IDENTIFICATION OF HUMAN CYTOTOXIC T-LYMPHOCYTE-DEFINED OSTEOSARCOMA GENE THAT ENCODES A TRANSCRIPTIONAL REGULATOR, PAPILLOMAVIRUS BINDING FACTOR

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Introduction

It is a great challenge to improve prognosis of patients with osteosarcomas who do not respond to current chemotherapy protocols. Peptide-based immunotherapy has brought about objective therapeutic responses in melanoma patients. However, such T-cell defined antigens have not been reported to date in osteosarcoma. Following attempts over a period of three years, we recently established an autologous tumor cell-CTL pair from a 16-year-old osteosarcoma patient (1). Using this pair, in the present study, we carried out cDNA library expression cloning and identified an antigen and an epitope, which sensitize the anti-autologous osteosarcoma CTL clone.

Materials and Methods

Establishment of osteosarcoma cell line and autologous CTL clone.

The osteosarcoma cell line, named OS2000, was established from a biopsy specimen of osteosarcoma in the femur of 16-year-old female. Peripheral blood mononuclear cells (PBMCs) were collected from the same patient before starting chemotherapy. The PBMCs were stimulated in vitro with irradiated OS2000 cells for 6 times every 10 days. Responding CD8+ T cells were cloned by limiting dilution and expanded as previously described (1). The specific cytotoxicity against OS2000 was measured in a standard 6h-chromium release assay.

Construction of cDNA library from OS2000 and screening using TcOScl-303

A cDNA library was prepared from OS2000 mRNA. By transfecting a pool of cDNAs into 293EBNA-B55 cells stably transfected with HLA-B*5502, 293EBNA-B55, followed by incubation with TcOScl-303, LDH released in the media was measured in a colorimetric assay (LDH release assay). After a cDNA clone was isolated, the sequence analysis was performed and compared with the GenBank database.

RT-PCR

Expression of papillomavirus binding factor (PBF) mRNA in sarcoma tissue specimens was determined by RT-PCR. Biopsy specimens of sarcoma included 20 cases of synovial sarcomas, 14 osteosarcomas, 12 Ewing sarcomas, eight malignant fibrous histiocytomas and another eight malignant peripheral nerve sheath tumors, and five liposarcomas. Also, three cases each of rhabdomyosarcomas, leiomyosarcomas and clear cell sarcomas were included.

Immunostaining

Expression of PBF protein in sarcoma tissue specimens was determined by immunostaining and Western blotting. For immunostaining and Western blotting, polyclonal antibody against PBF was generated by immunizing rabbits with a 15-mer peptide derived from PBF, once per week for six weeks. The serum was purified by using Protein A column. Biopsy specimens of sarcoma included 20 cases of osteosarcomas, six malignant fibrous histiocytomas, six chondrosarcomas, five liposarcomas and three leiomyosarcomas. Also, two cases each of synovial sarcomas, rhabdomyosarcomas, clear cell sarcomas, extraskeletal Ewing sarcomas, alveolar soft-part sarcomas, and one malignant peripheral nerve sheath tumor were included. Formalin-fixed paraffin-embedded sections of sarcoma biopsy specimens and autopsy specimens of normal tissues were deparaffinized and then boiled by microwave for antigen retrieval. The sections were blocked and stained by the standard ABC method. Hematoxylin was used for counterstaining.

Western blotting

The lysate of normal tissues, cell lines and biopsy specimens were separated on 7% SDS-polyacrylamide gels and transferred to a membrane. The membranes were blocked and probed with anti-PBF antibody or mouse anti-beta actin monoclonal antibody for 40 min at room temperature, respectively. Then the membranes were stained with peroxidase-labeled-second antibody and visualized.

Identification of the epitope for TcOScl-303

The plasmid containing truncated variants of the cDNA was generated and transfected with b 293EBNA-B55 cells, followed by testing TcOScl-303 recognition with LDH release assay. Candidate peptides in the region containing the epitope were synthesized. To assay antigenic peptide recognized by TcOScl-303, synthetic peptides pulsed 293EBNA-B55 cells were tested TcOScl-303 recognition with LDH release assay.

Results

The CTL clone TcOScl-303 showed specific cytotoxicity against OS2000 and they were selected for further experiments. After screening of total 1x10^5 cDNA clones, one positive cDNA clone was isolated that recognized by TcOScl-303. This cDNA clone, contained 1,908-bp nucleotides, encoded a DNA binding transcriptional regulator, papillomavirus binding factor (PBF), that was recently reported. PBF mRNA was expressed in 57/76 cases of sarcoma tissues, especially in 12/15 of osteosarcoma tissues. PBF protein was also expressed in 45/51 cases in nuclei of sarcoma tissues, especially in 16/20 of osteosarcoma tissues, but was weak and restricted in cytoplasm in normal tissues by immunostaining. No detectable reaction was observed in the lysate of normal tissues in Western blotting. TcOScl-303 appeared to recognize the epitope around the 3' end of PBF. Moreover, TcOScl-303 recognized the 293EBNA-B55 cells pulsed with synthetic 12mer peptide, CTACRWKKACQR, which was derived from PBF.

Discussion

We identified the papillomavirus binding factor (PBF) as an osteosarcoma antigen recognized by autologous CTLs using the cDNA expression cloning procedure. We found that a 12-mer peptide, CTACRWKKACQR, located at the Cterminus of PBF was a minimum requirement for recognition by TcOScl-303 in the context of the HLA-A*5502 molecule (2). These findings suggest that PBF may serve as a source of therapeutic peptides which are effective in further improving survival rates of patients with osteosarcoma, especially those who are unresponsive to current chemotherapy protocols.

References


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