Human Tumor Cells Killed by Anthracyclines Induce a Tumor-Specific Immune Response

Jitka Fucikova, Petra Krilikova, Anna Fialova, Tomas Brtnicky, Lukas Rob, Jirina Bartunkova, and Radek Špišek

Abstract

Immunogenic cell death is characterized by the early surface exposure of chaperones including calreticulin and HSPs, which affect dendritic cell (DC) maturation and the uptake and presentation of tumor antigens. It has also been shown that it is characterized by the late release of high mobility group box 1 (HMGB1), which acts through Toll-like receptor 4 (TLR4) and augments the presentation of antigens from dying tumor cells to DCs. Most of the data on immunogenic tumor cell death were obtained using mouse models. In this study, we investigated the capacity of clinically used chemotherapeutics to induce immunogenic cell death in human tumor cell lines and primary tumor cells. We found that only anthracyclines induced a rapid translocation of calreticulin, HSP70, and HSP90 to the cell surface and the release of HMGB1 12 hours after the treatment. The interaction of immature DCs with immunogenic tumor cells led to an increased tumor cell uptake and induces moderate phenotypic maturation of DCs. Killed tumor cell–loaded DCs efficiently stimulated tumor-specific IFN-γ–producing T cells. DCs pulsed with killed immunogenic tumor cells also induced significantly lower numbers of regulatory T cells than those pulsed with nonimmunogenic tumor cells. These data indicate that human prostate cancer, ovarian cancer, and acute lymphoblastic leukemia cells share the key features of immunogenic cell death with mice tumor cells. These data also identify anthracyclines as anticancer drugs capable of inducing immunogenic cell death in sensitive human tumor cells.

Introduction

Cancer affects half of the inhabitants of developed countries and kills one third of them. Primary tumors can often be completely removed with current cancer therapeutic modalities; however, micrometastases of dormant tumor cells frequently lead to the establishment of the distant metastases and to the relapse of the disease (1). In addition to the standard treatment of metastatic disease by combinations of chemotherapeutics, it would be beneficial for cancer patients to elicit a tumor-specific immunity that would control or slow the growth of residual tumor cells. A combination of chemotherapy with immunotherapeutic strategies aiming to induce tumor-specific immunity represents a challenging task because chemotherapy is generally considered to be immunosuppressive. Physiologic cell death by apoptosis is known to be nonimmunogenic or tolerogenic (2). Consequently, phagocytosis of apoptotic tumor cells has been long considered to be immunologically silent (3). However, recent studies have shown that tumor cells killed by some chemotherapeutics, such as bortezomib (4, 5), oxaliplatin (6), and anthracyclines (7), can induce a tumor-specific immune response. This immunogenic cell death is characterized by molecular events shared for all described chemotherapeutics. Anthracyclines have a major role in the treatment of leukemia, lymphoma, sarcoma, and uterine, ovarian, and breast malignancies. Despite their side effects, anthracyclines (8) are able to induce immunogenic cell death in mouse tumor cells. Within hours after the initiation of immunogenic cell death, preapoptotic tumor cells translocate calreticulin (CRT; refs. 9, 10) and HSPs from the endoplasmic reticulum to the cell surface together with other molecules that serve as “eat me” signals (phosphatidylserine). At the same time, following permeabilization of the plasma membrane, cells release the late apoptosis marker high mobility group box 1 (HMGB1; ref. 12) into the extracellular milieu. HMGB1 can bind several pattern recognition receptors (PRR), such as Toll-like receptor (TLR)2, TLR4 (8, 13), and receptor for advanced glycosylation end products (RAGE). The release of this protein seems to be required...
for optimal presentation of antigens from dying tumor cells, T-cell priming by DCs, and subsequent T-cell-mediated elimination of the tumor.

