Comparative Analysis of Necrotic and Apoptotic Tumor Cells As a Source of Antigen(s) in Dendritic Cell-based Immunization

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Abstract

There is considerable controversy as to whether necrotic (lysozyme) or apoptotic tumor cells serve as the superior source of multiple tumor-associated antigens (TAAs) to pulse dendritic cells (DCs) for immunotherapeutic applications. Here, we show that standard procedures to induce apoptosis by UVB irradiation unequivocally result in a mixed population of viable, apoptotic, and necrotic tumor cells, necessitating additional purification. We used highly enriched apoptotic versus lysate of B16 melanoma cells to examine whether or not there are important distinctions between these two sources of TAAs for loading of DCs. Our results demonstrate that although some differences exist between the two forms of TAAs in expression of heat shock proteins, as well as production of interleukin-12 by pulsed DCs, their respective capacities to mature DCs phenotypically, as well as to elicit both effective immune priming and antitumor therapeutic efficacy in vivo when presented by DCs, are equivalent.

Introduction

Killed tumor cells have been shown to be a source of TAAs for processing and presentation by DCs, both experimentally and clinically. Controversy exists with respect to the optimal form of killed tumor cells for stimulating effective immune priming and antitumor activity in DC-based vaccine strategies. Also, there is disagreement in the field with respect to the impact of killed tumor cell uptake on DC maturation and capacity to stimulate antigen-reactive T cells via the separate MHC class I and II pathways. Early reports argued that DCs that phagocytosed apoptotic cells (as opposed to necrotic cells) could exclusively cross-present antigens to CTLs (1). However, other studies demonstrated the induction of tolerance by DCs that had captured apoptotic cells (2). It was later shown that necrotic, but not apoptotic, cells could induce the maturation of immunostimulatory DCs to elicit immunity (3, 4). In some studies, apoptotic cells engulfed by DCs could induce maturation (5), whereas in others, only necrotic cells appeared to be effective (3, 6). It was also reported that HSPs were released only from tumor lysate but not from apoptotic cells; HSP was implicated for inducing the maturation of DCs. However, once stressed, HSPs were identified on apos as well (7). Whether or not additional maturation of DCs induced by killed tumor cells is necessary for effective antitumor immunity in vivo remains unclear, however, based on animal studies that successfully used fully viable tumor cells as the source of TAAs for DC pulsing (8). These seemingly discordant findings were set against a backdrop of studies that showed that DCs pulsed with lysate (i.e., necrotic cells) could induce both CD4+ helper T-cell and CD8+ CTL reactivity in vitro and mediate tumor regression in vivo, both in animals (9–11) and in humans (12–14). Moreover, lysate-pulsed DCs (11) and apoptotic tumor-pulsed DCs (15) both demonstrated enhanced antitumor therapeutic efficacy in vivo when combined with the systemic administration of IL-2. It is known that agents that induce apoptosis of nonsynchronized tumor cells often yield a population containing a mixture of viable, apoptotic, and necrotic cells in varying proportions, which can complicate interpretation when used as a source of TAAs for DC-based vaccines. Moreover, secondary necrotic cells are readily formed from apoptotic cells over time. Such a dynamic and variable process may explain to some extent the discrepant results obtained to date with pulsed DCs. In the current study, we exposed B16 melanoma cells to UVB light and used FACS sorting to obtain a highly enriched population of apoptotic cells, which were then immediately exposed to DCs. We directly compared these killed tumor cells to necrotic cells (freeze/thaw lysate) as a source of TAAs for processing and presentation by bone marrow-derived DCs.

Materials and Methods

Animals. Female (6–8 weeks old) C57BL/6 (denoted B6) mice were purchased from Harlan Laboratories (Indianapolis, IN), housed at the Animal Maintenance Facility at University of Michigan Medical Center, and were 8–12 weeks of age when used in the studies.

