants were selected and pooled. CEA secretion from the cells was verified by analysis of the transfectant culture supernatant for CEA protein. As determined by ELISA, 16 μg/L of recombinant CEA was secreted per 10^6 transfectants within 48 hrs, an increase over 40 times relative to the untransfected cells. The size of the recombinant CEA secreted by HT29 transfectants in our experiment is identical to that of reference CEA secreted from tumors and is fully antigenic. It seems that the C-terminal truncation does not affect CEA glycosylation in HT29 cells. It is predicted that human cancer immunotherapy using recombinant CEA expressed in this system would be more effective than the commercial protein which is usually prepared from bacterial or other heterologous expression systems. (Pathology Oncology Research Vol 11, No 4, 211–217)

Key words: CEA, GPI, site-specific mutagenesis, immunotherapy

Introduction

Carcinoembryonic antigen (CEA) is a human tumor marker with a wide clinical use especially for detection of recurrent or metastatic colorectal malignancy. Elevated blood CEA levels are found in many cancers including lung, colorectal, pancreas, breast, and gynecological neoplasms.[22] The physiologic role of CEA and its relevance to malignant transformation has not been elucidated yet.

Elevated blood CEA levels are related to the extent of the disease as well as to the stage and degree of tumor differentiation.[26] CEA might also play an instrumental role in cancer progression.[11] Both experimental and clinical data indicate evidence of a correlation between elevated blood CEA levels and the development of liver metastasis. The latter function has been suggested to be facilitated by soluble CEA through induction of cytokine production by Kupffer cells.[12]

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At the molecular level CEA is a 180 kD, heavily glycosylated cell surface protein with more than 50% carbohydrate content by mass. CEA consists of one N-terminal IgV-like domain and three sets of Ig-C like domains. CEA is anchored to the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor.[9] The C-terminal hydrophobic domain that is missing in the mature membrane-bound CEA plays a key role in the targeting and attachment of CEA to the cell surface. GPI anchor synthesis and its attachment to proteins, a process that includes the removal of the C-terminal GPI signal sequence from the proteins and its replacement by a pre-assembled anchor, occurs in the endoplasmic reticulum.[29]

Several immunotherapy trials against cancer using CEA as a target have been conducted in recent years.[15,24,30] Different approaches have been developed to induce anti-CEA immune response, including vaccination using immunogens containing the recombinant proteins, or tumor cell lysates containing the entire CEA molecule (for review see ref. 2 and 25). Immunization protocols using the whole CEA protein as the recombinant antigen have the advantage, over methods relying on a discrete number of epitopes, that they ensure presentation of a large repertoire of naturally processed epitopes, circumventing the MHC restriction imposed when using a discrete number of peptide epitopes. Moreover, antigen-presenting cells might be able to stimulate lymphocytes specific for all the potentially available epitopes encompassed by the entire sequence of CEA.

The widespread use of CEA as a tumor marker and as a targeted molecule for clinical applications in cancer imaging and therapy has increased the demand for its protein preparations.[4,21,32] Various methods have been applied for CEA protein isolation from serum of cancer patients and tumor tissues, including perchloric acid extractions,[5,10] heat inactivation,[31] detachment of cell surface-bound CEA by phosphatidylinositol-phospholipase C enzyme followed by purification by immunoadsorption and gel filtration to remove impurities.[7,17,20] In addition to complexity, these methods usually suffer from low recovery and heterogeneity of the purified proteins. It has also been shown that perchloric acid modifies the carbohydrate moiety of CEA and therefore its antigenic properties.[14] Enzyme extraction has the disadvantage of digestion of other GPI-proteins together with contamination with the enzyme itself; therefore, another step of purification would be necessary.

Recombinant DNA technology has provided an alternative effective and economic way for synthesizing important molecules. Therefore, we decided to apply this technique for CEA protein production. Our results showed that the recombinant secretory CEA (rsCEA) expression in a human colon carcinoma cell line is a highly effective way of large-scale production of fully glycosylated CEA that could be purified from culture medium by simple procedures.

