Highly Successful Therapeutic Vaccinations Combining Dendritic Cells and Tumor Cells Secreting Granulocyte Macrophage Colony-stimulating Factor

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ABSTRACT

In an attempt to induce potent immune antitumor activities, we investigated, within the rat 9L gliosarcoma model, distal therapeutic vaccinations associating three therapies: dendritic cell vaccination, intratumoral granulocyte macrophage colony-stimulating factor (GM-CSF) gene transfer, and tumor apoptosis induction. Vaccines of dendritic cells coinjected with processed GM-CSF secreting 9L cells induced systemic responses, resulting in the complete regression of distant preimplanted 9L tumor masses in, with the best strategy, 94% of male rats. All of the cured rats developed a long-term resistance to a rechallenge with parental cells. The curative responses were correlated with the detection of elevated specific cytotoxic activities and a CD4+ and CD8+ T cell-, and natural killer (NK) cell-mediated IFN-γ production. The survival rate of the rat seemed more directly linked to the amount of GM-CSF secreted by the transduced tumor cells, which in turn depended on the toxicity of the apoptosis-inducing treatment, than to the level of apoptosis induced. Unexpectedly, alive GM-CSF secreting 9L cells became apoptotic when injected in vivo. Thus we documented the positive role of apoptosis in the induction of therapeutic antitumor responses by comparing, at equal GM-CSF exogenous supply, the effects of dendritic cells coinjected with apoptotic or necrotic 9L cells. The data showed the superior therapeutic efficiency of combined vaccines containing apoptotic tumor cells. In conclusion, vaccinations with dendritic cells associated with apoptotic tumor cells secreting GM-CSF show a very high therapeutic potency that should show promise for the treatment of human cancer.

INTRODUCTION

Despite progress in classical therapies, most cancers are still incurable. In the future, successful treatments will need to combine different therapeutic strategies. Among them, cytokine-secreting tumor cell vaccines (1, 2) or dendritic cell vaccines (3) are the most promising. Numerous murine models together with a few clinical trials have revealed the potent ability of granulocyte-macrophage colony stimulating factor (GM-CSF) to enhance antitumor immunity by inducing coordinated cellular (CD4+ and CD8+ T cell-mediated) and humoral responses with a broad cytokine profile (4). Vaccination with irradiated tumor cells engineered to secrete GM-CSF stimulates the recruitment of granulocytes and large numbers of antigen-presenting cells (dendritic cells and macrophages) to the tumor site, suggesting the involvement of GM-CSF in the augmentation of tumor-antigen presentation (5). Moreover, GM-CSF induces the maturation of these dendritic cells (6). For gliomas, several studies have shown that delivering GM-CSF s.c. in conjunction with inactivated tumor antigens can initiate a systemic response that leads to the regression of distant peripheral or intracerebral tumors (7, 8). We have observed previously that the rat 9L gliosarcoma cells that were transduced with the mouse (m)GM-CSF gene completely lost their tumorigenicity when injected s.c. Moreover, s.c. vaccines of irradiated 9LmGM-CSF cells not only induced protective immune responses against s.c. or intracerebral challenges with parental 9L cells but also some therapeutic responses against a 9L tumor preimplanted s.c. a few days before. But, despite significant tumor growth delay, only a few animals were completely cured (9). Thus, in agreement with data published elsewhere, we concluded that GM-CSF gene-engineered vaccines had good prophylactic effects but were poorly effective against established tumors (7, 10). Therefore, we hypothesized that better therapeutic activity could be obtained by combining such GM-CSF gene transfer with other immunotherapeutic approaches.