Medium and Cytokines. CM was prepared as described previously (9). Recombinant murine granulocyte macrophage colony-stimulating factor (specific activity, >5 × 10^6 units/mg) and recombinant murine IL-4 (2.8 × 10^5 units/mg) were obtained from Immunex Corp. (Seattle, WA) and Schering-Plough Pharmaceutical Research Institute (Kennesaw, NJ), respectively.

Tumor Cell Line. B16-BL6 melanoma is a tumor of spontaneous origin that expresses a low level of MHC class I molecules and no detectable MHC class II molecules. Tumor cells were maintained by serial in vitro passage in CM and were used before the 10th passage.

Preparation of Tumor Cells for Antigen Pulsing of DCs. B16 melanoma cells were collected using trypsin/EDTA solution (Life Technologies, Inc., Grand Island, NY), were washed twice in PBS (Life Technologies, Inc.), and were resuspended in PBS at 1 × 10^6 cells/ml. For induction of apoptosis, the cells were first incubated 5 min on ice, then added to 100 mm^2 culture dish (Corning, Inc., Corning, NY). Tumor cells were exposed to UVB light (Gel Doc 2000; Bio-Rad, Hercules, CA) for 20 min. After exposure (equal to 200 mJ/cm^2), the B16 tumor cells were washed with HBSS and cultured in CM. Later (4 h), the nonadherent portion was harvested and used for additional experiments. For induction of lysate (i.e., necrotic cells), B16 melanoma cells were suspended in PBS and subjected to four cycles of rapid freeze/thaw exposures and spun at 700 rpm at 4°C for 10 min to remove cellular debris.
Collection of Apos. After UVB 20-min exposure, B16 melanoma cells were cultured in CM. Later (4 h), the nonadherent cell fraction was collected. Apos within this fraction (representing ~50% of the total cells by Annexin-V/PI FACS analysis) were then highly enriched by FACS sorting on a B-D Vantage (Becton Dickinson, San Jose, CA) based on forward light scatter gating. B16 melanoma cells that became apoptotic showed distinct change in their optical properties, with a decreased forward scattering. FACS-sorted apoptotic cells were immediately used in all experiments.

Detection of Apoptotic and Necrotic Cells. FACS-sorted apoptotic cells and freeze/thaw lysate of B16 melanoma were examined for the degree of apoptosis and necrosis using a standard FACS assay (R & D Systems, Inc., Minneapolis, MN), which detects binding of Annexin V-fluorescein and exclusion of PI (Annexin V/PI assay), as described previously (9, 10).

Phagocytosis Assay. Apos were washed with PBS and labeled with 7-AAD (20 μg/ml/10^6 cells) for 30 min. After labeling, the preparations were washed twice and cocultured with MHC class II mAb (PharMingen, San Diego, CA) labeled DCs at a 1:3 ratio. After 18 h coculture, the cells were harvested and washed with PBS. Phagocytosis by DCs was quantified by FACS with a B-D FACScaliber (Becton Dickinson) as the percentage of double positive staining cells. Because 7-AAD was not a reliable stain for labeling tumor lysate, we used standard transmission electron and confocal microscopy to visualize its uptake by DCs.

IL12p70 Assay. Bone marrow-derived DCs were left unpulsed or cultured with either apos or tumor cell lysate. After 18 h, culture supernatants were harvested for measurement of IL-12p70 production by standard ELISA with either apos or tumor cell lysate. After 18 h, culture supernatants were harvested for measurement of IL-12p70 production by standard ELISA.

Immunoblotting. The presence of the HSPs 70 and gp96 in viable cells, apoptotic cells, and lysate of B16 melanoma was examined by Western blotting by SDS-PAGE. The protein of interest was identified using anti-apoptotic cells, and lysate of B16 melanoma was examined by Western blotting by SDS-PAGE. The protein of interest was identified using anti-apoptotic cells, and lysate of B16 melanoma was examined by Western blotting by SDS-PAGE. The protein of interest was identified using anti-apoptotic cells, and lysate of B16 melanoma was examined by Western blotting by SDS-PAGE.

