Increased Immunogenicity of Colon Cancer Cells by Selective Depletion of Cytochrome c

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ABSTRACT

We and others have previously reported in an in vivo rat colon cancer cell model that cell death precedes and is necessary for the development of a specific antitumoral immune response. To sensitize colon cancer cells to death, we depleted cytochrome c by stable transfection with an antisense construct. Cytochrome c depletion sensitizes human and rat colon cancer cells to a nonapoptotic, nonnecrotic death induced by various stimuli. This increased sensitization to a necrosis-like cell death may be related to a decrease in cellular ATP levels and an increase in reactive oxygen species production caused by cytochrome c depletion. In vivo, depletion of cytochrome c decreases the tumorigenicity of colon cancer cells in syngeneic rats without influencing their growth in immune-deficient animals. Furthermore, decreased expression of cytochrome c in tumor cells facilitates in vivo “necrotic” cell death and the induction of a specific immune response. These results delineate a novel strategy to sensitize colon cancer cells to chemotherapy and to increase their immunogenicity in immuno-competent hosts.

INTRODUCTION

The ability of colon cancer cells to elicit a T-cell-dependent immune response was suggested to depend on their sensitivity to death, either by apoptosis or by necrosis (1, 2). We and others (3–5) have shown that expression of antiapoptotic proteins such as Bcl-2 and Hsp27 could decrease colon cancer cell immunogenicity in syngeneic animals, thus increasing their tumorigenicity. On the other hand, we have demonstrated that selective depletion of heat shock protein HSP70 could sensitize a poorly immunogenic and strongly tumorigenic colon cancer cell line to death and facilitate induction of an immunogenic response in syngeneic animals (6).

Apoptosis is a conserved, energy-dependent programmed cell death pathway used to eliminate cells in response to stress as well as excessive cells in developing multicellular organisms. One of the central molecules in apoptotic pathways is cytochrome c, which upon release from the intermembrane space of the mitochondria in response to a variety of apoptotic stimuli activates the caspase cascade in the cytosol (7, 8). In the mitochondria, cytochrome c is the only water-soluble component of the electron transfer chain and participates in the reduction of oxygen by cytochrome c oxidase, the terminal and putative rate-limiting step of electron transfer (9). Once in the cytosol and in the presence of ATP, cytochrome c binds the adaptor molecule apoptosis protease activating factor 1 (Apaf-1) with a high affinity and triggers its oligomerization to form the apoptosome complex in which apoptosis protease activating factor 1 (Apaf-1) with a high affinity and triggers its oligomerization to form the apoptosome complex in which caspase-9 is recruited and activated (10–13). Activated caspase-9 in turn cleaves and activates caspase-3, caspase-6, and caspase-7, which function as downstream effectors of the cell death program (14). The components of this pathway have been conserved throughout evolution of metazoan organisms (15). Murine embryos devoid of cytochrome c demonstrate profound developmental delay and do not survive beyond day 10.5 post coitum. Furthermore, cell lines derived from early embryos of the cytochrome c-deficient genotype have reduced proliferation and viability and need special media to support ex vivo culture (16).

Whether cytochrome c could be used as a specific target for modulating cell death and immunogenicity in tumor cells has not been investigated. The present study explores the consequences of cytochrome c down-regulation, obtained by the use of an antisense strategy, in rat and human colon cancer cells. We show that cytochrome c depletion sensitizes these cells to a nonapoptotic, nonnecrotic death induced by various stimuli in vitro and enhances their immunogenicity and tumorigenicity when injected to syngeneic animals. These results demonstrate the potential interest of targeting cytochrome c for treating colon cancers (17).