Materials and Methods

Materials

DNA purification kit was obtained from Bio-Rad (USA); Geneticin (G418) was from Gibco Life Science (UK); E. coli DH10β from Invitrogen (USA); phenol was purchased from Fluka Chemical Company (Switzerland); L-glutamine, and Dulbecco's Modified Eagle's Medium (DMEM) from Sigma Co. (St. Louis, USA). Agarose MP and dNTPs were from Roche Diagnostics (Mannheim, Germany). Penicillin, streptomycin, mineral oil, Pfu DNA polymerase, T4 DNA ligase, protein and DNA size markers were supplied and oligonucleotide primers synthesized by MBI Fermentas (Lithuania). Fetal bovine serum (FBS) was from Biochrom (Berlin, Germany). The CEA ELISA kit was from CanAg Diagnostics AB (Gothenburg, Sweden). HT29 human colon carcinoma cell line was obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran). Tissue culture plates and flasks were purchased from Nunc (Denmark). All other chemicals were obtained from Merck (Germany).

Construction of recombinant CEA cDNA clone lacking C-terminal domain

A cDNA encoding full-length human CEA was previously synthesized in our lab by performing RT-PCR on total RNA extracted from HT29/219 tumor cell line using CEA-specific primers. CEA cDNA, cloned in the unique EcoRI site of pBluescript SK plasmid (PSK), was subjected to PCR-based mutagenesis. We used Pfu DNA polymerase instead of Taq polymerase for higher fidelity of polymerization. 30 cycles of PCR reactions were performed according to the instructions provided by the enzyme supplier.

Two sets of primers (*Table 1*) were used to introduce a stop codon at position 2104 A/T (K670/stop) in CEA cDNA in 3 steps described in the following paragraph and also summarized in *Figure 1*.

1. Amplification of a 464-bp CEA fragment (fragment 1) using CEA S.1/CEA A.S.2 primers.

2. Amplification of a 996-bp CEA fragment (fragment 2) using CEA S.2/PSK A.S. primers.

3. After electrophoresis, fragments 1 and 2 were purified from 1.5% agarose gel. We ligated these two overlapping fragments to get fragment 3. For this purpose 30 ng of each of fragments 1 and 2 were mixed in another tube and used as a template for the third round of PCR reaction using CEA S.1/PSK A.S., external primers (see *Figure 1*).

4. The third PCR product (fragment 3, 1445 bp) and wild type CEA in PSK were double digested with two restriction enzymes of Eco 81 I and Xba I. The digestion products were gel-purified as before.

5. The 1424-bp fragment between the unique Eco81 I and Xba I sites of wild-type CEA in PSK was replaced by

Table 1. **PCR primersused for mutagenesis**

Primers	*Position*	*Sequence*
CEA S.1*	1657-1676	**5′** TTCACCTGTGAACCTGAGGC **3′**
		Eco 81 I
CEA A.S.2*	2121-2095	**5′** AGAGACTGTGATGCTCTAGACTATGGA **3′**
CEA S.2*	2086-2109	**5′** CGCAATAATTCCATAGTCTAG AGC **3′**
PSK A.S.**	672-695	**5′** GCCGCTCTAGAACTAGTGGATCCC **3′**
		Xba I

The arrowheads point to the unique restriction sites that have been used to digest and replace a 1396-bp fragment of PSK-CEA by the related mutant PCR fragment to make the truncated CEA construct. The underline shows overlapped bases between CEA A.S.2 and CEA S.2 primers, and bold letters show the mutation introduced in primers for making the stop codon mutation in CEA cDNA.

*Position of the primer in CEA cDNA,
**Position of the primer in PSK vector DNA

the corresponding mutant PCR fragment to make PSK-rsCEA construct. After transformation of ligation product into the competent E. coli DH10β and selection on ampicillin plate, PSK-rsCEA was purified from bacteria.

6. PSK-rsCEA was digested with EcoRI enzyme and recombinant secretory CEA cDNA was gel-purified and inserted into EcoRI site of the P91023B (Kauf) expression vector for expression in a eukaryotic cell line.

Cell culture and transfection

The human colon carcinoma cell line HT29 was grown as monolayer cultures in 3×10 cm tissue culture dishes containing DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2. At sub-confluence cells were co-transfected by calcium phosphate co-precipitation with 10 mg of P91023B containing rsCEA cDNAs and 1 mg pSV2neo plasmid.[1] Stable transfectants were selected by incubating cells in Geneticin (G-418 sulfate) (400 μg/ml) for 3 weeks. G-418-resistant colonies were pooled and enriched for stable transfectants.