All these findings were obtained using mouse models, and it is thus important to determine whether immunogenic tumor cell death can also be induced in humans and whether similar molecular events also occur in human cancer cells. Here, we report that anthracyclines are able to induce the expression of several immunogenic molecules on the surface of a wide spectrum of primary and secondary human cancer cell lines (leukemia, ovarian cancer, and prostate cancer), in contrast to other tested chemotherapeutic agents and irradiation, which is often used in immunotherapeutic protocols. The uptake of anthracycline-killed human tumor cells by DCs leads to the stimulation of the T-cell response and the induction of antitumor immunity.

Materials and Methods

Cell lines
Acute lymphoblastic leukemia cell lines were kindly provided by Childhood Leukemia Investigation Prague (CLIP; REH, HLA-A2 positive; DSMZ). Ovarian cancer cells [OV90, HLA-A2 positive; American Type Culture Collection (ATCC)], prostate cancer cells (LNCap, HLA-A2 positive; ATCC). All cell lines were cultured in RPMI 1640 medium (Gibco). All media were supplemented with 10% heat-inactivated fetal calf serum (FCS)/glutamine/penicillin.

Isolation of primary tumor cells
Primary ovarian cancer cells were obtained from patients undergoing surgery for ovarian cancer. The resected tumors were weighed, minced into small pieces (1–3 mm), and mechanically minced into smaller pieces in PBS + 2 mmol/L EDTA or an enzyme solution (Collagenase D), placed into the incubator (37°C), kept in the dark and incubated for 15 minutes before the addition of another 400 μL of 0.1% propidium iodide (PI) incubation buffer and subsequent analysis on a FACSAria flow cytometer (BD Bioscience) using FlowJo software.

Apoptosis induction and detection
Tumor cell death was induced by UV (UVA) light exposure (7 and 6 J/cm²). Cell death was assessed by Annexin V/fluorescein isothiocyanate staining. Briefly, 2 × 10⁵ cells per sample were collected, washed in PBS, pelleted, and resuspended in an incubation buffer containing Annexin V/fluorescein isothiocyanate antibody. The samples were kept in the dark and incubated for 15 minutes before the addition of another 400 μL of 0.1% propidium iodide (PI) incubation buffer and subsequent analysis on a FACSAria flow cytometer (BD Bioscience) using FlowJo software.

Flow cytometric analysis of HSP70, HSP90, and CRT on the cell surface
A total of 10⁵ cells were plated on 12-well plates and treated the following day with the indicated agents or were UV-irradiated for 6, 12, or 24 hours. The cells were collected and washed twice with PBS. The cells were incubated for 30 minutes with primary antibody diluted in cold blocking buffer (2% FBS in PBS), followed by washing and incubation with the Alexa 648-conjugated monoclonal secondary antibody in a blocking solution. Each sample was then analyzed by a FACSAria flow cytometer (BD Bioscience) to identify cell surface HSP70, HSP90, and CRT.

Detection of HMGB1 release
REH cells, OV90 cells, LNCap cells, primary ovarian cells, and leukemic blasts (10⁶) were plated in 1 mL full medium appropriate for the cell type. Supernatants were collected at different time points, dying tumor cells were removed by centrifugation, and the supernatants were isolated and frozen immediately. Quantification of HMGB1 in the supernatants was assessed by ELISA according to the manufacturer’s instructions.

Fluorescent microscopy
For surface detection of CRT, the cells were placed on ice, washed twice with PBS, and fixed in 0.25% paraformaldehyde in PBS for 5 minutes. The cells were then washed twice in PBS, and a primary antibody diluted in cold blocking buffer was added for 30 minutes. After 2 washes in cold PBS, the cells were incubated for 30 minutes with the appropriate Alexa 648-conjugated secondary antibody. The cells were fixed with 4% paraformaldehyde for 20 minutes, washed in PBS for 20 minutes, and mounted on slides.

For phagocytosis, the DCs were stained with Vybrant DiO cell labeling solution (Invitrogen). The tumor cells were stained with Vybrant DiI cell labeling solution (Invitrogen) and cultured in the presence of selected cytostatic agents or UV radiation for 24 hours. Immature DCs (day 5) were fed tumor cells at a DC/tumor cell ratio of 1:5. The cells were fixed with 4% paraformaldehyde for 20 minutes, washed in PBS for 20 minutes, and mounted on slides with ProLong Gold antifade reagent (Invitrogen).