MATERIALS AND METHODS

Cells, Plasmids, and Transfections. Human colorectal cancer cell lines HT29 and HCT116, rat colorectal cancer cell line PRO, and mouse melanoma cell line B16 were grown as monolayers in a controlled atmosphere (37°C, 5% CO2) using FCS-supplemented (10%, v/v) DMEM, EMEM, and RPMI, respectively (BioWhittaker, Fontenay-sous-Bois, France). When indicated, we also tested glucose-free RPMI (BioWittaker). Cells were stably transfected with a CDNA encoding cytochrome c introduced in an antisense orientation in pTARGET (Promega, Charbonnières, France). The same construct was used in human, rat, and mouse cell lines, due to the highly conserved sequence of cytochrome c. Exponentially growing cells were transfected with 5 μg of DNA vector mixed with 30 μl of Superfect react (Qiagen GmbH, Hilden, Germany), and pools of cells resistant to G418 (Sigma-Aldrich, Steinheim, Germany) were analyzed for cytochrome c expression by immunoblot.

Immunoblotting. Immunoblots were performed as described previously (18). In brief, whole cell extracts were prepared by lysing the cells with 2% SDS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and NaCl/Pi (pH 7.4). Protein concentration was measured in the supernatant by using the micro bicinchoninic acid (BCA) protein assay (Pierce, Ansinières, France). Proteins were separated in 8–12% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes (Bio-Rad, Ivry sur Seine, France) after blocking non-specific binding sites with 5% nonfat milk in PBS and 0.1% Tween 20, blots were incubated with specific antibodies (Abs), washed in PBS and 0.1% Tween 20, incubated for 30 min at room temperature with horseradish peroxidase-conjugated goat antimouse Ab (Jackson ImmunoResearch Laboratories, West Grove, PA), and revealed following the ECL Western blotting analysis procedure (Amersham Biosciences, Les Ulis, France). Autoradiographs were recorded onto X-Omat AR films (Eastman Kodak, Rochester, NY), a Bioprofil system (Vilber Lourmat, Marne La Valée, France) was used for quantification, and analysis was performed within the range of proportionality of the film. All of the Western blots were repeated three times. Abs used include a mouse anti-cytochrome c monoclonal Ab (mAb; Pharmingen, Le Pont de Claix, France), a mouse anti-apoptosis inducing factor (AIF) polyclonal Ab (Chemicon, Soufflaweysheim, France), and an antihuman HSP60 mAb (Stressgen, victoria, Canada).
Cell Death Analysis. Adherent cells (5 × 10^2 cells/well) were plated onto 6-well culture plates in complete medium for 24 h and then treated for 24 h with cisplatin (50 μg/ml), etoposide (100 μM), doxorubicin (50 μg/ml), or staurosporine (200 nm) in the presence or absence of 40 μg/ml zVAD-fmk (Bachem, Basel, Switzerland). The percentage of dead cells was determined by analyzing 10^5 cells stained with propidium iodide by flow cytometry, using a FACS Scan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Cell Cycle Analysis. Detached cells collected from the supernatant and adherent cells recovered by trypsinization were pooled, fixed with ethanol, washed in PBS, and resuspended in PBS containing RNase (100 μg/ml) and propidium iodide (10 μg/ml). After digestion of cellular RNA, cells were pelleted and resuspended in fresh PBS containing propidium iodide (10 μg/ml). Data were acquired on a FACS Scan flow cytometer (Becton Dickinson) and analyzed by using the Cellquest software.

ATP Concentration. Quantification of cellular ATP was performed by using the luciferin-luciferase method (ATP bioluminescence assay kit HS II, no. 1 699 709; Roche Diagnostic, Mannheim, Germany) according to the protocol provided by the manufacturer. Each point was measured in duplicate.

Caspase Activation in Cell-Free Extracts. Cell-free extracts (5–10 mg/ml protein), prepared as described previously (18), were incubated with 5 μM horse heart cytochrome c (Sigma-Aldrich) and 1 mM dATP (Pharmacia, Orsay, France). Caspase-3 and caspase-9 activities were determined by measuring the cleavage of N-acetyl-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-asparticamide, 7-amino-4-trifluoromethylcoumarin (DEVD-AFC) and N-acetyl-CBZ-L-leucyl-L-glutamyl-L-histidyl-L-aspartic acid amide, 7-amino-4-trifluoromethylcoumarin (LEHD-AFC) fluorometric substrates, respectively (Calbiochem, San Diego, CA). AFC released from the substrates were excited at 400 nm, and emission was measured at 505 nm.