Measurement of CEA released from cells

The stable transfected cells and untransfected controls were seeded as an aliquot of 10^6 cells in 6-cm cell culture dishes. The cells were grown as monolayers until confluence in 3 ml of serum-containing medium as described above. Culture media were aspirated every 48 hrs and centrifuged first at 3,000 ×g for 5 minutes to remove any floating cells and then the supernatant was transferred to a new tube and centrifuged for another 20 min at 4º C and 10,000 g to remove membrane vesicles and debris that might have been shed from the cells. Media were stored at –70º C until assayed. We determined levels of CEA release into media by an ELISA kit according to the manufacturer's instructions. At the time of confluence, the cells were counted and the amount of CEA released by cells was normalized to 10^6 cells.

PAGE analysis of secreted CEA

Samples of 40 μl of cell culture supernatants from control untransfected cells and those secreting recombinant CEA were run on a 7.5% SDS-PAGE gel using Laemmli discontinuous buffer system.[18] After electrophoresis, the proteins were detected by silver staining of the gel.

Results

In order to delete the C-terminal GPI-encoding domain (the last 33 amino acids) to obtain an actively secreted form of CEA, we introduced a new stop codon 99 base pairs upstream of the original stop codon in CEA cDNA. The construction of the recombinant CEA (mCEA) is summarized in three steps in *Figure 1*. For this purpose two sets of primers (*Table 1*) were designed to make two overlapping mutant fragments. Primers CEA S.2 and CEA A.S.2 have a mismatch to the wild-type CEA at position 2104. Therefore, after PCR using these primers, the amplified fragments would contain the specified stop-encoding mutation. PCR amplification using the primer sets CEA S.1/CEA A.S.2 and CEA S.2/PSK A.S gave rise to the expected 464- and 996-bp fragments 1 and 2, respectively (*Figures. 1,2a*). To ligate overlapping fragments 1 and 2, these two fragments were mixed after gel purification and used for the 3rd round of PCR reaction using the external primers CEA S.1 and PSK A.S for

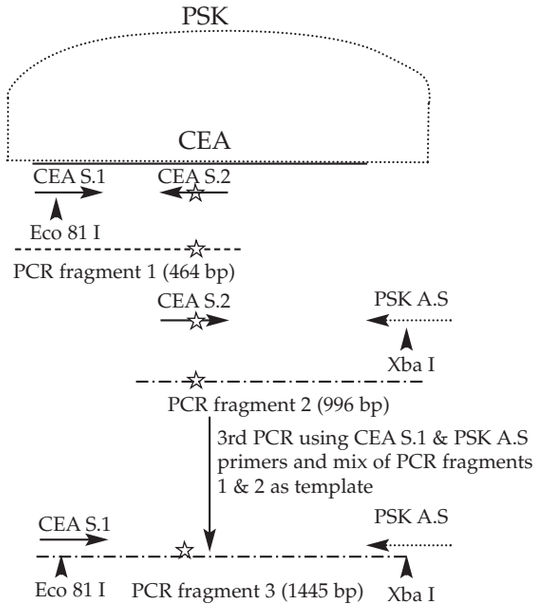

Figure1. *Schematic representation of PCR-based mutagenesis of CEA and the relative positions of primers used for amplification in PSK-CEA. The PCR fragment generated by each set of primers was shown by dashed line. Vertical arrowheads show the unique restriction sites used to replace the mutant fragment 3 with the corresponding native fragment in CEA. The star sign points to the stop codon-generating mutation introduced into primers CEA S.2 and CEA A.S.2, and in fragment 3.*

amplification. The expected PCR band of 1445 bp (*Figure 2b*) was gel-purified. This fragment which had the desired stop codon-generating mutation at the position corresponding to position 2104 of CEA cDNA, was double-digested by Eco81 I and Xba I enzymes, and gel-purified. To make the mutant CEA construct (rsCEA), PSK-CEA was also double-digested by the same restriction enzymes (Eco81 I/Xba I). The wild-type CEA fragment was replaced by mutant fragment by ligating double-digested, mutation-containing PCR fragment 3 to the double digest of PSK-CEA, using T4 ligase.

The resulting construct (rsCEA) was subcloned into EcoR1 site of the eukaryotic expression vector P91023B, and the clone with the right orientation was used for expression and transfection into a eukaryotic cell line.