Generation of tumor-loaded DCs and induction of tumor cell death
DCs were generated by culture of purified CD14+ cells isolated from buffy coats in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; Gentaur) and interleukin-4 (IL-4; Gentaur; ref. 14). Tumor cells were killed by culturing in the presence of a selected cytostatic agent (100 nmol/L) or by UV irradiation for 24 hours. The extent of apoptosis was monitored by Annexin V/PI staining. The cells were extensively washed before feeding to DCs. Immature DCs (day 5) were fed tumor cells at a DC/tumor cell ratio of 1:5. In some experiments, pulsed DCs were stimulated with 100 ng/mL of lipopolysaccharide (LPS; Sigma) for 12 hours.
**Evaluation of IFN-γ–producing tumor-specific T cells**

Unpulsed or tumor-loaded DCs were added to autologous T cells at a ratio of 1:10 on days 0 and 7 of culture. IL-2 (25–50 IU/mL; PeproTech) was added on days 2 and 7 of culture. The cultures were tested for the presence of tumor-specific T cells 7 to 9 days after the last stimulation with DCs. The induction of tumor-reactive, IFN-γ–producing T cells by tumor-loaded DCs was determined by flow cytometry. The T cells were stained with anti-human CD8/IFN-γ (17).

**Results**

We first tested the cytotoxic effect of a wide spectrum of clinically used cytostatic agents on the viability of REH (T-ALL), ovarian cancer (OV90), and prostate (DU145) cancer cell lines. The viability of tumor cells was repeatedly analyzed over the course of 48 hours by PI and Annexin V staining. Cytostatic agents that killed more than 50% of tumor cells after 24 hours were used for subsequent experiments to analyze the capacity of these agents to induce an immunogenic cell death (Fig. 1).

**Expression of immunogenic cell death markers HSP70, HSP90, and calreticulin by human cancer cell lines and human primary tumor cells**

Cytostatics were tested for their ability to induce the expression of the known immunogenic cell death markers HSP70, HSP90, and calreticulin in leukemic, ovarian, and prostate cancer cell lines and primary tumor cells. Significant expression of HSP70, HSP90, and calreticulin on T-ALL leukemia cells and T-ALL leukemic cells was detected 12 and 24 hours after the treatment with anthracyclines (doxorubicin and idarubicin; Fig. 2A and B). Increased expression of HSP70, HSP90, and calreticulin after the treatment with anthracyclines was accompanied by their translocation to the cell surface (Fig. 2C). The anthracyclines also induced significant upregulation of HSP70, HSP90, and calreticulin in the OV90 ovarian cancer cell line, in primary ovarian cancer cells freshly isolated from resected tumors, and in the DU145 prostate cancer cell line (Fig. 2D). We did not observe any significant upregulation of immunogenic cell death markers on ovarian, prostate, and ALL human tumor cells after UV light exposure.

**Anthracyclines induce HMGB1 secretion in human T-ALL, ovarian cancer, and prostate cancer cells**

We analyzed the release of a late-stage marker of immunogenic cell death HMGB1 in the supernatants of T-ALL, ovarian, and prostate cancer cell lines and primary T-ALL and ovarian tumor cells. Of the tested drugs, only anthracyclines induced significant release of HMGB1 in all tested human tumor cells (Fig. 3). Maximal release of HMGB1 nuclear protein was detected 24 hours after the induction of tumor cell death.

**Phagocytosis of anthracycline-treated tumor cells induces the expression of maturation-associated molecules on DCs**

The ability of DCs to activate the immune response depends on their maturation status and the expression of costimulatory molecules. We analyzed the phenotype of DCs that phagocytosed REH tumor cells killed by the indicated cytostatic agents or UV irradiation. The interaction of DCs with idarubicin- and doxorubicin-treated T-ALL cells induced the upregulation of CD83, CD86, and HLA-DR, although to a smaller extent than activation by LPS (Fig. 5). Activation of DCs with anthracycline-killed tumor cells and LPS together induced comparable expression of costimulatory molecules as treatment with LPS alone (data not shown).