Identification of Autophagic Vacuoles. The presence of autophagic vacuoles was analyzed by fluorescence microscopy after staining with monodensylcaverin added to the culture medium at a final concentration of 0.05 μM. After 15 min of incubation at 37°C, cells were collected by centrifugation and resuspended in culture medium, and 50 μl of cell suspension were applied to glass slides, coverslipped, and immediately examined under a Leitz microscope equipped with an epi-illuminator and appropriate filters (Leica, Bron, France). For each sample, 300 cells were examined.

Quantification of Reactive Oxygen Species (ROS) Content. Cells were incubated for 15 min at 37°C in the presence of 6.6 μM dihydro-ethidium (Sigma-Aldrich) and analyzed by flow cytometry using FACS Scan flow cytometer (Becton Dickinson). For each sample, 10^5 cells were acquired.

Flow Cytometric Measurement of the Mitochondrial Transmembrane Potential. Cells were treated or not with 50 μM of the uncoupler carbonyl cyanide-3-(trifluoromethoxy)phenyl hydrazone (ICN Laboratories) for 45 min at 37°C in standard medium containing with 150 μM tetramethylrhodamine ethyl ester perchlorate (Molecular Probes) for ΔΨm quantification and 2.5 μM/ml 4′,6-diamidino-2-phenylindole dihydrochloride (Molecular Probes) for determination of cell viability. We used a FACS Vantage (Becton Dickinson).

Immunoﬂuorescence. Cells were cultured on coverslips at 1.5 × 10^3 cells/well in a 24-well plate in the presence or absence of 1 μM staurosorpin (Sigma-Aldrich) for 16 h, fixed with paraformaldehyde 4% for immunofluorescence assays using either an anti-AIF (Ab 16501; Chemicon) or an anti-HSP60 (Ab SR-B805; Medical and Biological Laboratories) rabbit polyclonal antibody. Cells were permeabilized with 0.1% Triton X-100 and incubated with a goat antirabbit IgG conjugated with Alexa Fluor. At the same time, coverslips were stained with 2 μM Hoechst 33342 (Sigma-Aldrich).

Cell Growth Analysis in Vitro. Cells (2 × 10^4) were plated in tissue culture dishes (24-wells plates) on day 0, in RPMI or glucose-free RPMI and counted at indicated times using a hemocytometer. The results are the mean of four independent determinations for each time point.

Tumor Growth Analysis in Vivo. Exponentially growing cells were harvested, washed in PBS, and suspended in RPMI (10^6 cells in 200 μl) before s.c. injection into the anterior thoracic wall of syngeneic BDIX rats or in the back of nude rats. Tumor volume was evaluated weekly, using a caliper to measure two perpendicular diameters.

Histological Study of the Tumor Cell Injection Site. Animals were killed 15 days after cell injection. The site of tumor cell injection was resected and either fixed in formaldehyde (4% in PBS) and embedded in paraffin or embedded in Tissue-Tek (Miles, Elkhart, IN) and snap-frozen in methylbutane that had been cooled in liquid nitrogen. Apoptotic cells were labeled on 5 μm paraffin-embedded sections using an anticleaved caspase-3 Ab (Cell Signaling). Tumor cells on slides stained with hemalum-eosin were distinguished from inflammatory cells according to their size, their large nuclei with over-sized nucleoli, and their assembly in nodules. In addition, PRO tumor cells were immunolabeled with 12C mAb. An immunohistochemical study of tumor-infiltrating inflammatory cells was performed on acetone-fixed 5-μm cryostat sections. Mouse mAbs to rat macrophages (ED2), monocytic/dendritic cells and immature macrophages (ED1), CD8+ T-cells (OX8), CD4+ T-cells (W3/25), natural killer (NK) cells (3-2-3), and T-cell receptor αβ (R7/3) were obtained from Serotec (Oxford, United Kingdom). Sections were incubated with mAb and biotinylated sheep Ab to mouse IgG (Amersham Biosciences), subsequently incubated with streptavidin-peroxidase, and stained with aminochinolcarbazole.

