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Killing of Fas ligand-resistant renal carcinoma cells by interleukin-2- and BCG-activated effector cells

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Abstract Activated cytolytic effector cells like lymphokine-activated killer (LAK) and the recently described bacillus-Calmette-Guérin-activated killer (BAK) cells are thought to mediate antitumor effects against metastatic renal cell carcinoma (RCC) and superficial bladder cancer respectively. Perforin and Fas ligand (FasL) have been described as the major lytic principles in cellular cytotoxicity. Using a radioactive-release assay and specific inhibitors, we investigated the molecular mechanisms used by LAK and BAK cells in the lysis of renal carcinoma cells. In addition, we evaluated the susceptibility of RCC cells to FasL-mediated cytotoxicity. LAK and BAK cells effectively lysed the renal cancer cell line SK-RC-35 upon cell-cell contact. Both effector cell populations were shown to produce perforin and FasL as determined by reverse transcriptase/polymerase chain reaction (RT-PCR). Using fluorescence-activated cell sorting analyses and RT-PCR, we detected a marked Fas receptor (Fas, CD95) expression on RCC cells. However, RCC cells were shown to be resistant to killing by recombinant FasL and by lysis by BAK and LAK cells was not inhibited in the presence of anti-FasL antibody. In contrast, the cytotoxicity exerted by LAK and BAK cells was drastically reduced in the presence of the Ca\(^{2+}\)-chelating agent EGTA as well as concanamycin A, a specific inhibitor of perforin-mediated lysis. These results demonstrate that cytolysis of FasL-resistant RCC cells by activated immune cells is mediated via perforin. Our findings give further insights into the molecular mechanisms involved in the elimination of RCC by cytotoxic lymphocytes activated with biological response modifiers.

Key words Renal cell carcinoma · Immunotherapy · Perforin · IL-2 · Bacillus Calmette-Guérin

Introduction

Renal cell carcinoma (RCC) is the commonest neoplasm of the kidney and accounts for approximately 3% of adult malignancies [11]. Between 25% and 30% of patients have already developed distant metastases at initial presentation, which is associated with a 5-year survival rate of less than 10% [29]. Unfortunately, RCC is also largely resistant to conventional therapeutic approaches like chemotherapy [51]. Therefore, other treatment modalities, including immunotherapy, have been extensively studied. In vitro, the stimulation of peripheral blood lymphocytes with interleukin-2 (IL-2) has been shown to generate strong cellular cytotoxicity against a variety of tumor targets [1]. The cells showing this antitumor activity have been termed lymphokine-activated killer (LAK) cells and are thought to mediate the therapeutic effects of IL-2. Clinical trials with interferon-\(\alpha\) and IL-2 have been conducted with some success [6,10]. However, response rates are low and vary from 11% to 33% [11]. The molecular mechanisms of these biological response modifiers (BRM) remain poorly defined.

Bacillus Calmette-Guérin (BCG) is a very potent antitumor BRM in the treatment of superficial bladder cancer and has become clinically established [10, 28]. Recently, we were able to demonstrate that the in vitro stimulation of peripheral blood mononuclear cells (PBMC) with BCG leads to the generation of cytotoxic effector cells. These BCG-activated killer cells, termed BAK cells, rapidly lysed various tumor targets including bladder cancer cells [43]. BAK cells are of the CD8\(^{+}\)/CD56\(^{+}\) phenotype and differ from LAK cells insofar as their stimulation requires various cytokines as well as the presence of CD4\(^{+}\) Th1 cells and monocytes [44]. Furthermore, in contrast to LAK cells, BAK cells are more cytotoxic to malignant bladder tumor cells.
than to short-term cultured benign urothelial cells (our own unpublished observations).

To understand further the interaction of RCC with BRM-induced cytotoxic lymphocytes we analyzed the lytic pathways involved in the killing process. Perforin and Fas ligand (FasL), a member of the tumor necrosis factor (TNF) family, have been identified as the major molecules mediating cytotoxicity of killer lymphocytes [18]. Furthermore, the Fas system has been implicated in drug resistance and escape from immune recognition in numerous malignancies [9, 50]. Perforin is known as a pore-forming glycoprotein, released from lytic granules of the effector cell, that mediates target cell necrosis [19]. The molecule’s function requires the presence of Ca^{2+} ions [46]. Cytotoxic lymphocytes expressing FasL deliver their death signal through the receptor Fas, also termed APO-1 or CD95, and induce target cell apoptosis. This interaction is known to be Ca^{2+}-independent [34, 38]. In this study, the cytotoxicity of LAK and BAK cells against the established RCC cell line SK-RC-35 was investigated in vitro. We used different inhibitors to distinguish between the various lytic pathways. EGTA was added to determine whether lysis of renal carcinoma cells was Ca^{2+}-dependent. In addition, the widely used concanamycin A, which has been described as a powerful tool for specifically inhibiting perforin-mediated lysis, was used [15, 16, 26, 52]. To shed light on the role of the Fas system in RCC, we determined Fas expression of the target cells and FasL production by the effector cells. Our results demonstrate a dominant role for perforin in the lysis of renal carcinoma cells by stimulated cytotoxic effector cells, irrespective of a significant expression of Fas by the renal targets. Interestingly, we were for the first time able to show Fas resistance of renal cancer cells. Since many anticancer drugs, like doxorubicin, methotrexate, cytarabine or 5-fluorouracil with leucovorin, have been shown to induce tumour cell apoptosis via the Fas system, this finding might help to explain clinically observed drug resistance [9, 45].

