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SEREX analysis of gastric cancer antigens

Abstract Stomach cancer is the major malignancy in Japan and one of the most common cancers worldwide. To establish the basis for an immunotherapeutic approach to stomach cancer, we have initiated an analysis of stomach cancer antigens recognized by human immunoglobulin G (IgG) antibodies using SEREX, a powerful expression cloning method developed by Dr. M. Pfreundschuh's group. Five stomach cancer cDNA libraries have been screened with autologous patient sera: one moderately differentiated adenocarcinoma; two poorly differentiated adenocarcinomas; and two scirrhous-type poorly differentiated adenocarcinomas of Borrmann type 4, the most devastating form of stomach cancer. Based on the reactivities of clones with autologous IgG antibodies, an average of 50 independent clones from each library and a total of 297 clones were isolated. DNA sequencing revealed that these 297 clones were derived from 136 different genes. Comparison of the 136 genes to sequences in DNA databases showed that 95 are previously identified genes and 41 are newly identified in this study. The antigens are derived from various genes including a chimeric gene between E-cadherin and an unknown gene Y, AKT oncogene, genes overexpressed in stomach cancers, genes of which the transcripts are alternatively or aberrantly spliced, and genes known to be involved in autoimmune diseases. Thus stomach cancer patients can generate an immune response against a surprisingly diverse set of gene products. To identify antigens potentially useful in the diagnosis and therapy of gastric cancer, all 136 genes were tested for their reactivities with a panel of sera from 44 gastric cancer patients (17 women and 27 men, aged 35–81 years) and with a panel of sera from 100 control individuals with no previous history of cancer but some of whom had gastritis (55 women and 45 men, aged 30–69 years). Eleven antigens showed reactivity only with a certain proportion of cancer patient sera but not with any control sera. An additional 12 antigens elicited antibody production at a much higher frequency in cancer patients than in control individuals. To evaluate the clinical usefulness of these antigens we are now examining their expression in normal and malignant tissues.

Key words Cancer antigen · Gastric cancer · Autologous serum · Expression cloning · SEREX

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Introduction

Until recently, human cancer antigens have remained elusive and there were even doubts about their existence.

Molecular identification of a human melanoma antigen, MAGE-1, recognized by cytotoxic T lymphocytes (CTLs) by T. Boon and his colleagues in 1991 [30] ended the long-standing debate on the existence of human cancer antigens and began a new era of cancer immunology. Since then, several human cancer antigens recognized by CTLs derived from cancer patients have been identified using molecular and biochemical approaches [6, 11, 12, 22, 31]. MAGE-1 and its related antigens are expressed in a proportion of various cancers and normally only in testis, ovary, and placenta [30]. These are now termed cancer-testis (CT) antigens. Differentiation antigens that are expressed in cancer and in normal tissues from which cancer arises have been identified as cancer antigens by CTLs. Tyrosinase, gp100, and MART-1/Melan-A in melanoma are prime examples of such antigens [12]. A few antigens derived from mutated gene products such as CDK4 mutant and β-catenin mutant have also been identified [16, 32]. Clinical trials targeting CT and differentiation antigens have already commenced [14, 17].

SEREX

The cancer antigens defined by CTLs so far are clearly too few to allow immunotherapy for the diverse types of human cancer. For the identification of cancer antigens by CTLs, both CTLs and the target cancer cells must be established in vitro. However, establishing cultured cell lines from cancer tissues is very difficult. Cell lines can be established with any regularity only from melanoma, brain, and renal cancers. For this technical reason, the majority of cancer antigens defined so far by CTL assay have been from melanoma. To overcome this limitation, a new serological method for the identification of cancer antigens called SEREX was developed by the group of Michael Pfreundschuh at the University of Saarland, Germany, in 1995 [19].

The method combines a molecular cloning procedure with autologous typing of cancer cells with patient serum [15]. A cDNA expression library is constructed using mRNA isolated directly from cancer tissue. This library is screened with autologous serum, and clones producing recombinant proteins reactive with IgG antibodies in the patient's serum are isolated. Identification of antigens is done by sequencing cDNA inserts. As SEREX does not require cultured cancer cell lines, it allows investigation of cancer antigens in any type of cancer, provided that cancer tissue and serum from the same patient are available. The initial SEREX study identified various cancer antigens including melanoma antigens originally defined by CTL assay such as MAGE-1 and tyrosinase [19].

