Dead or Dying: Necrosis versus Apoptosis in Caspase-deficient Human Renal Cell Carcinoma

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Abstract

The antitumor effect of immuno- and chemotherapeutic agents is executed through stimulation of apoptotic programs in susceptible cells. Apoptosis induced in tumor cells requires activation of members of the caspase family of proteases. Deficient expression or activation of caspases may account in part for the failure of many current anticancer therapies. However, recent studies suggest that cell death can proceed in the absence of caspases. We investigated the susceptibility of human renal cell carcinoma (RCC) lines to two distinct modes of cell death, apoptosis and necrosis. RCC lines displayed almost complete resistance to apoptosis in response to the intracellular zinc chelator, N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), which instead induced dramatic accumulation of nonapoptotic necrotic cells. Conversely, TPEN was a potent inducer of apoptosis in caspase-competent normal kidney cells (NK-72) and Jurkat T lymphocytes. Resistance to apoptosis in RCC lines correlated with almost complete loss of caspase-3 expression and variable down-regulation of caspase-7, caspase-8, and caspase-10. These data may explain the resistance of RCC to drugs inducing apoptosis and have important consequences for further attempts to manipulate tumor cell death.

Introduction

The cytotoxic effect of most immuno- and chemotherapeutic agents in vitro and in vivo depends on induction of apoptosis in susceptible tumor cells (1, 2). Apoptotic cell death is characterized by a series of unique events including phosphatidyl serine exposure, cell shrinkage, and condensation and fragmentation of chromatin (3). There is strong evidence that sensitivity of various tumor types to current therapeutic methods critically depends on the expression and activation of multiple apoptosis-regulatory proteins. Down-regulation of the CD95 receptor/ligand system, deficient expression of caspase family members, or overexpression of antiapoptotic Bcl-2 protein have all been demonstrated in drug-resistant tumors (1, 4, 5). Recently, substantial evidence has implicated mitochondria in apoptotic signaling pathways. Alterations in mitochondrial membrane structure and function and opening of mitochondrial permeability transition pores result in the release of cytochrome c into the cytosol, where it exerts its activating effect on caspases (6, 7). It is now clear that although inhibition of caspases can prevent nuclear apoptotic changes, it does not always prevent irreversible loss of cellular function. Convincing data provide direct evidence that upon changes in the mitochondrial permeability transition, the availability of members of the caspase family determine the choice between apoptotic and necrotic modes of cell death. Alterations of mitochondrial function induce apoptosis when downstream caspases can be activated; however, in cells with reduced expression or activation of effector caspases, mitochondrial dysfunction results in necrotic cell death (8). In this work, we investigated the susceptibility of apoptosis-resistant RCC cells to necrotic modes of cell death. To investigate this, we used RCC lines in which expression of several caspase family members was found to be diminished with near complete loss of caspase-3 expression. We determined that these cell lines were almost completely resistant to apoptosis in response to the intracellular zinc chelator TPEN, although TPEN treatment resulted in a significant increase in the number of dead cells in culture. Using the pan caspase inhibitor Z-VAD.fmk, we could prevent apoptotic but not necrotic cell death in normal kidney (NK-72) and Jurkat cell lines. Therefore, apoptosis-resistant tumor cells may be eliminated through an alternative nonapoptotic pathway providing new targets for antitumor drug development.

Materials and Methods

Cell Lines and Culture Conditions. Three established and well-characterized RCC lines (RCC-7, RCC-26B, and RCC-48; Ref. 9) were cultured in RPMI 1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% FCS (Hyclone, Logan, UT), l-glutamine (2 mM), gentamicin (50 μg/ml), sodium pyruvate (1 mM), and nonessential amino acids (0.1 mM).

Antibodies and Reagents. Antibodies used in Western blotting for caspase-3 and caspase-7 were obtained from Transduction Laboratories (Lexington, Kentucky). Antibody to caspase-8 was purchased from PharMingen (San Diego, CA). Antibody to caspase-10 was purchased from Alexis Corp. (San Diego, CA). Anti-actin antibody and TPEN were obtained from Sigma Chemical Co. (St. Louis, MO). Secondary horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit antibodies were purchased from Amersham (Arlington Heights, IL). Broad spectrum caspase inhibitor Z-VAD.fmk was purchased from Calbiochem (San Diego, CA).

