Chemotherapy Induces Tumor Clearance Independent of Apoptosis

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Abstract
Dysregulation of apoptosis is associated with the development of human cancer and resistance to anticancer therapy. The ultimate goal of cancer treatment is to selectively induce cancer cell death and overcome drug resistance. A deeper understanding of how a given chemotherapy affects tumor cell death is needed to develop strategically designed anticancer agents. Here, we use a xenograft mouse tumor system generated from genetically defined cells deficient in apoptosis to examine the involvement of multiple forms of cell death induced by cyclophosphamide (CP), a DNA alkylating agent commonly used in chemotherapy. We find that although apoptosis facilitates tumor regression, it is dispensable for complete tumor regression as other forms of cell death are activated. Sporadic necrosis is observed in both apoptosis-competent and deficient tumors evident by tumor cell morphology, extracellular release of high mobility group box 1 protein, and activation of innate immune cells in CP-treated tumors. Our findings indicate that in apoptosis-deficient tumors, necrosis may play a fundamental role in tumor clearance by stimulating the innate immune response. [Cancer Res 2008;68(23):9595–600]

Introduction
Apoptosis, or Type I programmed cell death, plays a crucial role in the clearance of cells that have potentially harmful genetic mutations. The ability of a cell to evade apoptosis is a crucial step in tumorigenesis (1–3). Despite the fact that human cancers are defective in their apoptotic pathways, DNA alkylating agents remain among the most effective chemotherapeutic agents used clinically (4), suggesting that alternative cell death pathways are activated. Five main types of cytoidal and cytostatic mechanisms have been described in the context of cancer therapy: apoptosis, necrosis, mitotic catastrophe, senescence, and autophagy (2, 5–7). However, a comprehensive understanding of tumor cell death has been lacking due to the complexity of a tumor’s response to chemotherapy. Presently, most mechanistic studies of the anticancer activities of DNA alkylating agents have been conducted using cultured cells. It is therefore important to develop an in vivo system to evaluate the contribution of the alternative cell death pathways in anticancer therapy. Apoptosis is mainly controlled by two sets of molecules: the Bcl-2 family of proteins and the caspases.

Deletion of the proapoptotic Bcl-2 family members Bax and Bak is sufficient to prevent apoptotic events initiated by mitochondrial membrane permeabilization (8). We used these cells to address the question as to how conventional chemotherapy induces cancer cell death in vivo.

Materials and Methods
Cell lines. Immortalized wild-type and bax−/− bak−/− murine embryonic fibroblasts (MEF; ref. 8) were retrovirally transformed with E1A and K-Ras. The cells were s.c. injected into the back of 6- to 8-wk-old male athymic nude mice (Taconic Farms). When tumors formed, they were excised from the mice, minced, and digested with trypsin-EDTA (0.05%) and collagenase A (1 mg/mL) to generate stable tumor cell lines.

Antibodies. Bax (Santa Cruz), Bak (Upstate), Caspase 3 (Transduction Lab), cleaved Caspase 3 (Cell Signaling), γH2AX (Cell Signaling), high-mobility group box 1 (HMGB1; Abcam), β-tubulin (Sigma), IL-1β (R&D systems), F4/80 (Serotec), Neutrophil (Serotec), phycocerythrin (PE)-conjugated anti–Mac-1 (BD Biosciences), Alexa 488–conjugated anti–Mac-1 (BD Biosciences), APC-conjugated anti-F4/80 (eBiosciences), PE-conjugated anti–Gr-1 (BD Biosciences), and Alexa 488–conjugated anti–Gr-1 (Serotec).

Electron microscopy. The tumor samples for transmission electron microscopy (TEM) were collected immediately after sacrificing the mice to ensure tissue integrity. TEM was performed according to standard protocol by the Central Microscopy Imaging Center at Stony Brook University.

Xenograft mouse tumor experiments. Tumors were established by injecting 1 × 10⁶ tumor cells into the midflanks of nude mice. When palpable tumors formed, mice were randomly grouped and either left untreated or treated via i.p. injections of 170 mg/kg of cyclophosphamide monohydrate (CP; Sigma) every 5 d. The tumor length (l) and width (w) were measured every 4 to 5 d with electronic calipers. Tumor volume (v) was calculated using the formula: v = (l × w²)/2. The animals bearing untreated tumors were sacrificed post tumor inoculation before tumors reached 4 cm³ in size in compliance with the Stony Brook University Institutional Animal Care and Use Committee guidelines.

