Antigen-presenting Cells That Phagocytose Apoptotic Tumor-derived Cells Are Potent Tumor Vaccines

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Abstract

We have reported recently that treatments combining injections of apoptotic bodies from tumor cells and interleukin 2 led to tumor regression and induced specific protection. In the present study, we show that tumor-bearing rats were cured with an 80% success rate by injection of antigen-presenting cells (APCs) that had phagocytosed apoptotic bodies derived from poorly immunogenic tumor cells, whereas phagocytic cells exposed to nonapoptotic tumor cell extracts were essentially without effect. In addition, curative vaccination using APCs that had phagocytosed apoptotic bodies generated a tumor-specific cytotoxic T-cell response and long-term protection from parental tumor challenge. Thus, systems using the processing and presentation of antigenic molecules by professional APCs after phagocytosis of apoptotic bodies appear to offer new possibilities for antitumor treatment.

Introduction

Many human cancers have a high incidence and a poor prognosis because of resistance to classical chemotherapy and radiotherapy. Manipulation of the immune system appears to be a promising means of achieving their eradication, although most human cancer cells are not sufficiently immunogenic to trigger an immune response in vivo. Whole-cell tumor vaccines, although capable of producing effective but partial regression of tumor, are mostly devoid of intrinsic immunogenicity and do not trigger sufficient long-term memory responses. One possible reason for this lack of tumor cell immunogenicity is that most tumor antigens are either masked or unaccessed. An alternative strategy in vaccine therapy is to exploit tumor-specific peptide antigens (1) or develop peptide presentation by using “professional” APCs pulsed with tumor cell extracts (2, 3). However, the question still remains of the effect of such promising therapies when poorly or nonimmunogenic tumor cells are concerned, as described in most human cancers. Nair et al. (4) have shown previously that immunizations with unfractionated tumor extracts are efficient when presented by professional APCs. Similarly, we have demonstrated recently that in vivo treatments involving a dramatic increase of the apoptotic process from poorly immunogenic tumor cells led to remission of established peritoneal carcinomatosis (5). We proposed that phagocytosis of apoptotic tumor cells may favor antigen presentation by professional APCs (6).

It is now well-established that apoptotic cells provide effective antigens recognized by the immune system (7). Moreover, Inaba et al. (8) have shown recently that a protein from a phagocytosed cellular fragment is presented better than preprocessed peptide by MHC class II products of dendritic cells (8). These observations reinforce our hypothesis that the development of antigen processing and presentation of antigenic molecules through the use of APCs that phagocytosed apoptotic bodies from cancer cells offer new possibilities for triggering specific immunity against tumors. Phagocyte recognition and ingestion of intact cells undergoing apoptosis are key events in the program of cell death (9). Macrophages, the “professional phagocytes” that remove apoptotic cells and bodies, are capable of presenting epitopes from engulfed apoptotic cells (10). Likewise, immature dendritic cells can internalize apoptotic cells and present antigens stimulating class I-restricted lymphocytes (11). These recent data suggest that APCs that phagocytosed apoptotic tumor cells are potent models for use in antitumor cellular therapy. Our report shows that in vivo treatment of tumor-bearing rats with monocyte-derived cells that had phagocytosed apoptotic bodies from a poorly immunogenic tumor cell line led to tumor regression and induced more effective specific long-term protection than that of APCs that had phagocytosed nonapoptotic tumor cell extracts.

Materials and Methods

Animals. Rats of the BDIX strain, 4–6 weeks of age, were purchased from Ifa Credo (l’Arbresle, France) and housed and bred in our laboratory.

mAbs. mAbs OX 17 (class II), OX 18 (class I), ED3 (anti-sialoadhesin), OX62 (integrin on dendritic cells), R7.3b (T-cell receptor-αβ), OX41 (macrophages and some dendritic cells), OX33 (CD45-RA), NKRPI (E3.2.3, natural killer cells), 8A2 (CD11c, myeloid and dendritic cells), and ICAM-1 (CD54) were purchased from Serotec (Oxford, United Kingdom). OX7 (Thy-1) and OX42 (CD11b/c) were kindly provided by Dr. M.C. Cuturi (INSERM 437, Nantes, France) and H5 and 24F (respectively, anti-CD80 and CD86 in rat) by Dr. Yagita (Tokyo, Japan).