The CEA molecule is heavily glycosylated, and it was shown that there are considerable differences in the glycosylation pattern in CEA preparations of various origins.[6] The glycosylation of CEA could have profound effect on its biological activity and antigenicity. We speculated that the glycosylation repertoire and immunogenicity of CEA expressed in a tumor cell line should be more like the CEA expressed by tumors *in vivo*. Therefore, to prepare rsCEA as a target to induce anti-tumor immune responses in our future work, we chose HT29, a human colon carcinoma cell line to express the recombinant cDNA. P91023B-rsCEA was transfected into HT29. Eighteen geneticin-resistant colonies were pooled and their culture supernatant was assayed for CEA secretion by ELISA. ELISA showed that HT29 transfectants secreted 16 μg of CEA protein per 10^6 cells /72 hrs at sub-confluent stage. Untransfected HT29 cells also released CEA into culture medium spontaneously. However, under the same conditions, the control untransfected cells shed about ~0.4 μg CEA/10^6/72 hrs. Thus, the transfection of HT29 with rsCEA resulted in a 40-fold higher level of CEA secretion compared to that of control cells. The time-dependent CEA release and the size of CEA secreted by the transfected and control cells into medium were analyzed by ELISA and SDS-PAGE, respectively (*Figure 3*). The spontaneous CEA release from controls was constant by time, while a higher CEA protein accumulation in media was observed for the transfectants (*Figure 3a*). The apparent molecular mass of the CEA glycoform secreted by the transfectants was identical to that of reference CEA purified from tumors as verified by SDS-PAGE (*Figure 3b*).

The HT29 transfectants cultured under serum-free conditions continue to release rsCEA into medium, although less than in serum-containing media (data not shown). There is no contamination with serum proteins when rsCEA is collected from serum-free culture medium. This makes the purification procedure much easier.

The above described method provides a system which could be easily scaled up to produce the protein in essentially unlimited amounts and reproducible quality for clinical and research purposes.

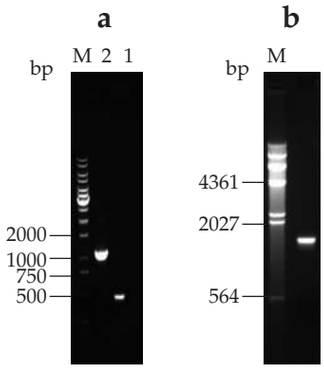

Figure 2. *PCR products of amplified PSK-CEA, using mutation-introducing primers.* ***(a)*** *Lane 1: amplified fragment 1 (464 bp) between nucleotides 1657-2121 in CEA cDNA using the primer set CEA S.1/CEA A.S.2. Lane 2: amplified fragment 2 (996 bp) using the primer set CEA S.2/PSK A.S* ***(see Figure 1). (b)*** *The PCR product of the 3rd PCR (1445-bp fragment 3) was generated using primers CEA S.1 and PSK A.S. This fragment has a new stop codon corresponding to position 2104 of CEA cDNA.*

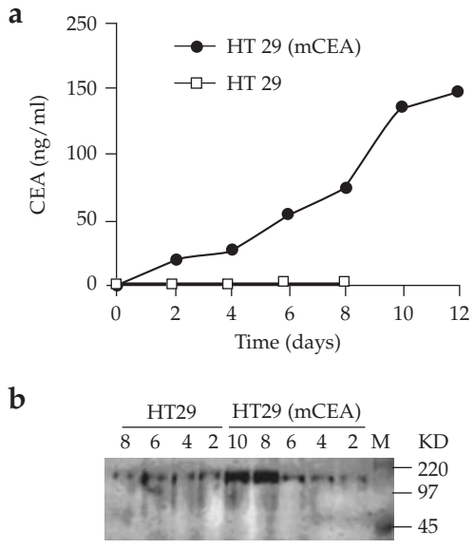

Figure 3. (a) *The time course of CEA release from transfected [HT29 (mCEA)] and untransfected (control) human colon carcinoma HT29 cells into culture medium. Culture media were collected and their CEA levels measured every 48 hrs.* ***(b)*** *PAGE analysis of CEA from transfected and untransfected cells. Cell culture supernatants were run on 7.5% polyacrylamide gel and stained by silver stain. The number on the top of each lane corresponds to the time (day) of cell culture supernatant collection. Recombinant mCEA protein has the same apparent Mw as the native one secreted from control HT29 cells. M: Size marker protein*

Discussion

All GPI-anchored proteins are synthesized with a C-terminal signal sequence, which is replaced by a GPI-anchor in a post-translational modification inside the cell. Based on this information we made a recombinant CEA cDNA which lacks the 3´ region encoding the last 33 amino acids, GPI-encoding signal. We applied PCR-based site-specific mutagenesis to truncate CEA protein by introducing a new stop codon 99 nucleotides upstream of the original stop codon in CEA cDNA. The target area of the template gene was amplified into two separate overlapping PCR fragments using two pairs of mutagenic primers. Then these two fragments were ligated as a single 1445-bp mutant fragment by a third round of PCR reaction. We have shown that this new stop codon is sufficient alone to convert the membrane-bound CEA into a fully secretory protein.