In theory, antigens defined by SEREX do not necessarily elicit a CTL response. Subsequent study, however, showed that one SEREX-defined CT antigen, NY-ESO-1 [5], induced CTL responses in cancer patients who were shown to produce high titered antibodies against this antigen [10]. The study demonstrated that at least a certain proportion of SEREX-defined antigens can be targets of CTLs. SEREX has now been applied to various cancers including melanoma, esophageal, breast, gastric, colon, lung, prostate, renal, and brain cancer and leukemias and lymphomas [1–5, 9, 13, 18–21, 24, 26–29, 33]. Over 1000 antigens have been identified by SEREX [4, 18, 27]. Of these, approximately 30% are unknown gene products. Many known gene products are recognized by the immune system of cancer patients, including nuclear transcription factors, metabolic enzymes, cytoskeletal proteins, stress proteins, and cell surface receptors.

Categories of SEREX-defined cancer antigens

The most interesting antigens are those showing cancer-restricted expression or cancer-restricted immunogenicity [4, 18, 27]. SEREX-defined antigens probably relevant to the etiology, diagnosis, and therapy of cancer can be classified into seven categories, as shown in Table 1. SEREX has dramatically increased the number of cancer antigens belonging to the following categories: 1) CT antigens; 2) differentiation antigens; 3) antigens derived from mutated gene products; 4) fused products that result from chromosomal translocation, known to occur in certain hematologic and soft tissue malignancies; 5) products of amplified or overexpressed genes assumed to be involved in the development of cancer; 6) products of spliced variants; and 7) products of retroviral origin. CT antigens are expressed by some tumors and normally only by testis, ovary, and placenta. This very restricted expression pattern makes CT antigens attractive targets for cancer vaccines. More than 10 CT antigens have been identified and their clinical value is now being extensively evaluated.

Recently the outcome of vaccination of melanoma patients with the CT antigen MAGE-3 has been reported [14]. Differentiation antigens can also serve as vaccine targets. Trials of vaccine against melanoma differentiation antigens such as tyrosinase and gp100 have been conducted in melanoma patients [17]. Vacci-

Table 1 Categories of SEREX-defined human cancer antigens

Antigen category	Example	Initially isolated in
Cancer-testis	NY-ESO-1	Esophageal cancer
	SSX-2	Melanoma
	CT7	Melanoma
Differentiation	Tyrosinase	Melanoma
	Galectin-4	Colon cancer
Mutational	P53	Colon cancer
Fusion	E-Cad/unknown gene Y	Gastric cancer
Amplified/ overexpressed	AKT	Gastric cancer
	Carbonic anhydrase XII	Renal cancer
	KOC3	Melanoma
Splice-variant	Restin	Hodgkin disease
	NY-CO-37/38	Colon cancer
Retroviral	HERV-K10	Renal cancer

nation with antigens of this category, however, requires caution because of possible immunoreactivity with normal tissue. Antigens resulting from mutations, translocations, or splicing abnormalities are ideal targets because most are cancer-specific events and may play essential roles in the development and maintenance of cancer. The frequency of these events in cancer is a decisive factor in determining their clinical value. Antigens of overexpressed products or of amplified genes can also be good targets if the level of expression in cancer is significantly greater than in normal tissue. Products of endogenous retroviruses aberrantly expressed in cancer are immunogenic in cancer patients, but the pattern of the expression in cancer and normal tissues is still unclear at this moment. SEREX is still at a very early stage of development, and only a few antigens have gone through the vigorous procedures to evaluate eligibility as targets for cancer vaccines. Only one antigen, NY-ESO-1, has entered clinical trial. It is likely that only a small proportion of cancer antigens has been identified, leaving the majority for future discovery.

The SEREX database

An international SEREX collaborative group was established in 1996 by the Ludwig Institute for Cancer Research, involving investigators at the University of Saarland, Ludwig Institute branches in New York, Melbourne, and London (University College), the Aichi Cancer Center (Nagoya, Japan), Krankenhaus Nordwest (Frankfurt, Germany), and Moscow State University (Russia). A SEREX database (http://www-ludwig.unil.ch/SEREX.html) has been organized and made available to the public.

Background to the present SEREX study

Gastric cancer is the major malignancy in Japan and one of most common cancers worldwide. Due to its insensitivity to chemo- and radiation therapy, the only treatment effective for gastric cancer is surgery, and the development of adjunct therapy is desperately needed. Furthermore, despite enormous efforts, conclusive causative genetic abnormalities in gastric cancer have not been found. To establish the basis for an immunotherapeutic approach to gastric cancer and to use immunological recognition as a way to gain insight into the genetic events involved in malignant transformation, we have initiated an analysis of gastric cancer antigens using SEREX.