**Phagocytosis of anthracycline-treated tumor cells induce tumor-specific T cells**

To investigate whether tumor cells expressing immunogenic cell death markers induce antitumor immunity, we evaluated the ability of tumor cell–loaded DCs to activate tumor cell–specific T-cell responses. Leukemic cells killed by selected cytostatic agents or by UV irradiation were cocultured with immature DCs with or without subsequent maturation with LPS. These DCs were then used as stimulators of autologous T cells, and the frequency of IFN-γ–producing T cells was analyzed 1 week later after restimulation with tumor cell–loaded DCs. DCs pulsed with REH cells killed by
anthracyclines induced a greater number of tumor-specific CD4\(^+\) and CD8\(^+\) IFN-\(\gamma\)-producing T cells than DCs pulsed with UV light–exposed cells in all experiments (\(n = 5\), even in the absence of additional maturation stimulus (LPS; Fig. 6).

In addition, we also tested the frequency of regulatory T cells (Tregs) induced in DC and T-cell cocultures. DCs pulsed with REH cells killed by anthracyclines had a lower capacity to expand Tregs than both immature DCs and LPS-activated DCs.
Figure 2. Anthracyclines induce the expression of HSPs on human tumor cells. A, the kinetics of HSP70, HSP90, and calreticulin expression on the T-ALL cell line (REH) and leukemic blasts isolated from T-ALL patients treated by the indicated cytostatics. The expression of the indicated markers is shown as a fold change of mean fluorescence intensity (MFI) when compared with untreated cells. The summary of a total of 5 experiments is shown. *, *P < 0.05. B, representative histograms of one of the experiments showing the expression of HSP70, HSP90, and calreticulin after 24 hours of treatment by tested cytostatics. C, confocal microscopy images of cells treated for 24 hours with anthracyclines and stained for HSP70, HSP90, and calreticulin.
Figure 2. (Continued) D, the kinetics of HSP70, HSP90, and calreticulin expression on an ovarian cancer cell line (OV90), primary ovarian cancer cells, and a prostate cancer cell line (DU145) after treatment with cytostatics. The expression of the indicated markers is shown as a fold change in the MFI when compared with untreated cells. The summary of a total of 5 experiments is shown. *, \( P < 0.05 \) for comparison with irradiated tumor cells.
We also sorted the induced CD4<sup>+</sup>CD25<sup>high</sup> T cells to test their inhibitory capacity in an allogeneic mixed lymphocyte reaction (MLR), and we did not see any significant difference in the inhibitory activity of Tregs induced by tumor cells killed by various cytostatics (data not shown).

**Discussion**

There is considerable interest in understanding the biochemical features of immunogenic versus nonimmunogenic death of tumor cells induced by anticancer therapies. Recent studies identified several markers accompanying immunogenic cell death. Our recent study reported the induction of immunogenic cell death in primary myeloma tumor cells by bortezomib, a specific inhibitor of the 26S proteasome subunit. Immunogenicity of tumor cells correlated with the expression of HSP90 on the surface of myeloma cells killed by bortezomib (4, 5). Cell surface expression of HSP90 was critical for the immunogenicity of killed tumor cells because activation of DCs was cell-contact dependent, and the specific blockade of HSP90 abolished the immunogenicity of myeloma cells. In addition to the study on primary myeloma tumor cells, upregulation of maturation-associated markers was reported for murine DCs.