Ex Vivo Cytotoxicity Assays. Macrophages (ED2+) NK cells (3-2-3+), and CD8+ T cells (OX8+) were purified from 18-d-old tumors obtained from rats given injections of either control PRO or cytochrome c antisense PRO cells (19) and then cocultured with parental PRO cells at a 1:4 ratio for 48 h before measuring parental PRO cell viability by using a crystal violet colorimetric assay.

RESULTS AND DISCUSSION

Selective depletion of cytochrome c modifies colon cancer cell sensitivity to death insults. HT-29 and HCT116 human colon cancer cells, and PRO rat colon cancer cells were stably transfected with either a construct encoding cytochrome c cDNA in an antisense orientation or an empty vector. Cytochrome c expression was measured in pools of populations resistant to G418 (Fig. 1A). Antisense transfected cells in which cytochrome c protein level was strongly decreased (up to 9-fold), as compared with both control-transfected and parental cells, were selected for subsequent experiments. Expression of the cytochrome c antisense construct did not affect the expression of other mitochondrial proteins involved in the apoptotic process such as AIF and HSP60 (Fig. 1A). These cytochrome c-depleted cells were viable and required no special media for survival. In glucose-free medium, cell growth was strongly delayed in the control cells and almost completely inhibited in cytochrome c-depleted cells, indicating that a decrease in cytochrome c content enhanced PRO cell sensitivity to glucose-depletion-induced growth arrest. However, in complete medium, cytochrome c depletion did not significantly influence tumor cell growth in culture (Fig. 1B) or their cell cycle distribution by flow cytometry (Fig. 1C).

Even though cytochrome c depletion did not induce an increase in spontaneous cell death under normal culture conditions, it increased the sensitivity of the three studied cell lines to death induced by exposure to commonly used anticancer drugs such as cisplatin, etoposide, and doxorubicin as well as to staurosorpin. As expected, death induced by the tested anticancer agents and staurosorpin was prevented by cotreatment with 40 μM zVAD-fmk in parental (not shown) and control-transfected (Fig. 1D) cells, but the large spectrum caspase inhibitor failed to inhibit the death process in cytochrome c antisense-transfected cells treated in similar conditions (Fig. 1E).

Accordingly, we failed to detect any significant cleavage of two peptides that mimic caspase target sites, i.e., DEVD-AFC and LEHD-AFC, in cytosolic extracts of cytochrome c-depleted cells treated with cisplatin or staurosorpin (Fig. 2A). On the other hand, addition of cytochrome c and dATP activated caspases similarly in cytosolic extracts of both control and cytochrome c-depleted cells (Fig. 2B).

In control cells, death stimuli induced the release of cytochrome c from the mitochondria to the cytosol (Fig. 3A), which was hardly observed in cytochrome c-depleted cells (Fig. 3A). Surprisingly, AIF was only minimally released in these cells with no difference observed between control and cytochrome c-depleted cells (Figs. 3A and B). Cytochrome c down-regulation did not affect the mitochondrial

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Transmembrane potential \( \Delta \psi_m \) (Fig. 3C) or prevent the \( \Delta \psi_m \) decrease induced by exposure to staurosporine or menadione (Fig. 3D).