Dendritic cells are potent antigen-presenting cells specialized in the initiation of T-cell immunity (11). The use of dendritic cell vaccines as adjuvant to induce tumor-specific CD8+ cytotoxic and CD4+ helper T-cell responses is supported by many animal studies as well as human clinical trials (12). In malignant gliomas, dendritic cell therapy has been shown to be safe and able to overcome, at least partially, the immunosuppressive state associated with those tumors and to result in substantially prolonged survival or even cure in animals (13). A multitude of dendritic cell antigen-loading strategies may be effective in inducing tumor-specific cytotoxic T-lymphocytes responses (14). But today, cross-priming is considered to be essential for generating immunity to tumors that are themselves poor antigen-presenting cells (15). Animal studies have shown that dendritic cells are sufficient for, and probably exclusive in, mediating MHC class I cross-presentation of exogenous antigens to CD8+ T cells (5). For example, a few years ago, Albert et al. (16) reported the unique capacity of dendritic cells and not macrophages, pulsed with apoptotic cells but not with necrotic cells, to present viral antigens on MHC class I molecules and to stimulate thereby antigen-specific cytotoxic responses. Dendritic cells can take up antigens in the form of exosomes from either living cells (17) or phagocytosed tumor cells dying from necrosis and/or apoptosis (18, 19). But there is still controversy as to whether necrotic or apoptotic tumor cells serve as a superior source of tumor-associated antigens to pulse dendritic cells for immunotherapeutic applications (20–22). Nevertheless, both types of immunogens (whole tumor cell lysates or apoptotic tumor cells), consisting of a panel of antigens instead of specific peptides, expand the scale of potentially available MHC-restricted tumor peptides and also provide MHC class II epitopes needed for the induction of CD4+ T cells, well-known as important regulators of sustained antitumor immunity (23, 24).

In the present study, we investigated, within the rat 9L gliosarcoma model, a new therapeutic approach to cure animals from a preimplanted tumor, combining three of the most promising existing anticancer treatments: cytokine (i.e., GM-CSF) gene transfer, dendritic cell vaccination, and tumor apoptosis induction. Our goal was to analyze carefully the respective role of each factor in the in vivo initiation of potent antitumor curative immune responses.
MATERIALS AND METHODS

Animals. Male inbred Fischer 344 rats, purchased from Charles River Laboratories (l’Arbresle, France), were housed at the Animal Facility at the University of Brussels Medical Center, in accordance with European Community guidelines, and used at the age of 10 to 12 weeks.

DENDRITIC CELL AND GM-CSF-SECRETING TUMOR CELL VACCINES

Cytokine Secretion Assays. Mouse GM-CSF production was measured by a specific ELISA, according to the manufacturer’s instructions (Biosource), within culture supernatants from 9LmGM-CSF cells either nontreated or γ-irradiated (80 Gy), mitomycin C- or cisplatin-treated, or freeze/thawed that were collected 24, 72, or 120 hours after treatment, and kept frozen until assayed. Interleukin-12 (p40 and p70) production was similarly tested in cell culture supernatants from dendritic cells cocultivated for 48 hours with 9L mGM-CSF cells either nontreated or treated as indicated above, in absence or in presence of lipopolysaccharide.

Tumor-Specific Cytotoxic Immune Responses. Several tumor-bearing rats or cured rats from different vaccinated groups were killed after 40 days, and the spleens were recovered. Enriched T-cell suspensions were purified by passage on nylon woolfiber columns and stimulated at 5.10^6 cells/mL in 2 mL dendritic cell medium with 10^5 irradiated (80 Gy) 9L cells. After 5 days of culture, the activated effector cells were tested for a lytic activity against the specific 9L target, or a third party syngeneic target (MATB, a mammary adenocarcinoma) or the K562 NK target in a standard 4-hour ^51Cr-release assay. Results are expressed as percent specific lysis at various effector to target cell ratios.