Cell Culture. DHDK12Trb (PROb), A15, and C6 rat cell lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Cergy Pontoise, France). Cells were routinely checked for Mycoplasma contamination by Hoechst 33258 labeling.

Generation of Apoptotic Cells. Apoptotic bodies were prepared and purified as described previously (5). Briefly, PROb cells were treated for 3 days with sodium butyrate (5 mM). Floating dead cells were removed every day and stored at 4°C until use. Purified apoptotic bodies were stained with annexin V-FITC (Ann-V) and propidium iodide and characterized as annexin V-FITC-positive/propidium iodide-negative by FACSscan analysis (Becton Dickinson, Grenoble, France).

Phagocytic Cell Culture. Monocyte-derived phagocytic cells were harvested from blood or obtained by washing the peritoneal cavity of a BDIX rat previously injected with 5 ml of sterile Brewer’s thioglycolate (Difco, Detroit, MI). Peripheral blood mononuclear cells and thioglycolate-induced PECs were purified on Ficoll-Hypaque gradient (Seromed, Berlin, Germany). Lymphocytes from peripheral blood mononuclear cells were depleted by cellular adhesion in culture for 4 h and treated for 7 days with 0.5 ng/ml of GM-CSF (Bioadvance). The BACs and PECs were maintained in culture for subsequent analysis. PECs or BACs (2.5 X 10⁶ cells) were cocultured for 4 h with or without apoptotic bodies (500 μg) or tumor cell extracts (500 μg) in complete RPMI. To avoid adhesion of cells to plastic culture flasks, they were seeded on plates precoated with 10 mg/ml poly 2-hydroxy-ethyl methacrylate (Sigma, France). Cells were then cultured overnight with 150 units/ml of IFNγ before injection.
Immunofluorescence Analysis. Labelings were performed on PECs and BACs fixed with formaldehyde 1% (15 min at room temperature), rinsed twice in PBS containing 0.1% of BSA, and saturated with rat serum (30 min at room temperature). Cells were then rinsed and seeded at 100,000/well in 96-well plates prior to incubation with mAbs diluted in PBS/BSA 0.3% for 30 min at 4°C. Specific mAbs were detected using an anti-mouse IgG antibody conjugated to FITC (Immunotech, Marseille, France). Finally, staining intensity was analyzed with a FACScan.

Vaccinations with APCs. For curative treatments, groups of four rats received a single i.p. injection of 10⁵ rat colon tumor cells. Ten days later, when carcinosarcoma was established, treated rats received three injections over 5 days of 10⁶ PECs (or BACs) or PECs (or BACs) that had phagocytosed either tumor cell extracts (PECs⁵CR) or apoptotic bodies (PECs⁵AB). The first vaccine was combined with one injection of interleukin 2 (6 × 10⁵ IU/kg; Chiron, Amsterdam, the Netherlands), twice a day for five days. Rats were evaluated daily until death.

Activation of Splenocytes and Cytotoxic Assays. One hundred and fifty-five days after the first injection of tumor cells, cured rats received a new s.c. challenge with parental tumor cells. Tumor growth was estimated weekly until 6 weeks when rats were sacrificed. Splenocytes from vaccinated or naive rats or from rats that developed s.c. tumors were removed aseptically and depleted of RBCs with EL buffer (Qiagen). Splenocytes (2.5 × 10⁶) were activated with 10⁶ irradiated PROb cells (15,000 rad) in 10 ml of RPMI completed with 0.1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO), 25 mM HEPES (GIBCO), 1% non-essential amino acids (GIBCO), and 10% fetal calf serum (GIBCO). The cells were cultured at 37°C. Cytotoxicity was quantified with a 4-h ⁴¹⁵⁶c release assay using PROb and C6 cell lines as targets. Briefly, target cells were labeled with ¹¹¹Indium for 15 min at 37°C in RPMI 1640 alone. Serial dilutions of effector cells in complete medium were mixed with 10,000 target cells in V-bottomed, 96-well plates. After 18 h, cells were centrifuged, the supernatants were then harvested, and specific ¹¹¹In release was determined. The percentage of specific ¹¹¹Indium release of quadruplicate was calculated as:

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\frac{\text{Average experimental cpm - average spontaneous cpm}}{\text{Average maximum cpm - average spontaneous cpm}} \times 100
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In spontaneous release was always <15% of maximal release.