Patients and methods

Patients

This study was approved by the institutional review boards of the Aichi Cancer Center and Aichi Prefectural Hospital. Tumor specimens and blood 20 mL were obtained at surgery from 44 gastric cancer patients (17 women and 27 men, mean age 61.0 years [range 35–81 years]) who agreed to participate in this study. Of the 44 patients, 16 had stage I, five stage II, 13 stage III, and 10 stage IV disease. Blood 5 mL from 100 individuals with no previous history of cancer was also obtained after giving written informed consent. This control group consisted of 55 women and 45 men, ranging in age from 30 to 69 years (mean 53.3 years). Forty-five had a previous history of gastritis, nine of polyp, nine of gastric ulcer, and seven of duodenal ulcer.

SEREX method

Messenger RNA directly isolated from tumor was used for construction of a cDNA library in the λ-ZAP Express phage vector [19]. Autologous serum diluted to 1:100 was used for screening of the library. Clones that produced recombinant proteins reactive to antibodies in serum were identified by horseradish peroxidase-conjugated goat anti-human IgG and visualized with the avidin-biotin complex (ABC) method. Isolated phage clones were converted to phagemids and subjected to DNA sequencing using an ABI PRISM Model 377 automated DNA sequencer (Perkin Elmer, Norwalk, CT, USA) at the BioResource Center, Cornell University, Ithaca, NY, USA. DNA sequence data of each clone were analyzed for their similarity to previously identified genes in the Genbank/DDBJ/EMBL database by a Blast program (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD, USA), and to SEREX-defined genes in the SEREX Database (Ludwig Institute for Cancer Research, Epalinges, Switzerland). Sequence similarity to our own clones was analyzed by a DNASIS software program (Hitachi, Yokohama, Japan).

Many key procedures were developed for SEREX. Human sera normally contain abundant antibodies against bacterial products. Since the selection is based on the reaction of antibodies in patient serum to recombinant proteins expressed in *Escherichia coli*, antibacterial antibodies in the serum would become obstacles. Accordingly, the antibodies to bacterial proteins and λ phage-related proteins are extensively absorbed by tandem columns containing Sepharose 4B cross-linked to lysates of *E. coli* or of *E. coli* infected with λ phage. To exclude low-titer, naturally occurring IgM autoantibodies in human sera from the library screening, an antibody specific to human IgG is used as a second reagent. Restricting the screening to IgG class antibodies also ensures that at least helper T cells are involved in the recognition of detected antigen molecules. Tumors are often infiltrated by B lymphocytes that produce IgG cDNA clones in a library. Accordingly, prescreening of IgG-producing clones is included to remove them.

Results and discussion

SEREX analysis of five cases of gastric carcinoma derived from tumors of various histologic types and grades was carried out (Table 2). The screening of each cDNA library with autologous serum was continued until approximately 50 positive clones were isolated from each library. In total, 297 positive clones were obtained, representing 136 distinct genes, 95 of which were previously known (Table 3). Thus gastric cancer patients can generate an immune response against a surprisingly diverse set of gene products, such as secreted proteins, cell surface proteins, and nuclear transcription factors. Of the 136 gene products, 21 were identified in two or more gastric cancer libraries and 25 were identified in SEREX analysis of other tumor types. No CT antigens were isolated, consistent with the low

Table 2 Gastric cancer tumor samples analyzed by SEREX (*Mod diff* moderately differentiated, *scirr* scirrhous type)

Patient (sex)	Age (years)	Stage	Histology	Library size	Library screened	Ig clone frequency	No. positive clones	No. antigens
SM (M)	74	IIIa	Mod diff	2.5×10^6	3.5×10^5	1/250	55	27
CK (F)	65	IV	Poorly diff	1.1×16^6	4.5×10^5	1/300	37	18
SS (M)	61	IV	Poorly diff	1.7×10^6	1.5×10^5	1/600	56	28
KM (F)	45	IV	Poorly diff scirr	2.0×10^6	3.0×10^5	1/800	60	35
YS (M)	50	IV	Poorly diff scirr	1.5×10^6	3.0×10^5	1/35	89	40

Table 3 Gastric cancer antigens identified by SEREX

Patient*	Identified by SEREX		Novel genes	Novel but similar to non-human genes	Fusion genes	Known human genes	Autoimmune related
	In the same cancer type	In other cancer types					
SM	7	2	4	4	0	7	3
CK	2	4	3	1	1	7	0
SS	3	5	5	3	0	12	0
KM	6	5	10	2	1?	10	1
YS	3	9	8	1	0	18	0
Total	21	25	30	11	1 + 1?	54	4

* Patients are listed in the same order as in Table 2

frequency of CT gene expression in gastrointestinal cancer [4, 20].