RESULTS

Coinjecting Dendritic Cells with Irradiated 9LmGM-CSF Cells Strongly Increases the Survival of Tumor-bearing Rats. We have previously published, within the rat 9L gliosarcoma model, that the transfer of the mGM-CSF gene inside tumor cells led, with s.c. implantation, to a complete loss of tumorigenicity. Moreover, s.c. vaccines of irradiated 9LmGM-CSF induced not only protective immune responses against s.c. or intracerebral challenges with parental cells but also some therapeutic responses against 9L cells implanted s.c. some days before. But, despite significant tumor growth delay, only a few rats (6%) were completely cured (9). In the present study and as illustrated in Fig. 1, we have greatly increased the therapeutic efficacy of irradiated 9LmGM-CSF cell vaccines by coinjecting bone marrow-derived dendritic cells generated from naive Fischer 344 rats. Indeed, with this combination, up to 60% of the vaccinated rats (P < 0.001) rejected a preimplanted tumor, whereas all of the non-treated rats or rats vaccinated with dendritic cells alone died within 50 days. Furthermore, although a tumor never appeared in some rats, in others we observed regression of tumor masses even when measuring more than 1 cm^2. This effect was repeatedly seen in all of the experiments done. As shown in Fig. 2, the in vivo therapeutic responses were linked to the induction, in all of the cured vaccinated rats, of highly effective 9L-specific cytotoxic responses (Fig. 2A). Almost no nonspecific lytic activities against a third-party syngeneic tumor (MATB, a mammary adenocarcinoma) were detected (Fig. 2B). But, in few rats, NK-type cytotoxic responses were observed as reflected by the moderate to high levels of K562 cell killing (Fig. 2C). As a whole, the cytotoxic activities underlined in cured vaccinated rats.
were correlated with the production of high amounts of IFN-γ by 9L-activated splenic T cells: 1,448 ± 156 pg/mL for cured vaccinated rats versus 10 ± 5 pg/mL for control nonvaccinated rats (Fig. 2D). Flow cytometric analysis showed that in cured vaccinated rats the IFN-γ was equally secreted by about 40% CD4+ or CD8+ T cells but less by NK cells (~20%; Fig. 2D, inside). Cold-target inhibition assays done with hot 9L cells and cold K562 cells confirmed that the anti-9L activity in panel A was mediated by T cells (Fig. 2; data not shown). All tumor-free rats, even those cured since >1 year, became resistant to a challenge with 10^5 or even 10^6 parental 9L cells but not with 10^3 MATB cells, indicating the emergence of tumor-specific memory immune responses (data not shown).

Fig. 1. Coinjecting dendritic cells with irradiated 9LmGM-CSF cells strongly increases the survival of tumor-bearing rats. Rats were vaccinated s.c. in the right flank with 3 × 10^6 dendritic cells (□) or 5 × 10^6 irradiated 9LmGM-CSF cells (●) or mixture of both (▲) at days 4, 11, and 18 after the implantation of 10^5 9L cells, s.c. in the left flank. Control rats (●) were nonvaccinated. Results are from three independent experiments for the group dendritic cells alone, four for the group 9L GM-CSF irradiated alone, and six for the control and dendritic cells +9L GM-CSF irradiated groups. **, significantly different (P < 0.01). (DC, dendritic cells)

Fig. 2. In vivo induction of curative therapeutic responses was correlated to in vitro detection of specific cytotoxic responses and IFN-γ secretion. Splenic T cells were harvested, forty days after 9L inoculation, from untreated control rats (●) or from cured vaccinated rats (▲) and stimulated in vitro with irradiated 9L cells. Five days later, the cytolytic activity was tested against the specific target 9L (A), a third-party syngeneic target MATB (B), and the NK target K562 (C) in a standard 4-hour 51Cr-release assay. Each curve represents an individual rat. Aliquots of supernatants from same cultures were collected after 72 hours and assayed for IFN-γ content by ELISA (D). Results are from five control rats and seven vaccinated rats; bars, ±SD. Intracytoplasmic IFN-γ production was investigated, by flow cytometry, among CD4+ or CD8+ T cells and NK cells after similar 3-day cocultures between splenic T cells and irradiated 9L cells (D, inside). **, significantly different (P < 0.01).
Both Tumor Apoptosis and GM-CSF Secretion Seem Required to Sustain the Therapeutic Effects of the Combined Vaccines. Next, we assessed the respective roles of apoptosis induction and GM-CSF secretion in the successful efficacy of the combined therapeutic vaccinations. For this purpose, we compared in vivo the capacities of dendritic cells coinjected with apoptotic versus necrotic, GM-CSF secreting versus nontransduced 9L cells, to reduce the regression of a preimplanted tumor. As illustrated in Fig. 3, the therapeutic effect of the combined vaccines was increased in the presence of at least some apoptotic tumor cells secreting GM-CSF. Indeed, by vaccination with dendritic cells and solely necrotic (freeze/thaw) 9LmGM-CSF cells rather than dendritic cells and apoptotic (irradiated) 9LmGM-CSF cells, the percentage of cured rats decreased from 60 to 22% (P < 0.05). Furthermore, when we injected mixtures of non-GM-CSF-secreting 9L cells and dendritic cells, we observed that with irradiated (apoptotic) cells the long-term survival decreased from 60 to 20% (P < 0.01) and from 22 to 0% (P < 0.05) for necrotic tumor cells.