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High-Level Enrichment of Apoptotic B16 Melanoma Cells after FACS Sorting. To induce apoptosis of B16 melanoma cells, we used UVB light exposure. In preliminary experiments, we compared different agents reported to induce apop, including betulinic acid, sodium butyrate, γ irradiation, recombinant TNF-related apoptosis-inducing ligand protein, and the Fas pathway, and concluded that UVB exposure was preferable (data not shown). We determined that 4 h after 20-min UVB exposure was optimal, with ~40–50% of B16 melanoma cells showing early apoptosis by the Annexin-V/PI FACS assay. In addition to being Ann+/PI−, tumor cells undergoing early apoptosis were found to also have a distinct light scatter profile on FACS analysis. As shown in Fig. 1a, FACS sorting based on light scatter could provide a highly enriched population of UVB-induced, apos. In contrast, freeze/thaw tumor lysates were composed exclusively of necrotic cells (i.e., Ann+/PI+). Both B16 melanoma lysate and FACS-sorted apoptotic cells were then immediately cultured with DCs.

DC Engagement of Killed Tumor Cells, Phenotype, and IL-12 Production. We labeled highly enriched apos and DCs with 7-AAD and anti-MHC class II mAb, respectively, to ascertain the level of uptake of the killed tumor cells by DCs when measured as dual-staining events by FACS analysis. At 4 h after coculture, 45% of DCs demonstrated uptake of apos (data not shown). We evaluated the status of cultured apoptotic cells at 4 h after sorting and found them to be unchanged for the Ann+/PI− phenotype. At 18 h after coculture, 65% of the DCs were shown to have engulfed apos by this detection method (Fig. 1b). DCs also engulfed tumor lysates as efficiently by both transmission electron and confocal microscopy analysis (data not shown). We examined whether or not changes occurred in surface phenotype of DCs after engulfment of killed tumor cells. As shown in Table 1, unpulsed DCs demonstrated a high-level expression of MHC class II and the costimulatory molecules CD40, CD80, and CD86, similar to what we had reported previously (9, 11). Although the percentages...
Day-4 bone marrow-derived DCs were left unpulsed or were cocultured with either B16 tumor lysate or highly enriched apoptotic cells. Later (18 h), the DCs were harvested and examined for surface expression of costimulatory and MHC class II molecules by flow cytometry. Mean channel fluorescence intensity was determined by CELLQuest software analysis. For IL-12 determination, day-4 bone marrow-derived DCs were cultured with different forms of killed tumor cells or were left unpulsed. Later (18 h), supernatants were harvested, and IL-12 secretion was determined by standard ELISA. These data are representative of three experiments with similar results.

Table 1 Phenytype of and IL-12 production by DCs loaded with different forms of killed tumor cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage and mean channel fluorescence expression of:</th>
<th>IL-12 p70 secretion (pg/ml ±/− SE)</th>
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<tbody>
<tr>
<td></td>
<td>CD40</td>
<td>CD80</td>
</tr>
<tr>
<td>Lysate-pulsed DC</td>
<td>92, 171</td>
<td>92, 62</td>
</tr>
<tr>
<td>Sorted Apo DC</td>
<td>90, 278</td>
<td>92, 80</td>
</tr>
<tr>
<td>Unpulsed DC</td>
<td>87, 42</td>
<td>91, 57</td>
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Fig. 2. Immunoblotting analysis of HSP content in tumor preparations. Proteins from B16 melanoma lysate supernatant, FAC-sorted apoptotic cells, and viable cells were examined by SDS-PAGE. The blots were probed with monoclonal antibodies to HSP70 and gp96 as indicated. Detection of gp96 was compared in cell preparations made by: (a) sonication; and (b) lysis buffer, as described in “Materials and Methods.”

of unpulsed and tumor-loaded DCs expressing these markers were not notably different from each other, the intensity of surface expression (i.e., mean channel fluorescence) was modestly enhanced, particularly for CD40 and MHC class II, after uptake of either tumor lysate or highly enriched apos (Table 1).