(Fig. 7). We also sorted the induced CD4<sup>+</sup>CD25<sup>high</sup> T cells to test their inhibitory capacity in an allogeneic mixed lymphocyte reaction (MLR), and we did not see any significant difference in the inhibitory activity of Tregs induced by tumor cells killed by various cytostatics (data not shown).
that phagocytosed bortezomib-killed 67NR colon carcinoma cells (13). This report also showed increased immunogenicity of bortezomib-killed tumor cells in tumor protection experiments. In a mouse colon carcinoma model (CT26 cell line), Zitvogel and Kroemer screened an array of chemotherapeutic drugs for each drug’s capacity to induce immunogenic cell death (9). Evaluating the ability of tumor cells killed by tested drugs to serve as a protective vaccine in tumor protection experiments, they identified anthracyclines as compounds that induced immunogenic cell death, even in the absence of external activation signals (18, 19). Rapid translocation of the endoplasmic reticulum–resident chaperone protein calreticulin to the cell surface of dying tumor cells was identified as a molecular mechanism underlying the increased

Figure 4. The kinetics of phagocytosis of cytostatic-treated REH T-ALL cells by immature DCs. A, the percentage of phagocytosis at 12 and 24 hours is shown relative to the control at 4°C. B, dot plots of the representative experiments are shown. Killed REH cells were labeled with DiO and cocultured with DiD-labeled immature DCs. C, confocal microscopy analysis of the phagocytosis experiment. After 24 hours of coculture of killed tumor cells with immature DCs, the engulfment of tumor cells was verified by confocal microscopy. D, correlation between the expression of immunogenic cell death markers and the rate of phagocytosis at 24 hours. Summary of 7 independent experiments is shown.
Figure 5. The phenotype of DCs after interaction with cytostatic-killed REH cells. Day 5 immature DCs were cultured for 24 hours with REH T-ALL cells killed by irradiation or the indicated cytostatics. After 24 hours, the expression of CD83, CD86, and HLA-DR on DCs was analyzed by flow cytometry. The mean fluorescence intensity (MFI) and representative histograms are shown. *, $P < 0.05$ for comparison with irradiated tumor cell–loaded DCs.
immunogenicity of tumor cells (9, 10). Calreticulin translocation enhanced the phagocytosis of tumor cells by DCs, and blockade of calreticulin abolished anthracycline-induced immunogenicity of killed tumor cells in mice. Upon exposure to anthracyclines, calreticulin translocates very quickly to the outer leaflet of the cell membrane, whereas other tested chemotherapeutics fail to induce calreticulin translocation and thus did not induce immunogenic cell death. The identification of HSPs as markers of immunogenic cell death is also in accordance with murine studies showing that cell surface HSPs represent a potent immunogenic signal and promote the development of autoimmunity. Immunostimulatory activity and increased immunogenicity of tumor cells enriched in HSPs after induction of hyperthermia has also been reported in various animal tumor vaccination models as well as in the human in vitro model (20, 21). Together, the common theme from these independent observations is that the expression of HSPs on the surface of dying cells may be a marker for immunogenic forms of cell death and deliver an activating stimulus to DCs.

Recently, Apetoh and colleagues conducted elegant studies that led to the discovery of another soluble endogenous danger signal (6, 8, 12). They reported that TLR4 deficiency compromised the immunogenicity of tumor cells and identified HMGB1 as a specific ligand of TLR4 that is released from dying tumor cells at the stage of late apoptosis. HMGB1

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**Figure 6.** The induction of tumor-specific T cells by cytostatic-killed REH T-ALL cells without the need for an exogenous DC maturation stimulus. Monocyte-derived DCs were pulsed with REH T-ALL cells killed by irradiation or tested drugs and then used for the stimulation of autologous T cells for 2 weeks. The number of IFN-γ producers in cultures with unpulsed DCs or DCs pulsed with REH T-ALL cells was analyzed by intracellular IFN-γ staining. The data show a summary (top) and representative staining (bottom) of 5 independent experiments.
is a nonhistone chromatin-binding protein that influences transcription and other cell functions. HMGB1 is actively secreted from inflammatory cells or released from necrotic cells (22, 23). The identity of its receptor is still controversial, but it signals through TLR2 and TLR4 as well as RAGE (24–26). Depletion of HMGB1 from tumor cells abolished TLR4-dependent DC-mediated presentation of tumor antigens. The relevance of this study is further illustrated by the finding that breast cancer patients with the TLR4 allele variant that reduces the affinity of TLR4 for HMGB1 had a higher incidence of metastasis after conventional treatments than patients with the wild-type allele (8).