Irradiation or Chemotherapeutic Agents Heterogeneously Induce Apoptosis in 9LmGM-CSF Cells. Apoptosis induction in 9LmGM-CSF was tested by measurement of caspase-3 activation and phosphatidylserine translocation like we previously described for Fig. 1, except that the tumor cells were induced.

Propidium iodide, were submitted to one cycle of freeze/thaw, they were exclusively and necrotic tumor cells. In contrast, when 9L or 9LmGM-CSF cells were treated with cisplatin (a chemotherapeutic inducer (35%) followed by mitomycin (10%) and cisplatin (5%). These data also show that not only irradiation but also chemotherapeutic molecules such as mitomycin C or cisplatin were able to induce apoptosis in 9LmGM-CSF cells. However, as indicated by the percentages of cells in early apoptosis (annexinV-FITC/propidium iodide−, 72 hours after treatment), the level of apoptosis induced varied greatly according to the agent: γ-irradiation was the best inducer (35%) followed by mitomycin (10%) and cisplatin (5%). These data also show that all of the agents in vitro used to induce apoptosis resulted in a mixed variable proportion of viable, apoptotic, and necrotic tumor cells. In contrast, when 9L or 9LmGM-CSF cells were submitted to one cycle of freeze/thaw, they were exclusively propidium iodide+, i.e., in necrosis only.

Vaccination Efficacy Does Not Depend on the Rate of Apoptosis Induced. Interestingly, vaccines of dendritic cells coinjected with 9LmGM-CSF cells in vitro treated with cisplatin (a chemotherapeutic drug identified as the lowest apoptosis inducer) led to cure the highest number of rats, i.e., 94% (Fig. 4B). Similar vaccinations with dendritic cells and 9LmGM-CSF cells either irradiated or treated with mitomycin C allowed tumor eradication in 60 and 75%, respectively, of the rats. On the other hand, when using (as an expected negative control for apoptosis) a vaccine composed of dendritic cells admixed with untreated 9LmGM-CSF cells, we were surprised to observe an 88% cure of the tumor-bearing rats. Control vaccines consisting in untreated 9LmGM-CSF cells, in the absence of dendritic cells, had no therapeutic efficacy.

Altogether, the data suggest that the number of apoptotic cells in the vaccine did not correlate with the therapeutic outcome, which seemed rather more related to the number of viable cells remaining. However, as shown in Fig. 4C, TUNEL assays (a) and antiactive caspase 3 immunostainings (b) done on regressing 9LmGM-CSF tumor masses revealed the presence of many positive cells, indicating that 9LmGM-CSF cells lost in vivo their tumorigenicity in a s.c. location, by an apoptotic mechanism. Therefore, tumor apoptosis induction was inescapable for a vaccine combining dendritic cells and alive 9LmGM-CSF cells.

