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Michael E. Grossmann · Monique Wood · Esteban Celis

Expression, specificity and immunotherapy potential of prostate-associated genes in murine cell lines

Abstract The TRAMP-C1 (C1) and TRAMP-C2 (C2) cell lines were derived from a prostate tumor that arose in a mouse from the transgenic adenocarcinoma mouse prostate (TRAMP) model. However, their similarity to primary prostate tumors and therefore their usefulness in immunotherapy studies has not been clearly defined. We showed using RT-PCR that these cell lines exhibited a variety of prostate-specific genes expressed by human prostate tumors that may be used as tumor-associated antigens for immunotherapy. Interestingly, several of these genes are also expressed in cell lines that are not prostatic in origin. The prostate cell lines were also shown to grow in an androgen-independent manner, to be capable of expressing MHC class I and to be susceptible to specific lysis by cytotoxic T lymphocytes. Therefore, these cell lines will provide us with the ability to evaluate immune responses to and tolerance of prostate-specific protein peptides in an animal model.

Prostate cancer is the second leading cause of cancer deaths in men in the United States with 31,500 deaths estimated to occur in 2001 [1]. Surgery and radiation therapy remain the treatment of choice for the early (localized) stages of prostate cancer. Although approximately 80% of the early diagnosed and treated patients survive for more than 5 years, many patients progress to the advanced metastatic stages. Advanced metastatic prostate cancer is treated using androgenic therapy either through bilateral orchiectomy or hormonal therapy using estrogens or analogs of hypothalamic luteinizing hormone-releasing hormone. However, the median survival for these patients is in the range of only 24 to 42 months [2]. It is therefore clear that new approaches to treating advanced disease and for preventing potential relapses of prostate cancer need to be developed.

Immunotherapeutic maneuvers designed to treat cancer, such as in vivo vaccination, are more likely to succeed in patients with early disease or following removal of primary tumor mass for elimination of micrometastases [3]. This is because the patient’s immune system will not be compromised by disease progression or by antineoplastic therapies that are often immunosuppressive. Several proteins appear to be produced preferentially by the epithelial cells of the prostate that could be immunotherapeutic targets [4–9]. Some of these are prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA) and the homeobox gene Nkx3.1. Most transformed prostate cells continue to produce these proteins and the levels in serum of PSA and PAP have been used to diagnose and monitor the progress of tumors and the effect of treatment [2, 3]. Recently, we have observed that a member of the Hox family gene, Hoxb-13, is also preferentially expressed in the prostate and in prostate tumors (Grossman et al., submitted for publication). Like many other normal cell constituents, Nkx3.1, PAP, PSMA, PSCA and Hoxb-13 are all likely to be processed by the prostatic epithelial cells with tumors generating peptide fragments that will associate with class I MHC molecules. Therefore, these prostate-specific proteins may be able to serve as T cell epitopes allowing cytotoxic T lymphocytes (CTL) to identify and eliminate prostate cancer.

The transgenic adenocarcinoma mouse prostate (TRAMP) model utilizes transgenic mice in which progressive forms of prostatic neoplasia spontaneously develop in 100% of the males [10]. This transgenic mouse model was established by utilizing the prostate-specific androgen-sensitive portion of the probasin gene.
promoter to drive transcription of the SV40 T antigen gene in the prostates in these mice. Mild to severe epithelial hyperplasia is observed as early as 10 weeks, invasive adenocarcinoma is observed as early as 18 weeks and metastatic disease can be observed in all mice by 28 weeks. The metastases are found in the periaortic lymph nodes, lungs, kidneys, adrenal glands and bones [11]. In addition, castration of TRAMP animals results in transient regression of their prostatic tumors followed by androgen-independent growth [12]. The tumor location, disease progression and switch from androgen-sensitive tumor growth to androgen-insensitive tumor growth following castration provide a far better correlation with the human condition than previous models.

The TRAMP-C1 (C1) and TRAMP-C2 (C2) cell lines were derived from a prostate tumor that arose in a TRAMP mouse. The cells have been shown to be tumorigenic in syngeneic C57BL/6 mice and they no longer express SV40 T [13]. In addition, it has recently been reported that C1 cells can be recognized and destroyed by the immune system upon blockade of CTLA-4 by antibodies [14]. However, in spite of the fact that the immune system can kill these cells the specific antigens that are recognized have not been identified. It is essential that the antigens be prostate-associated antigens (PAA) and not cell line-specific mutant antigens that would not be recognized as self by the immune system since most PAA would normally be recognized as self.

Thus, it appears that these lines may be useful for the study of prostate cancer disease intervention if they express prostate-specific proteins. Therefore, we characterized the expression of Nkx3.1, PAP, PSMA, PSCA and Hoxb-13 in C1 and C2 cells. In addition, we showed that these cell lines are suitable targets for CTL and that the prostate tumors in TRAMP mice generally express some but not all of the prostate-specific proteins identified in C1 and C2 cells. These results indicate that the C1 and C2 cell lines and the TRAMP tumor model should provide an additional valuable tool for the investigation of immunotherapy for prostate cancer.