Statistical Analysis. The overall significance of treatments was determined by the Kaplan-Meier method, and the significance of differences observed between survival rates was determined by the log-rank test.

Results

Therapies with APC That Had Phagocytosed Apoptotic Bodies. We showed previously that i.p. injection of apoptotic bodies and interleukin 2 provided long-term survival of 50% of tumor-bearing rats (5), whereas injection of interleukin 2 or apoptotic bodies alone had no influence on the survival rate. We have suggested that apoptotic bodies were phagocytosed by monocyte-derived cells that had become effective APCs (6). In the present study, we tested whether APCs from the peritoneal cavity or blood, which had phagocytosed apoptotic bodies, were capable of curing tumor-bearing rats. The experimental conditions simulated as closely as possible those prevailing in cancer patients. Rats were first injected i.p. with PROb adenocarcinoma cells that developed visible tumor within 7 days, as described previously (12). Vaccine with PECs, which had phagocytosed apoptotic bodies in the presence of interleukin 2, cured 10 of 12 tumor-bearing animals (Fig. 1A). Rats survived for >5 months and showed no signs of any detectable tumor. Fifty % of cured rats challenged with parental PROb cells failed to develop a tumor, having acquired specific antitumor immunity. As a control, all cured rats injected s.c. with A15, a syngeneic glioma cell line, developed detectable tumors within 2 weeks (data not shown). Likewise, injection of PECs or PECS⁵CR had a poor curative effect (only 1 of 10 and 2 of 12 were cured, respectively). Rats treated with PECs or PECS⁵CR were challenged s.c. with PROb tumor cells, and all displayed tumors within 2 weeks after injection.

Very similar data were obtained using rat BACs treated for 7 days with GM-CSF that had phagocytosed apoptotic bodies or tumor cell extracts in the conditions described previously for PECs (Fig. 1B). Five of six rats treated with BACs that had phagocytosed apoptotic bodies from tumor cells were cured and survived for >5 months. Two of six rats were cured when injected with BACs that had phagocytosed tumor cell extracts. One of six rats was cured by injection of BACs alone. Only rats treated with BACs⁵AB were tumor-resistant when challenged s.c. with parental tumor cells.

Phenotypic Expression of PECs and BACs. To characterize the APCs used in cellular therapies, we first studied the expression of surface molecules from PECs or BACs before culture in the presence of apoptotic bodies or cellular extracts (Fig. 2). Using the protocol described in “Materials and Methods,” we obtained essentially monocyte-derived cells after peritoneal injection of thioglycollate into the BDIX rat. As expected, a majority of cells (>80%) obtained from PECs were monocyte-derived (OX 41), with few (<5%) T-lymphocyte (TCRαβ)-positive and (<5%) B-lymphocyte (CD45RA)-positive cells. PECs expressed a panel of markers specific for macrophages and dendritic cells in rats, such as OX41 and OX42 (CD11b/c). Most PECs were related to ED3 macrophages with ~20% of cells expressing it at higher density. However, a small subset of the population could be related to dendritic cells (OX62). Also, few cells were slightly positive for Thy-1 or NKR-P1, which are specifically recognized, respectively, by the monoclonal antibodies OX7 (thymocytes/dendritic cells) and E3.2.3 (natural killer cells/dendritic cells; Ref. 13). Conversely, freshly isolated peripheral blood mononuclear cells, before culture, were T lymphocytes (75%) because they could be labeled with OX 19 mAb (data not shown). After 7 days of culture in the conditions described in “Materials and Methods,” remaining cells were largely monocytes/macrophages and dendritic cells, as shown by positive staining with mAbs. As far as macrophages/dendritic cells are concerned, the phenotype of BACs was comparable with that of PECs. However, the main difference concerned the expression of 8A2/CD11c, which is recognized as a marker for dendritic cells but not macrophages.