Various 9LmGM-CSF Cell Treatments Modulate GM-CSF Secretion But Not Dendritic Cell Maturation. What, then, is the key factor in the efficacy of the various vaccination conditions if it is not the degree of apoptosis induced? We hypothesized that it could be because of differences in GM-CSF secretion by differently treated 9LmGM-CSF cells or to an impact of the tumor treatment on the maturation state of the coinjected dendritic cells, which should be reflected on interleukin-12 secretion. Therefore, we first quantified (by ELISA) the amount of GM-CSF secreted by 9LmGM-CSF cells either nontreated, or γ-irradiated (80 Gy), mitomycin C- or cisplatin-treated, or freeze/thawed, at different time-points after treatment (24, 72, or 120 hours). Fig. 5A shows that the GM-CSF production was high for nontreated or cisplatin-treated tumor cells, low after irradiation or mitomycin treatment, and almost totally undetectable after one cycle of freeze/thaw. Interestingly, the GM-CSF secretion was linearly correlated with the viability of 9LmGM-CSF cells: the more toxic was the treatment, the less viable cells were and the less the GM-CSF production was. On the other hand, the amount of interleu-
kin-12 (p40 and p70) produced by dendritic cells cocultured during 48 hours with differently treated 9LmGM-CSF cells was found to be similar to the one of dendritic cells cultured in medium alone and much lower than that of lipopolysaccharide-activated dendritic cells (Fig. 5B). The dendritic cells incubated with processed tumor cells did not mature spontaneously in vitro, but their maturation on additional incubation with lipopolysaccharide was not prevented (data not shown). Altogether, these data suggest that the success of the combined therapeutic vaccines was directly linked to differences in GM-CSF secretion after tumor cell treatment.

At Equal GM-CSF Secretion, Tumor Apoptosis Induction Helps to Increase the Therapeutic Efficacy of the Combined Vaccines. To definitively document the positive role of apoptosis in the induction of the therapeutic antitumor responses, we investigated in vivo coinjections of dendritic cells and variously treated 9L cells with local delivering of recombinant GM-CSF. Our results show that administrating GM-CSF by repeated s.c. injections was not effective at all because all of the rats died within 60 to 90 days (Fig. 6).

However, when the GM-CSF was continuously delivered at vaccination site via osmotic minipumps, vaccines of dendritic cells coinjected with 9L cells led to a cure of 38% of the rats with irradiated cells (i.e., in apoptosis) but only 12% (P < 0.02) with freeze/thawed cells (i.e., in necrosis). Without GM-CSF supply, dendritic cell-9L vaccines resulted in death of rats within 60 days in case of necrotic tumor cells or within 80 days in case of apoptotic tumor cells. These data show that at equal GM-CSF administration, tumor apoptosis induction was better to increase the therapeutic efficacy of the combined vaccines. Nevertheless, the results also indicate that exogenous administration of GM-CSF, i.e., via minipumps, was far less effective (38% rats cured) than endogenous delivery by transduced 9L cells themselves (60 to 94% rat survival; see above).

DISCUSSION

The present study shows for the first time the great curative potential of an original immunotherapeutical strategy synergizing apoptotic
GM-CSF–secreting tumor cell vaccines and dendritic cell vaccines. These combined vaccinations induced systemic responses resulting in the complete regression of distant preimplanted 9L tumor masses in (with the best strategy) 94% of rats and the long-term resistance of all of the cured rats to a rechallenge with parental 9L cells. Importantly, these results were obtained in male rats where previous studies have failed to obtain good results as compared with female rats (28). Numerous murine models and few clinical trials have revealed the potent ability of GM-CSF to enhance antitumor immunity by a coordinated humoral and CD4+/CD8+ mediated cellular response with a broad cytokine profile (4, 29). Vaccination with tumor cells engineered to secrete GM-CSF has been shown to be an effective adjuvant, mainly because it enhances antigen processing and presentation by recruiting locally large numbers of antigen-presenting cells (30, 31). However, if GM-CSF gene-engineered cell vaccines were quite effective at protecting animals from a parental tumor injection (32, 33), they were recorded poorly effective against established tumors (9). By coinjecting dendritic cells with irradiated GM-CSF–secreting 9L cells, we have greatly increased the induction of specific and systemic therapeutic immune responses and thereby the long-term survival rate of the rats (from 8 to 60%, P < 0.0001). The curative responses were correlated with the detection of elevated specific cytotoxic activities and a CD4+, CD8+ T cell- and NK cell-mediated IFN-γ production. Moreover, because all of the cured rats failed to develop a tumor when challenged with parental tumor cells (but not with a third-party syngeneic tumor), they exhibit specific immunologic memory. Such acquired tumor-resistance has rarely been reported in other studies (34).