CEA is a glycoprotein with more than 50% carbohydrate content by mass. It was shown that the CEA expressed in bacteria had a lower antigenicity, presumably due to incomplete glycosylation or folding.[16] In contrast, the recombinant CEA in our experiment had the same apparent molecular weight as the native form secreted from human colon carcinoma cells. Therefore, it seems that the C-terminal truncation did not affect CEA glycosylation in the tumor cell line.

Complete or partial deletion of the C-terminal hydrophobic domain of GPI-proteins could result either in secretion, or retention and lysosomal degradation inside the cell.[3,28] However, lack of the C-terminal hydrophobic domain of CEA in our experiment did not affect the transport of CEA to the cell surface, but only prevented its anchoring to the plasma membrane. These results are consistent with those obtained by Terskikh et al.,[27] and also with reports on other GPI-anchored proteins.[28] While the amount of CEA released in the culture medium by untransfected cells was constant by the time, an accumulation of CEA was observed in the medium of transfected cells. This observation could be explained by the higher rate of CEA protein synthesis and release by the transfected cells than its degradation in the medium.

Various methods have been used for CEA preparation and purification, including perchloric acid extraction and gel filtration. There are some disadvantages of using these methods (see the Introduction). The yield of recombinant CEA in our experiment was 4 times more than that obtained by the method using PI-PLC enzyme to release CEA from tumor cells.[20]

Although the recombinant technology has been previously used for CEA production, the construct that we made and introduced in this study is a genuine novel construct, and it seems more efficient than the one made by Terskikh et al.[27] They used a more complicated method than the strategy we applied. To delete the C-terminal hydrophobic tail of CEA, they performed several restriction digestion and ligation steps to cut a relatively long stretch (78 bp) from the 3'-end of CEA cDNA.[27] The yield of recombinant CEA in our experiment was 4 times more than that obtained by Terskikh et al. This difference might be related to the construct itself, the efficiency of transfection, or different expression vector and cell line used for CEA expression.

CEA protein preparations are required as standard and reference antigen for CEA assays or antibody preparations for immunotherapy and gene therapy trials, as well as for other research purposes. CEA has been implicated in the process of human colon cancer liver metastasis. Injected intravenously into athymic nude mice, it has been shown to enhance experimental metastasis in the liver by human colorectal cancer cells.[12,13] The 3´-truncated CEA has been used for investigation of a cause-effect relationship between soluble circulating CEA and liver metastasis.[19] Our stable transfectants of HT29, which constitutively secrete higher amount of CEA than untransfected cells, would also provide another experimental model to evaluate the possible role of secretory CEA in the facilitation of liver metastasis in nude mice.

CEA has proved to be a suitable target antigen for cancer immunotherapy. Several anti-CEA immunization

strategies have been tested in *in vivo* tumor models.[2] Some of these strategies have been carried out to induce anti-CEA antibodies, whereas others can induce humoral and/or cellular immune responses.

It has been reported that the expression of CEA in normal and tumor cells differ in the pattern of glycosylation.[6] Recombinant CEA prepared from bacteria, insects,[30] or even from heterologous mammalian cells[8,27] had a lower mass and less glycosylation than CEA expressed by human tumors.[16,30] The sugar composition of many glycoproteins directly modifies their immune recognition.[23] Our approach to produce the same CEA glycoform and immunogenicity as the native CEA expressed by human tumors *in vivo* was to express the protein in human colon carcinoma cells.

A simple extraction procedure of secreted rCEA in serum-free culture medium greatly reduces contamination by other proteins and simplifies or eliminates further purification, depending upon the aim and purpose of CEA application. Because the recombinant CEA production in tumor cell line both quantitatively and qualitatively is advantageous and its extraction-purification is less time-consuming, it offers an excellent alternative to other methods.

To use the above-mentioned recombinant CEA protein in an immunotherapeutic trial, the immunogenicity and biological properties of this recombinant CEA protein is currently under investigation.

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