**Materials and methods**

**Cell culture**

The C1 and C2 cells were obtained as a gift from Dr. Norman Greenberg. They were grown in DMEM with high glucose (Life Technologies, Baltimore Me.), 2 mM l-glutamine (Life Technologies), 10% FCS (HyClone, Logan, Utah), 25 U/ml penicillin and 25 µg/ml streptomycin (Life Technologies). Prior to treatment with 50 mM sodium butyrate (NaB; Sigma, St. Louis, Mo.) and/or murine interferon-γ (IFN-γ; Endogen, Woburn, Mass.), T75 flasks of confluent C1 and C2 cells were split at a 5:1 ratio and replated in fresh T75 flasks.

**RT-PCR analysis**

Cells were trypsinized, RNA was extracted using an Rneasy Kit from Qiagen according to the manufacturer’s instructions and concentrations were determined using a Beckman DU 64 spectrophotometer. RNA in a final volume of 40 µl [3–8] was treated with 40 U RNase-free DNase I (Boehringer Mannheim, Indianapolis, Ind.) in 100 mM sodium acetate and 5 mM magnesium chloride (Sigma) for 1 h at room temperature then 10 min at 75 °C. For reverse transcription, the DNase-treated RNA was incubated in 40 µl with 5 mM magnesium chloride, 1 mM dNTP mix, 40 U RNase inhibitor, 1 µg random primers, 25 U AMV reverse transcriptase with its appropriate 5 × incubation buffer (Boehringer Mannheim), and water. The RNA mixture was incubated at 30 min for 42 °C and 5 min at 99 °C, then placed on ice. Negative controls were performed with each experiment without reverse transcriptase. PCR was performed in 25 µl with 0.8 mM dNTP mix, 2 U Taq DNA polymerase, its appropriate 10 × buffer (Boehringer Mannheim), cDNA, water and various primers (15 pM each).

The conditions for the PCR were 94 °C for 30 s, annealing temperature optimized for each primer for 1 min, and 72 °C for 2 min for 40 cycles. The primers used were: PSCA primer sequence (forward TTCTCCTGCTGGCACCCTAC, reverse GCAGCTCATCCTCTTCAAT), annealing temperature 58 °C; PSMA primer sequence (forward CACCTGTGCTGGTCTTTAACCGG-GAACCTT, reverse GAGAGAGAGGCCGCTATTGGTGGCCAC-CT), annealing temperature 54 °C; Hoxb-13 primer sequence (forward CCAAGCGCTGTGAGCCAACTGACATGC, reverse GTTCTGTTACCTTGGCACAACACATCTGCT), annealing temperature 56 °C; PAP primer sequence (forward TCTGGAGAAGTTTGCAGGACGTCTGGA, reverse TCAGTGGCTGTA-CCCCAGGGTTCTTAA), annealing temperature 60 °C; Nkx3.1 primer sequence (forward AGACACGCTAGAACCAGGTGC-TGATGAC, reverse AGACAGTACAGTTAGGGTAGTAG-GGATGAC), annealing temperature 56 °C; and L-19 (as positive control) primer sequence (forward CTTAGAGCTCAA-GGAATGTG, reverse GGACAGATCCTGTTAGTCTC), annealing temperature 50 °C. PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

**Cell proliferation assays**

The MIT assay was performed using a Cellititer 96 assay (Promega, Madison, Wis.). In brief, the cells were trypsinized, counted and plated in triplicate at a density of 5 × 10^3/well in a 96-cell plate with medium that contained various concentrations of dihydrotestosterone (DHT). The cells were then grown for 72 h before assay. To obtain live cell counts, C1 and C2 cells were plated at a density of 1 × 10^4/well in six-well plates and incubated for 96 h in medium with various concentrations of DHT. Cells were then washed, trypsinized and counted using trypan blue exclusion.

**MHC class I analysis**

Cells that had been treated with NaB and/or IFN-γ were trypsinized, aliquoted at a concentration of 1–5 × 10^6 per tube and washed twice with PBSA comprising 1 × phosphate-buffered saline (Life Technologies), 2% calf serum (Life Technologies), and 0.1% sodium azide (Sigma). The cells were then incubated with mouse serum for 10 min at room temperature followed by incubation with H-2 K or H-2 Db antibodies (Pharmingen, San Diego, Calif.) for 10 min at room temperature. The cells were then washed twice with PBSA and stored in 0.5% paraformaldehyde (Sigma) until they could be analyzed on a FacScan.

**CTL lysis assay**

SV40-specific CTL were generated using naive splenocytes (8 × 10^6/well) from C57BI/6 mice incubated with 8 × 10^7/well C57SV cells that had been irradiated with 10,000 rad in 24-well flat bottomed plates. The cells were grown in 200 µl/well Iscove’s basal medium (Life Technologies) with 5% fetal calf serum (HyClone), 1 × 10^-5 M betamercaptoethanol (Sigma) and 10 µg/ml gentamicin (Sigma). Additional irradiated C57SV cells (8 × 10^6/well) and 50%