Statistical analysis. Data are represented as mean ± SE. Statistical analyses were performed using Microsoft Excel.

Results
Cyclophosphamide induces tumor regression in vivo independent of key apoptosis regulators. To study the contribution of different cell death pathways in the antitumor activity of chemotherapy, we first established an in vivo system to compare tumors derived from apoptosis-competent and deficient cells. E1A and K-Ras oncoproteins were used to transform genetically defined MEFs isolated from wild-type and bax−/− bak−/− mice (Fig. 1A). Moreover, the antiapoptotic protein Bcl-xL was expressed in wild-type cells (Fig. 1B). Stable tumor cell lines were generated from these MEFs that maintained their respective genotypes (Fig. 1A and B).

The stable tumor cells were injected into nude mice to test for their response to chemotherapeutic treatment in vivo. Tumors

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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developed from all three cell lines (Fig. 1C). Nine days after tumor implantation, CP was injected into the tumor-bearing mice i.p. at 170 mg/kg every 5 days. In response to CP, tumors derived from all three MEF lines stopped growing and progressively regressed, indicating that in vivo, DNA alkylating damage possesses antitumor activity independent of key apoptosis regulators Bax and Bak, and is unblockable by Bcl-xL (Fig. 1C).

To compare the response of apoptosis-proficient and deficient tumors to CP treatment in the same environment, the wild-type and \(bax^{-/-} bak^{-/-}\) tumor cells were labeled with green fluorescent protein (GFP) and red fluorescent protein (RFP), respectively, and injected bilaterally into the same animal. The tumors were monitored by a fluorescence imager (Fig. 1D). The treated wild-type and \(bax^{-/-} bak^{-/-}\) tumors both grew for an additional 3 to 4 d after the first CP treatment, then began to regress. The treated \(bax^{-/-} bak^{-/-}\) tumors showed an initial delay in response to CP compared with the wild-type tumors, but most of these tumors eventually resolved to undetectable limits. Overall, in two separate experiments, 92% (11 of 12) of the wild-type tumors and 50% (6 of 12) of the \(bax^{-/-} bak^{-/-}\) tumors regressed to a size that was not detectable by fluorescence imaging. The 6 remaining treated \(bax^{-/-} bak^{-/-}\) tumors were still detectable at the end of the study (after day 50) but had progressively decreased from their peak volume.

Importantly, when CP was discontinued on 6 mice whose wild-type and \(bax^{-/-} bak^{-/-}\) tumors regressed to a nondetectable limit, these mice remained tumor free for up to 9.5 months before they died due to natural causes. These results show that: (a) Bax/Bak-mediated apoptosis contributes to a more rapid tumor regression, as the \(bax^{-/-} bak^{-/-}\) tumors showed a delay in response; and (b) the apoptosis machinery may be dispensable for tumor regression in response to DNA alkylating damage.
Figure 2. Cyclophosphamide induces sporadic tumor necrosis. E1A and K-Ras–transformed wild-type and $\text{bax}^{-/-}\text{bak}^{-/-}$ tumor cells were injected bilaterally into the same nude mouse to allow tumors to form. When palpable tumors formed, the mice were treated with CP (170 mg/kg) every 5 d, or left untreated (Fig. 1). Tumors were obtained at various time points after CP treatment. A, tissues from tumors left untreated or treated with CP were subjected to TEM analysis. Representative pictures are shown. Apoptotic cells (indicated by condensed chromatin), necrotic cells (indicated by disruption of cell structure, lack of chromatin condensation, and formation of a large amount of vacuoles), and leukocytes are observed (A, apoptotic cells; L, leukocytes; N, necrotic cells). Note that cells with large and pleomorphic nuclei indicative of senescence or mitotic catastrophe are present in the CP-treated tumors. B, sections from tumors untreated or treated with CP were assayed for cleaved Caspase 3, and for apoptotic DNA fragmentation using a TUNEL assay (Chemicon International). The dark brown staining indicates positive cells (apoptotic). Representative pictures of untreated tumors and tumors treated with CP for 1 d are shown. Similar results were obtained from tumors treated with CP 2, 3, 4, and 5 times and are not shown. Quantification was performed by counting positive cells under a microscope in randomly selected areas. Columns, mean of five independent countings; bars, SE. C, tumor lysates from an untreated and two independent CP-treated animals (each received one treatment) were prepared and subjected to immunoblotting analysis for cleaved Caspase 3 and phosphorylated H2A.X. HMGB1 was probed as a control for equal loading. D, paraffin-embedded sections were made and assessed with IHC using an anti-HMGB1 antibody. Nuclear staining is present in the untreated tissue, whereas cytoplasmic and extranuclear staining of HMGB1 is found in the tumor tissue treated twice with CP, indicative of necrosis. Similar results were obtained from tumors treated with CP 1, 3, 4, and 5 times and are not shown.
agents, as the bax<sup>−/−</sup>bak<sup>−/−</sup> and Bcl-xL overexpressing tumors still regressed despite their defect in the apoptotic pathway.