As most of the data on immunogenic tumor cell death were obtained using mouse models, we investigated whether analogous mechanisms also apply to human cancer cells. We tested the capacity of clinically used cytostatics to induce immunogenic cell death in human tumor cell lines and primary tumor cells derived from prostate cancer, ovarian cancer, and acute lymphoblastic leukemia.

We show that only anthracyclines induced a rapid translocation of calreticulin, HSP70, and HSP90 to the cell surface and the release of HMGB1 12 hours after the treatment in all 3 models, as documented by cytometric analysis, confocal microscopy, and ELISA. The interaction of immature DCs with immunogenic tumor cells led to an increased tumor cell uptake and induced moderate expression of maturation-associated markers on DCs. As in the mice studies published by the group of Zitvogel and Kroemer, the rate of phagocytosis in our study very closely correlated with the intensity of calreticulin expression and, although to a lesser degree, with the intensity of HSP expression.

DCs loaded with anthracycline-killed tumor cells efficiently stimulated tumor-specific IFN-γ-producing T cells, even in the absence of other maturation stimuli such as LPS. DCs pulsed with killed immunogenic tumor cells also induced significantly lower numbers of Tregs, identified as CD4\(^+\)CD25\(^{high}\) and FoxP3\(^+\), compared with nonimmunogenic tumor cells, which may be relevant for the design of cancer immunotherapy studies. To test that by using the phenotypic markers of Tregs, we indeed identify the cells with inhibitory potential, we sorted the induced CD4\(^+\)CD25\(^{high}\) T cells to test their inhibitory capacity in an allogeneic MLR (27) and we did not see any significant difference in the inhibitory activity of Tregs induced by DCs loaded with tumor cells killed by various cytostatics (data not shown).
These data indicate that human prostate cancer, ovarian cancer, and acute lymphoblastic leukemia cells share the key features of immunogenic cell death with mice tumor cells, and we identified anthracyclines as anticancer drugs capable of inducing immunogenic cell death in sensitive human tumor cells. In mouse studies, Obeid and colleagues (10) also showed that γ-ray irradiation and UV light exposure induce a moderate expression of calreticulin on tumor cells and that irradiated tumor cells expressing calreticulin induce protection against subsequent challenge with live tumor cells. In this study, we show that killing of human tumor cells by UVA light exposure (7.6 J/cm²) does not lead to a significant upregulation of immunogenic cell death markers. We also tested tumor cells killed by γ-ray irradiation (20 and 75 Gy, data not shown), and we did not see any significant upregulation of immunogenic cell death–associated markers in accordance with previous studies in human tumor cells (5, 28). The absence of significant expression of immunogenic cell death–associated markers correlated with the low capacity of DCs pulsed with irradiated tumor cells to induce tumor cell–specific T cells in the absence of LPS.

Breakthrough studies that identified markers of immunogenic tumor cell death after chemotherapy treatment challenge the long-time perception of chemotherapy and immunotherapy as opposing and incompatible treatment modalities. The introduction of chemotherapy regimens and their thorough testing in well-designed clinical trials undeniably represents one of the greatest triumphs of modern medicine. For example, in childhood acute lymphoblastic leukemias, an invariably fatal disease in the 1960s, the introduction and subsequent improvement of chemotherapy protocols led to a cure rate of almost 90% in the last decade. Despite the continuous introduction of new drugs and further improvements of chemotherapy protocols, it is likely that, at some point, chemotherapy will reach its limits, and clinical efficacy will plateau. Moreover, despite the undeniable success in the treatment of some malignancies, in some tumors, particularly in solid tumors, chemotherapy is rarely curative. A combination of treatment modalities has been a standard strategy for cancer treatment, the combination of surgery with chemo- or radiotherapy being a classical example. Effort should be made not only to design modern immunotherapeutic strategies but also to incorporate immunotherapy approaches into current chemotherapy protocols (29, 30). Chemotherapy and immunotherapy should not be henceforth considered antagonist forms of therapy, and it is conceivable that their rational combination could substantially improve the prognosis of cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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