In contrast to cell death in control cells, death in cytochrome c-depleted cells was not associated with chromatin condensation measured by Hoechst staining (Fig. 4A), DNA laddering (not shown), or phosphatidylserine exposure on the outer leaflet of the plasma membrane as measured by annexin V staining (Fig. 4B). After a 24-h exposure to either cisplatin or staurosporine, annexin-V\(^{+}/\Pi^{+}\) (apoptotic) cells were 2–3-fold lower in cytochrome c-depleted cells compared with the controls. However, the number of annexin-V\(^{+}/\Pi^{+}\) was 3-fold higher in the cytochrome c-depleted cells, suggesting plasma membrane disruption (Fig. 4B). We then looked at the presence of autophagic vacuoles in cytochrome c-depleted and control cells by monodensylcadaverin staining. The number of monodensylcadaverin-stained vacuoles was not increased in cytochrome c-depleted cells exposed to cisplatin as compared with control cells treated in the same conditions (Fig. 4C). Altogether, these results suggested that cytochrome c depletion increased the ability of colon cancer cells to die in response to various stresses such as anticancer drugs and that the death process observed in cytochrome c-depleted cells was neither apoptosis nor autophagy. Actually, disruption of the plasma membrane suggested that these cells died by necrosis. Mitochondria are a major source of cellular ATP (20). The cellular level of ATP has been proposed to play a role in the mode of cell death, e.g., an ATP depletion induced by culturing cells in the absence of glucose to block glycolysis-dependent ATP production and in the presence of oligomycin B to directly inhibit mitochondrial ATP production can induce a shift from apoptosis to necrosis in response to various stimuli (21–24). Using a chemiluminescent assay based on luciferase-catalyzed, ATP-dependent oxidation of luciferin, we observed that cytochrome c depletion was associated with a decrease in cellular ATP content (Fig. 5A). Mitochondria are also a source of ROS (25). As with ATP depletion, an increase in ROS production can induce a shift from apoptosis to necrosis (e.g., when caspases are inhibited in tumor necrosis factor-\( \alpha \)-treated L929 cells; Ref. 26) or a shift from differentiation to necrosis (e.g., when caspases are inhibited in TPA-treated U937 cells; Ref. 27). By using dihydroethidium in a flow cytometry assay, we observed that cytochrome c depletion increased superoxide anion production induced by exposure to staurosporine and menadione (Fig. 5B). The decrease in ATP cellular content and the increase in stress-induced ROS production induced by down-regulation of cytochrome c could contribute to the shift from an apoptotic to a necrotic mode of death in response to various toxic insults.
Depletion of Cytochrome c Inhibits PRO Cell Tumorigenicity in Syngeneic Animals and Induces a Specific Antitumor Response.

The mechanism of cell death has been proposed to influence the subsequent immune response to the tumor. In view of the observed switch from apoptotic to necrotic cell death in cytochrome c-depleted PRO cells exposed to stresses, we analyzed whether cytochrome c depletion would affect tumor growth in vivo. Wild-type (PRO-Wt), control transfected (PRO-C), and cytochrome c-depleted (PRO-AS) PRO cells were injected s.c. into syngeneic BDIX rats (1 × 10⁶ cells/rat). The two control cell populations (PRO-Wt and PRO-C cells) yielded progressive and lethal tumors that developed with a similar kinetics, whereas two distinct populations of PRO-AS cells (see Fig. 1A) gave rise to tumors that slightly progressed during the first 2 weeks and then regressed completely by 4–5 weeks after tumor cell injection (Fig. 6A). When injected s.c. into athymic nude rats, the four cell populations gave rise to tumors that developed with similar kinetics (Fig. 6B). Altogether, these observations suggest that down-regulation of cytochrome c endowed the normally immune-tolerated PRO cells with immunogenicity, thus decreasing their tumorigenicity. Similar observations were made in a very different cellular model, the melanoma B16 cell line. Parental or control-transfected B16 cells when injected in syngeneic C57black6 mice induced highly tumorigenic tumors, whereas B16 clones in which cytochrome c had been down-regulated by the antisense strategy induced very small tumors with a strong delay in their progression (not shown).

To further demonstrate that regression of PRO-AS tumors was associated with induction of a specific immune response, rats were given injections of PRO-Wt, PRO-C, or PRO-AS cells and rechallenged 15 days later with PRO-Wt cells on the contralateral side. Although a second tumor appeared in every animal bearing a PRO-Wt or a PRO-C tumor, none of the rats initially given injections of PRO-AS cells developed a second tumor (Fig. 7A). When the rats were rechallenged with syngeneic GV1A1 glioma cells instead of PRO-Wt cells, all of the animals developed a second progressive
tumor, including those initially given injections of PRO-AS cells (Fig. 7B). When injected into athymic nude rats, PRO-AS cells induced tumors that did not regress and failed to protect from a challenge with parental cells on the contralateral side (not shown). Altogether, these results suggested that cytochrome c depletion in a colon cancer cell line facilitated induction of a specific, T-cell-dependent immune response.