Our first data indicated that both apoptosis and GM-CSF secretion seemed required to sustain the therapeutic effects of the combined vaccines. Indeed, the percentage of cured rats was drastically reduced when the vaccines were made of dendritic cells and non-GM–CSF secreting 9L cells or of dendritic cells and solely necrotic tumor cells. But, as shown later, the crucial parameter was not the degree of apoptosis induced by the pretreatment but rather the toxicity of the latter, which influenced the level and duration of GM-CSF production. Indeed, the more toxic the treatment the less the secretion of GM-CSF by the transduced tumor cells and the worse the therapeutic response observed. Moreover, in agreement with the work of Shi et al. (35) but contrary to Kurane et al. (36), we observed that the GM-CSF protein expressed by tumor cells after gene transfer was a superior stimulant than exogenous GM-CSF given in the tumor microenvironment by repeated injections or osmotic minipumps (8). This could be explained in part by the short half-life (few hours) of recombinant GM-CSF (37). Nevertheless, the real influence of tumor apoptosis induction was difficult to evaluate within our 9L GM-CSF tumor model because we unexpectedly observed that alive 9L GM-CSF cells became apoptotic when injected s.c. in vivo. A similar mechanism should operate for the majority of other GM-CSF–transduced cell lines like Renca (38) or DHD/K12 (10), which also lose their tumorigenicity when injected in vivo (9).

There is still controversy in the literature as to whether necrotic or apoptotic tumor cells are the best sources of antigens to pulse dendritic cells for immunotherapeutic applications. In agreement with the works of Henry et al. (34), Strome et al. (39), and Scheffer et al. (40) but in opposition with the study of Herr et al. (20), our results are in favor of apoptosis rather than necrosis. Indeed, equal exogenous GM-CSF supply, the coinjection of dendritic cells with irradiated (apoptotic) 9L cells was more efficient than the coinjection of dendritic cells with freeze/thawed (necrotic) 9L cells to induce therapeutic responses. Nevertheless, because all of the agents we used in vitro to induce apoptosis within adherent 9LmGM-CSF tumor cells resulted in a mixed proportion of viable, apoptotic and necrotic cells (see Fig. 4A), it is likely that, as proposed by Kotera et al. (21), having both apoptosis and necrosis on the one hand induce MHC class I-restricted cytotoxic T lymphocytes activation (16, 19) and on the other hand give danger signals essential for dendritic cell maturation and subsequent optimal T-cell stimulation (27).

A few studies have shown, for malignant gliomas, that delivering the GM-CSF cytokine s.c. in conjunction with inactivated tumor antigens can initiate a systemic response that leads to regression of distant peripheral and intracerebral tumors (7, 8). Other studies have validated dendritic cell vaccinations (13) or even coupled injections of dendritic cells with irradiated tumor cells (41) as glioma immunotherapy. But, as far as we know, this is the first study revealing the high efficacy of a vaccine combining dendritic cells and irradiated (apoptotic) tumor cells secreting GM-CSF. We hypothesize that the dendritic cells injected as part of the vaccine initiate, as expected and shown elsewhere, a tumor-specific immune response (42). Meanwhile, the GM-CSF secreted by the coinjected transduced tumor cells, which allows the recruitment of endogenous dendritic cells and stimulates their maturation and migration, could thereby favor a possible exchange of tumor antigens between the injected dendritic cells and the endogenous dendritic cells. This last point is supported by two recently published studies. The first one showed the role of tumor cell apoptosis in tumor antigen availability in draining lymph nodes (43).---
The second study showed that the participation of endogenous dendritic cells was needed to obtain an optimal in vivo expansion of the T-cell responses that were induced by dendritic cells given as vaccines (44).

In conclusion, vaccinations with dendritic cells associated with apoptotic tumor cells secreting GM-CSF show a very high therapeutic potency we also validated in the murine Renca renal cell carcinoma model (manuscript in preparation). This original strategy should show promise for the treatment of human cancer.

REFERENCES


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