Measurement of Apoptosis and Necrosis. DNA fragmentation was detected using The Phoenix Flow Systems, Inc. (San Diego, CA) APO-BRDU kit, according to the protocol provided with the kit. Briefly, cells were harvested, washed in PBS, resuspended in 1% paraformaldehyde for 15 min on ice, rinsed twice with ice-cold PBS, and fixed in 70% cold ethanol overnight. The fixed cells were washed twice in wash buffer, incubated with 50 μl of DNA labeling solution containing 10 μl terminal deoxynucleotidyl transferase reaction buffer, 0.75 μl of terminal deoxynucleotidyl transferase enzyme, 8 μl of Br-dUTP, and distilled H2O. Cells were rinsed prior to resuspending with fluorescein-PRB-1 antibody solution and analysis by flow cytometry in the presence of PI/RNase solution (0.5 ml). All analyses were performed on 3,000 to 10,000 events on the FACScan (Becton Dickinson) using an argon ion laser (Cytofics) with 15 mW of 488 nm excitation. Live gating of the forward and

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4 The abbreviations used are: TPEN, N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine; FACS, fluorescence-activated cell sorter; RCC, renal cell carcinoma; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
orthogonal scatter channels was used to exclude debris and to selectively acquire cell events. All values presented are based on percentage of cells as determined by light scatter. Individual fluorescence populations were determined through the use of acquisition and contouring/quadrant analysis software (Cell Quest, Becton Dickinson). Determination of dead cells was performed by FACS analysis of PI-stained nuclei as described previously (10).

Apoptosis was also determined by conventional light microscopy for morphological changes. Specifically, cytospin samples were assessed for the cellular and nuclear changes characteristically associated with apoptotic cell death (cell shrinkage, chromatin condensation, and karyorrhexis).

Western Blot Analysis. Cells were lysed as described previously (11) directly in buffer [50 mM Tris (pH 7.6), 150 mM NaCl, and 1% Triton X-100] containing protease inhibitors 5 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Samples were placed on ice for 20 min with occasional vortexing. Protein concentration was measured with a commercial kit (Bio-Rad, Richmond, CA). Equivalent amounts of proteins from whole-cell lysates (30 μg) were mixed with an equal volume of 2× Laemmli sample buffer, boiled and resolved by electrophoresis in 10% SDS-polyacrylamide gels (SDS-PAGE). The proteins were transferred from the gel to a nitrocellulose membrane using an electroblotting apparatus (Bio-Rad; 15 V, 3 mA/cm² for 24 min). Membranes were incubated in blocking solution containing 5% nonfat dry milk, then in Tris-buffered saline overnight to inhibit nonspecific binding. The membranes were then incubated with specific antibody (1–3 μg/ml) for 2 h. After washing in Tris/0.1% Tween 20 for 30 min, membranes were incubated for another 30 min with horseradish peroxidase-conjugated secondary antibody. The membranes were then washed and developed with enhanced chemiluminescence (ECL Western Blotting kit; Amersham). For densitometry scanning, the developed X-Omat AR film was placed on a white light box by Fotodyn, and its image was captured by a high resolution CCD camera (Sierra Scientific). Image 1.57 was the program used to analyze the density of each band by graphically plotting the images and calculating the area under each peak.

Results and Discussion

Resistance to Apoptosis Correlates with Reduced Levels of Caspase Expression in Human Renal Cell Carcinoma. Resistance of tumor cells to anticancer therapies may result from failure to activate apoptotic pathways in response to drug treatment. The anti-tumor effect of CTLs, an important effector of the cellular immune response, is executed primarily through the stimulation of the apoptotic program in target cells via the perforin/granzyme B or CD95/CD95 ligand pathways. Two basic apoptotic pathways have been described previously. The first involves the ligation of death receptors, resulting in the recruitment of adaptor proteins and caspase-8 and caspase-10.

![Graph showing caspase expression](image-url)
caspase-10, which then act to cleave and activate downstream caspases. In the second, cellular stress triggers mitochondrial release of cytochrome c, followed by activation of caspase-9. Both pathways converge on downstream effector caspases such as caspase-3 and caspase-7 (12, 13).

Human tumor cell lines derived from patients with RCC and other malignancies demonstrate resistance to apoptosis induced by soluble CD95-L or antibody to CD95, despite expressing CD95 (14, 15). Whereas drug-induced apoptosis in tumor cells is believed to be mediated in part by the CD95 receptor/ligand system (16), tumor cell resistance to apoptotic signals mediated via death receptors correlates with resistance to apoptosis induced by anticancer drugs (15). Furthermore, failure of tumors cells treated with cytotoxic agents to undergo apoptosis corresponds to total resistance to cell death in response to these agents. Treatment of caspase-deficient RCC tumor cell lines with conventional chemotherapeutic agents such as etoposide, paclitaxel, methotrexate, 5-fluorouracil, and gemcitabine failed to induce either necrosis or apoptosis (data not shown). Therefore, we investigated whether renal carcinoma cell lines resistant to CD95-triggered apoptosis were sensitive to apoptosis induced by the intracellular zinc chelator TPEN, which mediates programmed cell death independently of CD95 signaling events. This may be explained by the effects of zinc on mitochondrial function (17).