Depletion of Cytochrome c Results in Increased Tumor Cell Death in Vivo. Hemalum-eosin staining of tumor sections, performed on days 5 and 15 after tumor cell injection in BDIX rats, identified foci of dead cells in PRO-AS tumors, but failed to identify such foci in PRO-Wt or PRO-C tumors (Fig. 8A). Furthermore, in agreement with our in vitro data, PRO-AS tumor sections did not stain with an Ab specific for the active caspase-3 (Fig. 8B), suggesting a caspase-independent in vivo cell death.

Interestingly, an extensive immunohistochemical analysis of tumor sections obtained at day 15 after PRO cells injection in BDIX rats, in which tumor cells were identified by using the 12C mAb, demonstrated that PRO-AS tumor nodules were infiltrated with 3-2-3/ED2 macrophages; ED1+ monocyte/dendritic cells; and R7/3 (T-cell receptor), OX8+ (CD8), and W3/25+ (CD4) T cells. Conversely, in control tumors, NK cells were almost absent, and macrophages and T cells remained at the periphery of control tumor nodules (Table 1; Fig. 9). These observations suggested that immune cells infiltrating tumors induced by cytochrome c-depleted cells could be responsible for their regression.

Increased Cytotoxicity of Macrophages Infiltrating Tumors Induced by Cytochrome c-Depleted Cells. To determine whether these infiltrating immune cells were responsible for the regression of PRO-AS tumors in syngeneic animals, we isolated NK cells (3-2-3/ED2), macrophages (ED2+), and CD8 T cells (OX8+) from tumors that developed in rats given injections of either control or cytochrome c-depleted cells (five tumors in each group), and we tested the ex vivo...
cytotoxic activity of these cells toward parental PRO cells. We observed that NK cells and CD8-positive T cells isolated from tumors obtained from PRO-AS cells were not significantly more cytotoxic than those isolated from tumors obtained with PRO-C cells. In contrast, macrophages isolated from tumors obtained with PRO-AS cells were strongly cytotoxic compared with those isolated from control tumors (Fig. 10). Thus, macrophages that infiltrate PRO-AS tumors and remain at the periphery of PRO-C tumors (Table 1; Fig. 9) appear to be responsible for the death of cytochrome c-depleted tumor cells in vivo.

**Concluding Remarks.** Deletion of cytochrome c-encoding gene induces early embryonic death in mice, probably as a consequence of mitochondrial oxidative phosphorylation breakdown (16). Similarly, cell lines generated from cytochrome c-deficient mice are not viable unless grown in conditions that compensate the deficit in mitochondrial respiration (16). In the present study, we show that a partial depletion in cytochrome c is not toxic per se in colon cancer cells but strongly sensitizes these cells to a variety of death stimuli in vitro. In addition, cytochrome c depletion was observed to enhance tumor cell immunogenicity in syngeneic animals, thus decreasing their tumorigenicity. Altogether, these observations suggest that decreasing cytochrome c expression could be an attractive strategy to sensitize colon

![Figure 8](image8.png)  
**Fig. 8.** Depletion of cytochrome c increases PRO cell sensitivity to death in vivo. Sections of tumors obtained 5 (D5) and 15 (D15) days after s.c. injection of PRO-C and PRO-AS1 cells were stained with hemalum-eosin (A) or an anti-active caspase-3 antibody (B). One representative experiment out of seven is shown.

![Figure 9](image9.png)  
**Fig. 9.** Cytochrome c depletion induces changes in tumor infiltrating inflammatory cells. Tumor sections were performed 15 days after s.c. injection of PRO-C and PRO-AS1 (PRO-CytcAS1) cells in syngeneic BDIX rats. Macrophages, CD8+ lymphocytes, and NK cells were labeled by using ED2, OX8, and 3-2-3 (3/2/3) antibodies, respectively.

