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From sequence of tumor liberated protein (TLP) to potential targets for diagnosis and therapy

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Abstract

A preliminary analysis of immunoprecipitation followed by Western Blotting (WB) shows corin and TLP precipitate at the same level (approximately 50 KDa) and are recognized by the same antibodies. In parallel the tests of immunoprecipitation were improved by the use of cell extracts derived from lung cancer cells A549 and NCI-H23 with the aim of obtaining a precipitate containing only the TLP. In fact the partial amino acid sequence of TLP shows a high homology with the sequence of human corin (only one amino acid is different) and is present in lung cancer under different isoforms. It is known that human corin is expressed mostly outside the cells and the protein extract derived from the extracellular medium and from the cells transfected with the plasmid, which overexpresses corin, shows several bands analysed on SDS-PAGE that are equivalent to the bands (about 50-100 KDa) observed in the WB analysed by anti-TLP.

Introduction

While surgery, radiotherapy and chemotherapy are able to cure many cancers, new approaches are required to improve radical curative therapy. A possible route is to utilize the latest achievements made in research on the immunology and genetics of cancer [1]. Cancer immunotherapy [2], or the manipulation of the naturally occurring oncolytic immune reaction, is based on the observation that neoplastic cell antigens stimulate the onset of specific humoral and cellular antibodies both in animals and humans [3]. Certain difficulties that have been encountered reflect the lack of well-purified antigens and/or their ability to unblock cell immunity in the cancer patient.

Two ways are known to enhance the host's immunity: aspecific activation (BCG in primis) and specific activation (to stimulate oncolytic circulating and cell antibodies). Moreover, some researchers have performed therapeutic trials with antigens, from autologous and homologous human cancer cells, obtained by various purification procedures [4,5].

The first observation by Tarro *et al.* [6] demonstrated that when TLP is extracted from a tumor, purified in the laboratory, and reintroduced into the patients body, it boosts the immune system's cancer responsive capabilities [7]. As lung cancer accounts for the largest number of cancer deaths in the Western world, TLP may have the potential to greatly improve the cure rate and or serve as a lung cancer vaccine (Table 1) [8].

Corin is a cardiac serine protease that activates natriuretic peptides. It consists of an N-terminal cytoplasmic tail, a transmembrane domain, and an extracellular region with a C-terminal trypsin-like protease domain. The transmembrane domain anchors corin on the surface of cardiomyocytes. To date, the function of the corin cytoplasmic tail remains unknown [9]. Corin shows high homology with TLP and is present in various isoforms in the lung [10. If the fragments cut with thrombin proved to be the same, the data would support the hypothesis that TLP and corin are the same protein. At the same time a plasmid was used that allows us to transfect and over-express human corin with the purpose of assessing whether the two proteins are actually the same protein or are different by Western blotting (using anti-TLP and anti-corin antibodies).

Table 1. Tumor liberated protein from lung cancer and perspectives for immunotherapy.

TLP AS A TUMOR–ASSOCIATED ANTIGEN
• 50 KD PROTEIN OVEREXPRESSED IN LUNG TUMORS AND OTHERS EPITHELIAL ADENOCARCINOMAS
• IMMUNIGENIC IN HUMANS AS EVIDENCED BY SERUM ANTIBODIES

Materials and methods

Antigen synthesis and antibody production

The synthesis of the TLP-derived peptide RTNKEASI [23] and two different rabbit polyclonal anti-RTNKEASI immune sera were carried out at Rockland Immunochemicals Inc. (Gilbertsville, PA, USA) and at Biogenes GmbH (Berlin, Germany). The serum anti-TLP produced at Rockland was further purified against RTNKEASI peptide by chromatography.

Cell culture

The human cell lines A549, CA46, HL60, MCF7, MRC-5, Hela and PC3 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). A549, MRC-5, HL60, Hela, and MCF-7 cell lines were cultured in Glutamax Dulbecco's Modified Eagle's

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Medium (Gibco, life technologies), CA46 cell line in Glutamax RPMI 1640 (Gibco, life technologies), and PC-3 cell line in Glutamax HAM's F-12 (Gibco, life technologies), supplemented with 10% fetal bovine serum (Biochrom GmbH, Berlin, Germany) at 37°C in a humidified atmosphere of 5% CO_2 in air, according to the ATCC recommendations.

Western blot

Cell pellets were prepared from A549, CA46, HL60, MCF7, MRC-5, Hela and PC3 cell lines and lysed as described previously.

Cell culture supernatant from $5X10^6$ A549 cells was incubated with ice-cold 10% trichloroacetic acid (TCA) on ice for 30 min and then centrifuged at 4°C at 14000 rpm for 30 min. The pellet was incubated with ice-cold 90% aceton at -20°C for 20 min. After centrifugation, the pellet was dried at 65°C for 30 min.

Proteins (30 µg) were resuspended in 4X Laemmli sample buffer (100 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 200 mM dithiothreitol (DTT) and 0.01% bromophenol blue), boiled for 5 min and resolved by 8% acrylamide (Applichem GmbH, Darmstadt, Germany) SDS-PAGE gel. The proteins were blotted onto activated polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Mass), which were incubated in blocking buffer (nonfat dry milk 8% + PBS + 0.1% Tween-20) and then probed overnight at 4°C with the anti-RTNKEASI serum 1:1000 (Biogenes, Berlin, Germany; Rockland Immunochemicals, PA, USA) or ß-actin 1:20000 (Biolegend, San Diego, CA). After 1 h of incubation with the corresponding horseradish peroxidase-conjugated secondary antibody diluted 1:30000 in PBS + 0,1% Tween-20 + BSA 5%, the immunoreactive bands were detected by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK).