**Apoptosis is not the sole cause for CP-induced tumor regression.** The observation that bax<sup>−/−</sup>bak<sup>−/−</sup> tumors regressed after CP-treatment suggests that apoptosis may be dispensable for chemotherapeutics-induced tumor regression. We performed TEM analysis to examine the form of cell death (Fig. 2A). After CP treatment, both apoptotic cells and necrotic cells at various stages were observed in wild-type tumors. In bax<sup>−/−</sup>bak<sup>−/−</sup> tumors, no apoptotic cells were apparent, whereas necrotic cells were commonly observed in CP-treated tumors. Some nuclei in the treated cells seemed to be larger and pleomorphic, indicative of senescence and/or mitotic catastrophe (9). In both wild-type and bax<sup>−/−</sup>bak<sup>−/−</sup> tumors treated with CP, lymphocytes, characterized by an electron-dense cell body and high nucleus/cytoplasm ratio (10), were also observed (Fig. 2A).

To further determine if apoptosis occurred in tumor cells following CP treatment, frozen and paraffin-embedded tumor sections were subjected to immunohistochemistry (IHC) for cleaved Caspase 3 and terminal transferase dUTP end labeling (TUNEL) assay, both hallmarks for caspase-dependent apoptosis. The treated wild-type tumors displayed positive staining for cleaved Caspase 3 and TUNEL, whereas the treated bax<sup>−/−</sup>bak<sup>−/−</sup> tumor showed virtually no positive staining (Fig. 2B). The same effect was observed by immunoblotting for cleaved Caspase 3 using tumor lysates (Fig. 2C). An antibody against phosphorylated H2A.X (γH2A.X) revealed that both wild-type and bax<sup>−/−</sup>bak<sup>−/−</sup> tumors incurred a similar extent of DNA damage after CP treatment (Fig. 2C). Together with those shown in Fig. 1, these results indicate that although apoptosis contributes to CP-induced tumor regression, tumor cells deficient in apoptosis can still die via alternative forms of cell death.

**DNA alkylating damage triggers sporadic necrosis in vivo.** TEM analysis indicated that necrosis may occur in CP-treated tumors (Fig. 2A). To further examine this, IHC was performed on CP-treated tumor tissues using an antibody against HMGB1 protein. HMGB1 is a nuclear protein that binds tightly to chromatin in apoptotic cells, whereas during necrosis, it is released into the extracellular environment (11). IHC analysis revealed nuclear staining of HMGB1 in untreated tumor tissues. In contrast, extracellular HMGB1 staining was observed in both wild-type and bax<sup>−/−</sup>bak<sup>−/−</sup> CP-treated tumors (Fig. 2D), indicating that necrosis occurs in response to CP in both apoptosis-proficient and deficient tumors. It is interesting to note that this CP-induced necrosis is different from necrosis that is often observed in solid tumors with overgrowth. The latter constitutes the “necrotic centers” composed of large amounts of necrotic cells resulting from limited oxygen and nutrient supplies due to tumor overgrowth and lack of vascularization. CP-induced necrotic cells were scattered and evenly distributed throughout the tumor mass, as judged by both TEM and HMGB1 staining (Fig. 2A and D), thus is called “sporadic necrosis”.

To further confirm that CP can induce necrosis, we used a biologically active metabolite of CP, mafosfamide (MAF; ref. 12) for in vitro cell culture studies. Similar to CP in vivo, MAF induced DNA damage indicated by the increase of γH2A.X (Fig. 3C).
Treatment of wild-type, Bcl-xL–expressing, and \( \text{bax}^{(-/-)}\text{bak}^{(-/-)} \) tumor cells with MAF induced cell death in all three cell lines (Fig. 3A). Wild-type cells were more sensitive than Bcl-xL–expressing cells, which were more sensitive than \( \text{bax}^{(-/-)}\text{bak}^{(-/-)} \) cells, indicating that Bax/Bak deficiency suppresses apoptosis more efficiently than Bcl-xL overexpression (Fig. 3A). This was also indicated by the levels of Caspase 3 cleavage, which correlated to the ability of a cell to die by apoptosis. Whereas apoptosis was virtually blocked in \( \text{bax}^{(-/-)}\text{bak}^{(-/-)} \) cells, Bcl-xL only shifts the balance between the anti- and proapoptotic Bcl-2 proteins and thus did not completely block apoptosis (Fig. 3D). Regardless, microscopic analysis revealed that the dead cells displayed features of necrosis such as plasma membrane dilation and disruption in all three cell lines (Fig. 3B). Additionally, HMGB1 was found in the cell culture medium from both MAF-treated apoptosis-proficient and deficient cells, indicative of necrosis (Fig. 3D). The more rapid release of HMGB1 in wild-type cells is probably due to “secondary necrosis” because apoptotic cells are not engulfed and cleared as they are in vivo.