Of the genes isolated, two showed possible etiological significance for gastric cancer. One was derived from a fusion gene product between E-cadherin (E-Cad) and a novel gene designated gene Y. E-Cad is an adhesion molecule involved in the regulation of various cellular functions, including normal differentiation and tumor invasion [7]. Mutations in E-Cad have been identified in gastric and other cancers, and inherited mutations have been related to familial gastric cancer [8, 25]. Nine independent cDNA clones encompassing the fusion gene were isolated and the combined sequencing data indicated that the fusion gene had E-Cad at the 5′ and an unknown gene Y at the 3′. Reverse-transcription polymerase chain reaction using a 5′ primer derived from E-Cad and a 3′ primer derived from gene Y showed that the amplification product was restricted to the tumor and was not detected in autologous nonneoplastic tissue or allogeneic normal or tumor tissues. This finding indicates a somatic translocation event involving E-Cad and gene Y, an event possibly contributing to the origin or progression of the cancer. Although this translocational event was not found in five other cases of gastric cancer in this small series or reported in the literature, a larger panel of gastric cancer specimens should be evaluated to assess the frequency and significance of this genetic alteration.

A second gene, recognized by the serum of a different gastric cancer patient and also related to gastric carcinogenesis, is the AKT1 (PKB) oncogene. The AKT1 gene is thought to promote cell survival by modulating antiapoptotic signals, and AKT1 gene amplification has been reported in a primary gastric cancer [23]. AKT1 expression was elevated in five of eight gastric cancers, and one of the five patients with amplified AKT1 expression had an anti-AKT1 antibody response. Overexpression of this gene presumably forms the basis for its immunogenicity in cancer patients.

Certain genes predominate as the source of antigens recognized by the patients, i.e., multiple independent isolates derived from the same gene. From the SM library, an unknown SG24 (nine clones), RPB-Jκ (seven clones), and an unknown SG132 (four clones) were isolated. From the CK library, the E-Cad/unknown gene Y chimeric gene (nine clones), HIV TATA element modulatory factor (five clones), and the follistatin-related protein of the activin family (four clones) were isolated. In the SS library, kinectin (nine clones), inducible poly(A) binding protein (five clones), and unknown SS114 (four clones) were prominent antigens. In the KM library, an unknown SG24 (seven clones) predominated as in SM. From the YS library, TATA-binding protein (13 clones), RPB-Jκ gene (11 clones), and HSP 60 (6 clones) were repeatedly isolated. The reasons for and significance of the immunodominance of these antigens in gastric cancer patients are now being explored.

For efficient selection of antigens potentially useful in the diagnosis and therapy of gastric cancer, all 136 antigens were examined for cancer-restricted immunogenicity. Each antigen was tested for its reaction with a panel of sera from the 44 gastric cancer patients and from the 100 control individuals. In most previous similar studies, sera from young and healthy volunteers were used as controls. It must be stressed that control sera in the present study were from age-matched individuals who had no previous history of cancer, but some had nonmalignant diseases such as gastritis, polyp, or ulcer. Antigens were divided into the following three groups based on the reactivity to the sera: 1) shared cancer antigens that reacted with a certain proportion of

cancer patient sera but not with control sera; 2) unique cancer antigens that reacted only with the serum of a patient in whose cDNA library the antigen was originally identified; and 3) autoantigens that reacted with both cancer patient and control sera. A total of 11 shared cancer antigens were identified and they are the most promising as vaccine targets.

Unique antigens may be the result of recognition of mutations occurring in tumors as in the case of the E-Cad/unknown gene Y fusion gene product. Although unique antigens have only limited value as vaccine targets, they may provide clues to understanding the etiology of gastric cancer, especially those isolated multiple times from a cDNA library. Among autoantigens identified in the present study, certain antigens elicited antibody production in cancer patient sera much more frequently than in sera from controls. For example, the frequency of antibody production to epithelial tropomyosin was 50% in gastric cancer patient sera but only 9% in that from controls. Similarly, 36% of patient sera produced antibody to an unknown SG24 gene, compared with 7% of control sera. Approximately 12 such antigens were identified. These antigens may also be useful in the clinical setting. Eleven shared cancer antigens and 12 autoantigens are now being examined for their expression in cancer and normal tissues. In addition, the basis for immune recognition of these antigens by sera from gastric cancer patients will be elucidated before considering them as candidates for vaccine targets.

Because SEREX technology is generally applicable to all tumor types and is less technically demanding than CTL cloning, it holds promise for greatly extending our understanding of the immune response to cancer. Vigorous evaluation of a promising list of cancer antigens defined by SEREX analysis is necessary. Once in clinical trials, monitoring immune responses in cancer patients against targeted antigens after vaccination is most critical, and methods for this are now being developed. Although immunotherapy for cancer is only a theory at present, solid scientific bases are now being established to make antigen-specific immunotherapy available to cancer patients.

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