For these studies, we treated three RCC lines with TPEN using a human normal kidney cell line (NK-72) and Fas-sensitive Jurkat T lymphocytes as controls. As shown in Fig. 1, after treatment with 25 μM TPEN for 24 h, all normal kidney cells and Jurkat T cells underwent apoptosis (97.9 and 99.9%, respectively) as assessed by TUNEL assay, whereas only a small number of tumor cells in each cell line tested had apoptotic DNA fragmentation. We next studied whether resistance to apoptosis

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in response to TPEN in tumor cells correlated with reduced levels of caspase expression. The expression of effector caspases (i.e., caspase-3 and caspase-7) as well as the expression of proximal members of caspase family (caspase-8 and caspase-10) was evaluated in RCC lines and control cells by Western blotting using specific anti-caspase antibodies. Results of this study are shown in Fig. 2A. In lysates from normal kidney cells (NK-72) and Jurkat T lymphocytes, the expression of all caspases evaluated was easily detected. In contrast, all RCC lines had significantly reduced levels of caspase-3 expression. Relative to levels of other caspases, expression in RCC lines was more variable, which was confirmed by densitometry scanning of Western blots (Fig. 2B). Reprobing the blots with an antiserum specific for actin confirmed loading equivalent amounts of proteins for all cell lines.

The data presented here demonstrate reduced but variable levels of caspase expression in RCC lines. The relative resistance to apoptosis in cases of RCC does not reflect simply diminished expression of any one particular caspase. However, because the activation of upstream initiator caspases is requisite for subsequent activation of downstream effector caspases, defects in any given member of the cascade predicts failure of subsequent downstream events.

TPEN Induces Necrosis in Apoptosis-resistant RCC Lines. Under normal conditions, alterations in mitochondrial membrane structure and function trigger apoptosis. However, under conditions in
which caspase expression or activation is precluded, mitochondrial dys-
function invariably leads to cell death, albeit by necrosis rather than ap-
apoptosis (8). Here we show that RCC lines with deficient expression of
certain members of the caspase family are almost completely resistant to
apoptosis induced by TPEN. Morphological studies show that caspase-
competent Jurkat T cells displayed nuclear fragmentation in response to
treatment with TPEN, whereas nuclei of caspase-deficient renal carci-
noma cells did not (Fig. 3A). However, although the nuclei of tumor cells
were not fragmented, they remained condensed, and all cells detached
from the plate after TPEN treatment with loss of cell viability (Fig. 3A).
To further investigate whether these cell lines underwent nonapoptotic
cell death, we used PI staining and FACS analysis. Cell membranes in
viable cells are impermeable to PI. In contrast, only dead cells whose
plasma membrane integrity has been disrupted, as is typical of necrotic
cells, incorporate PI. The RCC samples described in Fig. 1 were next
stained with PI and analyzed by flow cytometry. As demonstrated in Fig.
3B, in contrast to the slight effect on the induction of apoptosis, TPEN
Treatment resulted in substantial accumulation of PI-positive cells in all
RCC lines evaluated (RCC 7, 11.5% versus 49.3%; RCC 26B, 21.7% versus
64.3%; and RCC 48, 19.4% versus 50.5%). These data support the idea that
cell death can proceed in cells with reduced levels of caspase
expression or activation and raise the possibility that some pathways
leading to cell death do not require the action of any caspases. In these
instances, activation of specific caspases may be a byproduct of cell death
rather than an indispensable component. An exception is cell death
induced by ligation of death receptors, which is completely dependent on
proper caspase level and function (18). Our results indicate that although
tumor cells with almost complete loss of caspase-3 expression and
variable reduction in the expression of other caspases are almost com-
pletely resistant to apoptosis in response to TPEN, they are still capable
of dying from necrosis. Additional experiments aimed at understanding
the role that mitochondrial alterations play in mediating caspase-inde-
dependent cell death may have important implications for the development
of new therapeutic strategies to overcome the resistance of tumor cells to
apoptosis induced by conventional therapies. Careful monitoring of both
modes of cell death is critical for accurately evaluating the cytotoxic
response of antitumor agents.

**TPEN Induces Necrosis in NK-72 Cells and Jurkat T Lymphocytes When the Apoptotic Mode of Death Is Prevented.** It now
appears that cell death can proceed in the absence of caspase activa-
tion (19). Whereas inhibition of caspases can prevent nuclear ap-
apoptotic changes, it will not de facto prevent irreversible loss of cellular
function. Recent observations provide direct evidence that the ability
to induce apoptosis is a primary site for Bcl-2 regulation of apoptosis.
Thus, it is crucial to understand the role of specific participants of these pathways in life and death decisions.

Deficient expression of particular caspases documented in various
malignancies may give tumor cells a significant survival advantage
only in situations where apoptosis is initiated through death receptors
such as CD95 and tumor necrosis factor receptor-1. Nevertheless,
death in these tumor cells will invariably proceed when induced by
agents triggering mitochondrial changes directly. In contrast, tumor
cells overexpressing antiapoptotic proteins such as Bcl-2 and Bcl-x,
which act at the level of mitochondria (21), are protected from both
modes of death, apoptosis and necrosis. Additional studies of caspase-
dependent and -independent death pathways aimed at understanding
the role of specific participants of these pathways in life and death
decisions may provide new targets for antitumor drug development
and may help predict the efficacy of new therapeutic agents more
accurately.

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