Phagocytosis of apoptotic bodies was maximum during the first hours of coincubation with monocyte-derived cells but did not induce any immediate activation of the APC function. Twenty-four h after phagocytosis, accumulation of MHC class II molecules was only detectable.
inside APCs and not on their surface (data not shown). We showed previously that PECs can efficiently phagocytose tumor-derived apoptotic bodies, and that phagocytotic process activates PECs into APC (6). Two days after the phagocytic process, the majority of PECs and BACs developed the phenotype of professional APCs, with significant expression of presentation (MHC classes I and II) and costimulatory (B7) molecules (Fig. 3). As we reported previously for PECs (5), class I and class II expressions were increased after phagocytosis of apoptotic bodies and cellular extracts when compared to nonphagocytic cells (2- and 3-fold, respectively). Our present data show the emergence of PEC-derived APCs and particularly a subpopulation of cells that are B7.2high (20%; mean fluorescence intensity, 2990 versus 210; P = 0.001), suggesting that these cells are capable of efficient costimulation. After culture in the presence of GM-CSF and apoptotic bodies or cellular extracts, similar data were obtained for the phenotypic expression of BACs. It is interesting to note that, in contrast to PECs, the whole population of BACs was homogeneously positive for B7.2. Overnight addition of IFNγ had a stimulatory effect on the expression of MHC classes I and II and B7 molecules but dramatically reduced the phagocytic process of PECs and BACs (data not shown).

**Cytotoxic Activity of Splenocytes from Cured Rats.** After s.c. challenge of cured rats with PROb tumor cells, tumor development was observed in all rats treated with PECs or BACs that had phagocytosed tumor cell extracts. However, 50% of rats treated with PECs that had phagocytosed apoptotic bodies and four of the five rats cured with BACs that had phagocytosed apoptotic bodies did not develop tumor and rejected parental tumor cells. Four weeks later, splenocytes were extracted from vaccinated rats (with no tumor detectable), naive rats, and rats that developed s.c. tumors. Spleen cells were then activated in vitro with irradiated PROb or C6 tumor cells. As shown in Fig. 4, only those rats that rejected parental tumor cells injected s.c., i.e., rats treated with PECs or BACs that had phagocytosed apoptotic bodies were able to elicit a CTL response in vivo, resulting in the lysis of parental tumor cells. This was not the case for rats treated with PECs or BACs alone or those that had phagocytosed tumor cell extracts. As a control, a very low percentage of C6 cell lysis was detected, regardless of the origin of activated splenocytes.

**Discussion**

This study shows that cellular therapy with APCs that had phagocytosed apoptotic tumor cells has powerful therapeutic properties in animals bearing tumors. The curative vaccination elicited potent antitumor immunity and produced specific long-term protection against the challenge of parental tumor cells.

We showed previously that rat colon carcinoma PROb cells are weakly immunogenic (14) and quite resistant to natural killer/lymphokine-activated killer lysis (15). The efficacy of treatment of tumor-bearing rats, combining induction of butyrate and interleukin 2 (12), was related to the efficient production of apoptotic bodies from tumor cells, which was correlated with an increase of monocyte/macrophage recruitment (6). It seemed likely that the phagocytosis of apoptotic bodies activated these cells in APCs. In addition to their phagocytic functions, macrophages are capable of presenting exogenous antigens to T cells, depending on their state of activation. Bellone et al. (10) have shown recently that macro-

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**Fig. 2.** Phenotype of thioglycollate-induced PECs and peripheral blood monocytes (BACs) in culture. Antigen expression was analyzed by single-color immunofluorescence. Propidium iodide was used to exclude dead cells from analysis. Thin lines show the background with isotype-matched control antibody. Each staining was performed at least four times with similar results.

**Fig. 3.** Phenotype of the antigen-presentation capacity of monocyte-derived cells from blood (BACs) or thioglycollate-induced peritoneal cells (PECs) that had phagocytosed apoptotic bodies or cellular extracts. Phagocytes were kept in suspension overnight in culture medium with apoptotic bodies or cellular extracts (bold line), then rinsed, fixed, and stained with either different monoclonal antibodies (see "Materials and Methods") or with secondary antibody FITC-conjugate alone (thin line).