Peptide competition assay (PCA)

The anti-RTNKEASI serum was diluted 1:1000 in PBS + 0,1% Tween-20 + BSA 5% buffer and pre-incubated with a 500-fold molar excess of the peptide RTNKEASI or with control peptide KDSGNEQTFLPP for 1 h at room temperature followed by 1 h at 37°C with gentle rocking. The pre-incubated antibody samples were cleared from immune complexes by centrifugation and subsequently hybridized for 2 hours with A549 lysates transferred on PVDF membranes. After wash with PBS 0,1% + Tween-20 to remove unbound antibody, the membranes were processed according to the conventional western blot method.

Two-dimensional polyacrylamide gel electrophoresis

Protein extraction from A549 and MCF7 cell lines was performed as described previously. The first dimension isoelectric focusing was performed by using 7 cm immobilized pH gradient (IPG) dry strips with a linear pH 4-7 gradient (GE Healthcare). 50 µg of solubilized proteins were applied onto the strips in rehydration buffer (8M urea, 2M thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM DTT with 0.5% IPG buffer) with 1 µl bromophenol blue 1% and incubated over night at room temperature. Proteins were separated by the PROTEAN IEF system (Bio-Rad) using a programmed voltage gradient at 20°C with a current limit of 50µA per strip in the dark under the following conditions: 4 h at 250 V, 8000 V linear gradient to 15000 Vh, rapid 8000 V to 75000 Vh, for a total of 90 kVh. The first dimension strip was equilibrated in buffer A (0.05M Tris-HCl, 6 M urea, 20% glycerol, 2% SDS and 130 mM DTT) and buffer B containing 4.5% iodoacetamide instead of DTT for 10 minutes. For the second dimension, the equilibrated strips were applied to the top of 8% acrylamide (Applichem GmbH, Darmstadt, Germany) SDS-PAGE gel and sealed with 1% agarose prepared in SDS-Tris-glycine buffer with traces of BPB as a tracking dye to monitor electrophoresis. The resolved proteins were visualized directly by silver staining or transferred to a PVDF membrane (Millipore, Bedford, Mass) and incubated with the anti-RTNKEASI serum, according to the western blot procedure.

Results

Western blotting shows that Corin and TLP seem to precipitate at the same level (approximately 50 KDa) and are recognized by the same antibodies. Concurrently a plasmid was obtained from Prof, Qingyu (Cleveland, Ohio) that transfects HEK-293 cells and overexpress the human corin with the purpose of evaluating whether the two proteins are really the same proteins by Western blotting (using anti-TLP and anti-corin). In parallel the tests of immunoprecipitation were improved by the use of cell extracts derived from lung cancer cells A549 and NCI-H23 with the aim of obtaining a precipitate containing only the TLP. This result would allow a better sequence of the aminoterminal fragment of TLP and furthermore would allow to look in details the homologies between TLP and corin.

From a careful analysis of bibliography concerning both TLP and Human corin, analysed by Mass Spectrometry (in preparation) and from our data, TLP is present in more quantity within the cell and with more specificity in the non small cell lung cancer than in the small cell lung cancer as corin.

In fact the partial amino acid sequence of TLP shows a high homology with the sequence of human corin (only one amino acid is different) and is present in lung cancer under different isoforms. From the literature it is known that human corin is expressed mostly outside the cells and the protein extract derived from the extracellular medium and from the cells transfected with the plasmid, which overexpresses corin, shows a number of bands analyzed on SDS-PAGE that are equivalent to the bands (about 50-100 KDa) observed in the Western blots analyzed using anti-TLP.

Therefore the protein band identified as TLP through mass spectrometry reveales the molecular nature of at least one component of the previously described TLP complex.

Conclusion

Tumor liberated protein (TLP) is a new protein extracted from tumors in *vivo* and transformed cells in *vitro* (Figure 1)[8].

TLP is detectable in blood as well as in cancer tissue [11,12].

TLP is a tumor associated antigen of 50 KD monomer [13,14].

TLP is overexpressed in lung tumor [13,14] and other epithelial adenocarcinomas [15,16].

TLP is immunogenic in humans as evidenced by serum antibodies [17].

Preliminary information on lung tissue microarray is shown in table 2.

Research is ongoing to obtain the complete sequence of TLP, by proteomics approaches, in order to achieve adequate antigen preparations that might be used to generate assays for early diagnosis and, possibly, a specific anticancer vaccine [18].

The perspectives of TLP are the following:

Table 2. Sensitivity and specificity of TLP for antibodies.

TISSUE MICROARRAY PROFILE (a)		
NSCLC STAGE I TISSUE	POSITIVITY (%)	NEGATIVITY (%)
400	56.3 (225/400)	43.7 (175/400)
NORMAL LUNG TISSUE	POSITIVITY (%)	NEGATIVITY (%)
400	0 (0/400)	100 (400/400)
(a) Carried out by William C. Hyun, Ph.D., at the University of California San Francisco, Cancer Center, Laboratory Cell Analysis.		

– Since its sequences stimulate cytotoxic immunoresponse in humans and animal models, it is possible to design potential active and passive immunotherapies for non small cell lung cancer (NSCLC) and colorectal cancers (CRC) based on TLP epitopes and humanized antibodies [19,20.

– Fragments of TLP can be used to stimulate immune response to attack existing tumors [9,21].

– At risk populations could be inoculated with TLP fragments to stimulate immune response to undetected or newly developing tumors [22,23].

– Therefore the ability of the immune system to recognize TLP represents a main target for diagnosis and therapy in this field of research.

Conflict of interest statement

The author declares no conflict of interest.

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