We also evaluated the production of a Th1 cytokine, IL-12, by 1 × 10⁶ DCs pulsed for 18 h with various forms of killed tumor cells as a source of antigen(s). As shown by a representative experiment (of three performed) in Table 1, DCs cultured with highly enriched apoptotic B16 melanoma cells produced the highest amount of IL-12 p70 (528 +/− 71 pg/ml). On the other hand, DCs pulsed with tumor lysate produced 167 +/− 20 pg/ml, which was only slightly higher than that of unpulsed DCs (119 +/− 16). DCs cultured with unsorted apoptotic cells, which contained ~54% early apoptotic cells (i.e., Ann+PI−) and ~36% necrotic cells (i.e., Ann+PI+), produced 213 +/− 9 pg/ml of IL-12 p70 (data not shown). Similar patterns were noted for the secretion of IL-12 p40 (data not shown).

HSP Content of Viable, Apoptotic, and Necrotic Tumor Cells.

We next examined the level of HSP70 and gp96 expression in preparations of viable and killed B16 melanoma cells (Fig. 2). When comparing the supernatants of lysate and highly enriched apoptotic cells to that of viable B16 melanoma cells, only the supernatant of lysate contained both HSP70 and gp96. However, when we used lysis by sonication, HSP70 and gp96 could then be detected in highly enriched apoptotic cells and viable cells, respectively. Of interest, when lysis buffer was substituted in place of sonication, then gp96 could also be detected in highly enriched apoptotic cells. Collectively, these results underscored the importance of chosen methodologies for the detection of selected HSPs in viable and killed tumor cell preparations.

Comparison of Immune Priming Elicited by DCs Pulsed with Apoptotic versus Necrotic B16 Melanoma Cells.

We demonstrated previously that immunization of mice with lysate-pulsed DCs could mediate immune protection against challenge with viable B16 melanoma cells (17). To determine whether or not vaccine potency differences existed between DCs pulsed with highly enriched apoptotic cells versus lysate (i.e., necrotic cells), we used a suboptimal immunization strategy of two injections of 1 × 10⁶ killed tumor-pulsed DCs; we also challenged the mice with a greater dose of viable B16 melanoma cells (i.e., 2 × 10⁶). Fig. 3a shows the results of one representative experiment of three performed. When highly enriched apo-DCs were administered, B16 melanoma growth was inhibited by ≥70% of that in mice receiving HBSS alone (P < 0.001) or unpulsed...
DCs ($P < 0.01$). A similar level of tumor growth inhibition was observed after immunization with lysate-pulsed DCs ($P < 0.001$ versus HBSS, $P < 0.01$ versus unpulsed DCs).

**Treatment of Established Disease by DCs Pulsed with Apoptotic versus Necrotic B16 Melanoma Cells.** We next established s.c. tumor in B6 mice and began treatment of the palpable masses 7 days later. Again, we used a suboptimal vaccine strategy of two immunizations on days 7 and 14 to potentially uncover differences in the potency of pulsed DCs. Fig. 3b shows the results of one representative experiment of three performed. When highly enriched apo-DCs were administrated, B16 melanoma growth was inhibited by $\pm 50\%$ of that in mice receiving HBSS alone ($P < 0.05$). The same degree of tumor inhibition was obtained after the administration of lysate-pulsed DCs ($P < 0.05$). Similar results were obtained in two additional experiments compared with HBSS alone ($P < 0.05$) and unpulsed DCs alone ($P < 0.05$; data not shown).