**CP-induced cell death activates the innate immune response.** A fundamental feature of apoptosis is that apoptotic cells are quickly engulfed as an intact corpse in vivo and thus do...
not trigger proinflammatory responses. In contrast, cells dying by necrosis release intracellular contents into the extracellular environment and cause proinflammatory responses. In TEM analysis, we observed the presence of leukocytes in both the wild-type and bax<sup>−/−</sup>bak<sup>−/−</sup> CP-treated tumors, suggesting infiltration of innate immune cells (Fig. 2A). This is further confirmed by IHC of the tumor sections and flow cytometry using single-cell suspension prepared from the tumor tissues, using an antibody against F4/80, a pan macrophage marker, and an antibody against an allotropic marker of neutrophils (Fig. 4A; Supplementary Fig. S1A).

Both apoptotic and necrotic cells can attract phagocytes. However, it is generally accepted that phagocytes such as macrophages secrete immune-suppressive cytokines upon engulfing apoptotic cells, whereas those encountering necrotic cells secrete proinflammatory cytokines (13, 14). To examine this issue, we determined whether the leukocytes recruited to the tumor tissue produced proinflammatory cytokines. In both wild-type and bax<sup>−/−</sup>bak<sup>−/−</sup> tumors, CP treatment increased the number of interleukin-1β (IL-1β)-positive cells and the levels of both IL-1β and tumor necrosis factor α (Fig. 4B–D). To further investigate if macrophage activation contributes to tumor clearance, we used gadolinium chloride (GdCl<sub>3</sub>) that has been shown to deplete peripheral blood macrophages (15, 16). We tested the effect of GdCl<sub>3</sub> on E1A/K-Ras-transformed Bcl-xL–expressing tumor cells. Animals bearing these tumors were treated with CP alone, or in combination with GdCl<sub>3</sub> every 3 days by i.v. injection. A significant level of macrophage depletion in the peripheral blood was achieved in animals treated with GdCl<sub>3</sub> (Supplementary Fig. S1B). Around day 25, the tumors in mice treated with both CP and GdCl<sub>3</sub> began to increase in size (Supplementary Fig. S1C). This suggests a critical correlation of the innate immunity in DNA alkylating damage-induced tumor clearance. Taken together, these results strongly indicate that CP-induced tumor cell death is not exclusively apoptotic, but includes necrosis, and can activate a proinflammatory response.

Discussion

A greater understanding of how tumor cells die in response to chemotherapy is likely to reveal new approaches to induce tumor cell death. In the present study, we use a genetically defined apoptosis-deficient cell system to establish a xenograft mouse tumor model. We examine the contribution of different forms of cell death in tumor regression induced by CP. We find that although apoptosis facilitates a more rapid tumor regression, it is dispensable for complete tumor regression.

 Necrosis is often observed in solid tumor areas where vascularization lags behind tumor tissue growth. Using bax<sup>−/−</sup>bak<sup>−/−</sup> cells in which apoptosis is genetically inhibited, we find that sporadic necrosis is induced by CP treatment. Importantly, we notice features of necrosis not solely in the apoptosis-deficient bax<sup>−/−</sup>bak<sup>−/−</sup> cells but also in the apoptosis-competent wild-type cells. This is different from spontaneous necrosis resulting from tumor overgrowth and lack of vascularization, which promotes chronic inflammation and stimulates further tumor growth (17). We show that HMGB1 is liberated from the nucleus, consistent with previous studies showing that HMGB1 released by necrotic cells confers proinflammatory responses (11). Thus, sporadic necrosis is not merely a death mechanism observed in the absence of apoptosis. Rather, it may play an important physiologic role even in apoptosis-proficient cells by directly inducing tumor cell death and by activating the innate immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

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