**Fig. 4.** In vitro cytolytic activity of spleen T cells. T lymphocytes were harvested from cured and vaccinated rats , naive rats , or tumor-bearing rats when challenged with tumor cells after treatments , then activated in culture on irradiated PROb tumor cells (A and C) or on irrelevant C6 tumor cells (B and D), as described in "Materials and Methods." Five days later, the cytolytic activity of T cells was tested against PROb (A and B) or C6 cells (C and D) in a standard 18-h 111 In release. Results for one experiment of at least four are shown for each target.
phages, upon active phagocytosis, process apoptotic cells, yielding antigens that access their cytosol. They also suggested that a macrophage subpopulation involved in the physiological clearance of apoptotic cells may process and present tumor-associated antigens to T cells. Similarly, Albert et al. (11) demonstrated that ~20% of dendritic cells contain vesicles with apoptotic corpses after coculture with apoptotic bodies. They observed that dendritic cells, but not macrophages, acquired antigen from apoptotic cells and induced stimulation of class I-restricted CTLs. Rovere et al. (16) showed that dendritic cells internalized apoptotic cells and processed them for presentation to both MHC class I- and class II-restricted T cells when defective clearance of apoptotic bodies by scavenger macrophages occurred. In other words, they suggested that dendritic cells mature when challenged with a relative excess of apoptotic cells. In our experimental model, it was reported previously that thiglycollate-induced peritoneal exudate cells in the rat consist mainly of macrophages, although it cannot be excluded that a subpopulation of PECs is relevant to the dendritic lineage (17). In fact, it is still difficult to discriminate between the presence of macrophages or dendritic cells in the rat model. Through the use of combined markers, we presently and previously showed (6) that PECs in culture expressed phenotypes that contribute to their ability to activate T lymphocytes. We determined that the PECs were a combination of resident and activated peritoneal macrophages and dendritic cells, with some cells expressing a high rate of OX42 and OX41 markers common to dendritic cells and macrophages. Although CD90 (OX-7), CD11c, and OX62 (18) expressions were limited, it cannot be excluded that a subpopulation of PECs was composed of rat dendritic cells, according to the intense expression of MHC class II and B7.2 molecules. Similarly, BACs, originating from blood monocytes, expressed a high level of costimulatory, adhesion, and antigen-presenting molecules, suggesting that these cells are efficient professional APCs after 7 days in our culture conditions. Actually, it is tempting to speculate that the B7.2high cells are responsible for eliciting the tumor rejection immunity observed in our therapies. However, it does not exclude that subsets of macrophages are closed to dendritic cells in term of APCs after phagocytosis of apoptotic bodies by regulating them in the production of molecules appropriate to antigen presentation.

We showed previously in vitro that, in combination with low doses of interleukin 2, PECs that had phagocytosed apoptotic bodies stimulated naive T-lymphocyte proliferation, whereas PECs alone or after phagocytosis of tumor cell extracts did not (6). The present study shows that APCs that phagocytose apoptotic bodies may be particularly effective in presenting tumor-rejection antigens to the immune system because they express high levels both of the molecules needed for antigen presentation (MHC class I and II) and costimulatory molecules (B7.2). Systemic administration of low doses of interleukin 2 was not sufficient alone but enhanced the therapeutic efficacy of vaccines, as demonstrated previously (19). Spleen cells, extracted from cured rats treated with APCs that had phagocytosed apoptotic bodies and restimulated in vitro with PROB tumor cells, specifically lysed PROB cells. This was not the case for spleen cells from rats treated with PECs that had phagocytosed nonapoptotic cellular extracts. The fact that PECs that had phagocytosed tumor apoptotic bodies elicited a specific CTL response and long-term memory against tumor cells suggests an efficient cross-presentation of tumor-associated antigens. However, the in vivo presentation of tumor antigens still needs to be clarified.

Dendritic cell-tumor cell conjugate vaccines have several features of interest for potential applications to human immunotherapy (20). In this context, the similar data that we obtained with BACs that had phagocytosed apoptotic bodies from tumor cells could be more relevant for human therapies. We show that monocyte-derived cells that had phagocytosed apoptotic bodies are powerful antitumor weapons, even against poorly immunogenic tumor cells. Thus, the induction of apoptosis in tumor cells, and possibly the reorganization of the plasma membrane typical of apoptosis, could generate new or unmasked antigens and activate their presentation. It is also tempting to speculate that death by apoptosis provides a specific recognition of and phagocytic process for apoptotic cells by professional APCs. If our results can be extrapolated to human cancer, a simple protocol for treatment could be developed, involving only the ex vivo mixing of a patient’s APCs with apoptotic bodies derived from autologous cancer cells. This protocol implies no prior knowledge of which antigens are expressed by tumor cells, and no choice needs to be made concerning the best antigen to target. Thus, each individual would benefit from a tailor-made therapy.

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