Collectively, our data demonstrate that the antitumor efficacy of DCs pulsed with either apoptotic or necrotic (i.e., lysis) tumor cells was comparable. We opted to make the comparison in B16 melanoma because of its poor immunogenicity and aggressive growth behavior in vivo. We chose a suboptimal vaccine dosage schedule and higher tumor burden to better determine whether or not differences existed in antitumor potency between apoptotic cell- versus lysate-loaded DCs. Because agents that induce tumor apoptosis tend to do so in an incomplete manner, we used a method to provide a highly enriched population of apos for DC loading. Without enrichment, the UVB-exposed tumor cell preparation comprised a heterogeneous mixture of apoptotic, necrotic, and viable cells, which could, in part, explain the discrepant findings between studies reporting different effects of apoptotic and necrotic tumor cells on DC phenotype, maturation, and function (1–6, 8–15). In our experience, collection of only the non-adherent tumor fraction after UVB exposure did not provide an adequately enriched population of apoptotic cells (data not shown). We had noted earlier a property of changing light scatter of tumor cells undergoing morphological change as a result of apoptosis from exposure to chemotherapeutic agents. On the basis of this parameter, we were able to obtain by FACS sorting a highly enriched population of apoptotic B16 melanoma cells for DC loading. UVB exposure was chosen over other possible apoptosis inducers (e.g., TNF-related apoptosis-inducing ligand, TNF-α, and Fas pathway) to avoid potential indirect influences of these agents on tumor antigenicity and/or on DC function. Moreover, the use of FACS sorting based on light scatter parameter avoided potential complications in interpretation because of the use of antibodies or dyes.

It has been demonstrated that phagocytosis of particulate antigen is more efficient in immature DCs compared with mature DCs (9, 11). Although murine bone marrow-derived DCs already demonstrated a high level of surface expression of MHC and costimulatory molecules—an indication of a more mature phenotype—their capacity to engulf necrotic or apoptotic cells remained high enough. Some difference existed in the level of IL-12 produced by the pulsed DCs, which has been reported as another parameter of maturation. The amount of IL-12 production was greater by DCs pulsed with apos compared with lysate; this level of secreted IL-12 was similar in amount to that reported by others (18). However, although IL-12 has been implicated as an important cytokine for favoring the development of a Th1 immune response, its in vivo role in antitumor immunity elicited by DC-based vaccines is unclear. In our study, the level of IL-12 production by tumor lysate versus apoptotic cell-pulsed DCs did not correlate with antitumor efficacy in vivo. Moreover, TRP-2 peptide-pulsed DCs produced heightened levels of IL-12, yet, unlike lysate- or apoptotic tumor-pulsed DCs, failed to impact on the growth of pre-existing B16 melanoma in vivo (data not shown). Recently, Kuniyoshi et al. (19) have shown that production of IL-12 is not an important factor in the enhanced immunostimulatory ability of CD40L-treated human DCs in vitro. Although it has been reported that additional maturation of lysate-pulsed murine bone marrow-derived DCs could measurably enhance their antitumor activity in vivo (18), we have not observed such enhancement in our own studies. There was no discernible difference in DC phenotype when pulsing with highly enriched apos compared with lysate; both induced comparable elevations in the intensity of MHC class II, CD40, CD80, and CD86 molecule expression compared with unpulsed DCs.

DC maturation has also been linked to the expression of certain HSPs, which appear to differ in necrotic и apoptotic (3, 6, 7, 20). Thus, we also examined the presence of HSP70 and gp96 in the lysate versus apoptotic B16 tumor cell preparations used for DC loading. Some difference existed between the two populations of killed tumor cells, depending on the sample preparation method used for immunoblotting (i.e., lysis buffer versus sonication). Moreover, loading of equivalent amounts of protein required a 5–6-fold greater number of apoptotic B16 tumor cells compared with lysate or viable cells (data not shown). Despite these differences, there was no apparent correlation with vaccine efficacy in vivo between lysate- and apo-pulsed DCs.

The findings reported herein are obtained with DCs derived from murine bone marrow. Recently, it has been shown in the human that optimal cross-presentation of TAA via DCs requires both the uptake of apos as the source of antigens and a maturation signal provided by exposure to necrotic cells (3). This study and an earlier report (1) argued that human DCs loaded with necrotic cells do not induce CTLs in vitro, which is contrary to studies with murine DCs (4) and, in other reports, with human DCs (13, 14). These contrasting results again underscore the need to standardize both the composition of killed tumor cell preparations used for antigen loading, as well as the source and maturity of DCs.

References


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