**Materials and methods**

**Cell lines**

The human renal carcinoma cell line SK-RC-35 was maintained in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. This cell line was originally established at the Memorial Sloan-Kettering Cancer Center in New York, USA, and kindly provided by Ebert et al. [7].

The human acute T cell leukemia cell line Jurkat (ATCC-no. TIB-152) has been described to express Fas/APO-1 and was used to demonstrate the Fas resistance of SK-RC-35 cells [47]. The non-adherent cells were grown in the same medium as renal carcinoma cells.

**Effector cells**

PBMC from healthy human donors were isolated by density-gradient centrifugation on Ficoll-Paque (Pharmacia, Uppsalal, Sweden). Cells were adjusted to a concentration of 10^6/ml in RPMI-1640 medium containing 5% human AB serum, 100 U/ml penicillin and 100 µg/ml streptomycin. We used 800 U/ml human native IL-2 (kindly provided by H. Mohr, Blood Transfusion Service, Springe, Germany) and 3.75 × 10^4 cfu/ml reconstituted lyophilized BCG (Connaught substrate, Immucyst) to stimulate PBMC. Stimulated cell suspensions were cultured in six-well microtiter plates for 7 days at 37 °C and 5% CO₂. In all experiments, unstimulated cultured PBMC served as controls.

Reverse transcriptase/polymerase chain reaction (RT-PCR)

mRNA was isolated with magnetic oligo(dT) microbeads in a modified protocol from Jakobsen et al. [14]. In brief, 5 × 10^5 cells were incubated in 100 µl PBS for 60 min at 4°C. The cells were then washed twice in cold PBS containing 1% BSA, 100 µM EDTA and 10% FCS. The cells were then lysed with 50 µl PermiLyse (Immunotech, Marseille, France) for 20 min at room temperature. The cell lysate was then centrifuged at 2000 × g for 5 min at 4°C. The supernatant was transferred to a new tube and 2.5 µg total RNA was extracted with Trizol (Life Technologies, Karlsruhe, Germany). The RNA quality was checked by 1% agarose gel electrophoresis. The complementary DNA (cDNA) was reverse transcribed in a final volume of 20 µl containing 2 µg total RNA, 200 µM dNTPs, 5 µM of specific oligonucleotide primers, 0.5 U/µl RNase inhibitor (Roche, Mannheim, Germany) and 200 U of superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was amplified in a volume of 50 µl containing 2.5 µl of cDNA, 200 µM dNTPs, 5 µM of specific oligonucleotide primers, 0.5 U/µl Bst-FDA polymerase (New England Biolabs, Ipswich, MA, USA) and 5 X Bst-FDA buffer (New England Biolabs, Ipswich, MA, USA) for 30 cycles (40 s at 95°C, 45 s at 55°C, and 30 s at 72°C) with 2 × extension for each cycle. The PCR products were separated by agarose and ethidium bromide gel electrophoresis.

Flow cytometry

Single-colour immunofluorescence was performed on the renal carcinoma cell line SK-RC-35 indirectly stained with monoclonal antibodies and detected with a fluorescein-isothiocyanate (FITC)-conjugated secondary antibody. For each analysis 5 × 10^5 cells were incubated in washing buffer (phosphate-buffered saline containing 0.1% sodium azide and 3% human AB serum) with 1.25 µg/ml mouse anti-human Fas/APO-1 (the gift of P.H. Krammer, German Cancer Research Center, Heidelberg, Germany) on an isotype control buffer GAC for 30 min. After two washes a goat anti-(mouse Ig)-FITC conjugate was added for another 30 min at 4°C. Following two more washes in washing buffer, 1.5% formaldehyde was added and a total of 10,000 cells were analyzed on a FACStar Plus flow cytometer (Becton Dickinson, Heidelberg, Germany). Gating of debris, non-viable and aggregated cells was done according to forward/sideward scatter signals. We performed calculations on our data with the help of WinMDI software.

Cytotoxicity assay

A standard 51Cr-release assay was used to measure cytotoxicity. In brief, target cells were adjusted to a concentration of 5 × 10^5/ml and labelled with 25 µCi/ml Na^24CrO4 for 20 min at 37°C. After three washes, the labelled target cells were seeded out in 96-well microtiter plates. Effector cells were added at different effector/target (E/T) cell ratios in 200 µl/well unless otherwise stated in figure legends. The Ca^{2+}-chelating agent EGTA was added during coincubation to inhibit Ca^{2+}-dependent cytotoxicity. To inhibit perforin-mediated lysis, effector cells were preincubated with concanamycin A (Sigma) for 120 min. Thereafter, target cells were...