![Figure 10](image10.png)  
**Fig. 10.** Increased cytotoxicity of macrophages isolated from PRO-AS tumors. PRO parental cells were cocultured at a 1:4 ratio with macrophages (ED2-positive cells), NK (3-2-3-positive cells), or CD8 (OX8-positive) T cells recovered from control tumors (PRO-C tumors; □) or tumors induced by cytochrome c-depleted PRO cells (PRO-AS1 tumors; ■), with five tumors per group. After 48 h, tumor cell viability was measured by a crystal violet colorimetric assay (mean ± SD, n = 4).

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<th>Table 1 Immunohistochemical analyses of tumor-infiltrating inflammatory cells</th>
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<td>Semi-quantitative evaluation of antigen expression and location in tumor sections studied 15 days after s.c. injection of 1 × 10⁶ PRO-C and PRO-AS1 cells in syngeneic rats. Results were obtained from 10 sections for each tumor.</td>
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cancer cells to chemotherapy and to increase their immunogenicity in immuno-competent hosts.

The death of cancer cytochrome c-depleted cells is not inhibited by a broad spectrum caspase inhibitor used at concentrations that prevent apoptotic cell death in control cells (18, 28). Accordingly, no caspase activity was detected in these cells induced to die by various stimuli, even though caspases could be activated by cytochrome c when added to cell-free extracts in the presence of ATP (28, 29). Thus, specific down-regulation of cytochrome c is sufficient to prevent activation of the caspase cascade in response to the tested toxic insults, suggesting that no other protein can substitute for cytochrome c to promote oligomerization of Apaf-1 and caspase activation in these conditions. These results are consistent with observations made in murine embryonic fibroblasts from cytochrome c knockout mice when exposed to death stimuli under culture conditions that compensate for the deficit in mitochondrial respiration (16).

Colon cancer cells in which cytochrome c was down-regulated die by necrosis when exposed to a toxic insult, as indicated by the lack of mitochondrial release of AIF, involved in caspase-independent apoptosis, the absence of nuclear chromatin condensation, and the disruption of the plasma membrane. Previous observations suggest that the decrease in cellular ATP content that deregulates the cellular energy balance and the increase in ROS production in response to death stimuli may account for the switch from apoptosis to necrosis induced by cytochrome c depletion (23, 26, 27).

The tumorigenic and tolerogenic PRO model has proved to be a useful model to study the role of cancer cells death in the development of an antitumor response (30, 31). When injected in syngeneic rats, these cells give progressive, lethal tumors that do not illuminate the host against an additional challenge (32). Here, we show that depletion of cytochrome c is sufficient to provide these cells the ability to trigger a specific immune response. This capacity may be related to the increased sensitivity of PRO cells to death because tumor cell death has been shown to facilitate induction of a specific antitumor immune response (3, 4).

The mode of death involved in the immunogenic process remains a controversial issue (2, 33, 34). Although apoptotic tumor cell death could elicit a specific immune response in some conditions, the lack of plasma membrane disruption prevents the onset of an inflammatory response, and additional signals are required. In contrast, disruption of the plasma membrane of necrotic cells could generate signals that recruit, load, activate, and mature appropriate subsets of antigen-presenting cells, thus stimulating macrophage antitumor activity that eradicates the tumor (2, 35). Thus, cytochrome c depletion in colon cancer cells could facilitate induction of an efficient and specific immune response toward these cells by favoring necrotic death. Whether chemical agents that would deplete cellular ATP content and/or increase ROS production in tumor cells could also trigger tumor cell necrosis and induce a specific immune response merits additional investigation.

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28. Bruey JM, Ducasse C, Bonnourd P, et al. Hsp27 negatively regulates cell death by necrosis when added to cell-free extracts in the presence of ATP (28, 29). Thus, specific down-regulation of cytochrome c is sufficient to prevent activation of the caspase cascade in response to the tested toxic insults, suggesting that no other protein can substitute for cytochrome c to promote oligomerization of Apaf-1 and caspase activation in these conditions. These results are consistent with observations made in murine embryonic fibroblasts from cytochrome c knockout mice when exposed to death stimuli under culture conditions that compensate for the deficit in mitochondrial respiration (16).

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