Leveraging the Immune System during Chemotherapy: Moving Calreticulin to the Cell Surface Converts Apoptotic Death from "Silent" to Immunogenic

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Abstract

In contrast to prior belief, tumor cell apoptosis is not necessarily silent but can be immunogenic. By tracing how anthracyclines and γ-irradiation trigger immunogenic cell deaths, we found that they were causally connected to the exposure of calreticulin on the tumor cell surface, before apoptosis in the tumor cell itself occurred. Furthermore, we showed that calreticulin exposure was necessary and sufficient to increase proimmunogenic killing by other chemotherapies. Our findings suggest that calreticulin could serve as a biomarker to predict therapy-associated immune responses, and that tactics to expose calreticulin might improve the clinical efficacy of many cancer therapies. [Cancer Res 2007;67(17):7941–4]

Introduction

The chemotherapy of cancer is often conceived as analogous to the antibiotic treatment of microbial infection. A complete and permanent cure can be obtained by an anticancer agent that would kill all tumor cells including cancer stem cells and micrometastases. As in bacterial or viral infection, however, it is plausible that an immune response elicited against the residual cancer cells might contribute to the complete eradication of micrometastases and cancer stem cells (1, 2). Driven by this consideration, we speculated that chemotherapeutic regimes that would stimulate an immune response against tumor cells should be particularly efficient (3, 4). Indeed, some peculiar chemotherapeutic regimens can stimulate the immune system as a warranted side effect, by acting on the immune effectors themselves. As an example, low-dose cyclophosphamide can deplete regulatory T cells, thus deinhibiting anticancer immune responses in mice and humans (5, 6). Imatinib mesylate can stimulate dendritic cells (DC), natural killer cells, and a cell type with mixed DC/natural killer characteristics that may act as an antitumor effector (7). Another possibility to stimulate anticancer immune responses is to kill tumor cells in a fashion that they are recognized by the immune system (refs. 8–12; Fig. 1*A*).

According to current knowledge, cell death, as it physiologically occurs—at a pace of several million events per second in the healthy human adult—is nonimmunogenic. This absence of immunogenicity may be related to the fact that apoptotic cells emit "eat-me" signals to neighboring cells that lead to the efficient phagocytic removal of apoptotic corpses, in a way that no immune response is elicited or that self-tolerance is reinforced (13). Genetic defects in the phagocytic removal of apoptotic cells could thus lead to autoimmune disease (14).

Driven by these considerations, we investigated whether some chemotherapeutic agents might elicit a type of cell death that, at variance with physiologic cell death, would be immunogenic. Murine CT26 colon cancer cells or MCA205 fibrosarcoma cells were treated with a panel of 20 different cell death–inducing agents *in vitro* and the dying cells were injected s.c. into immunocompetent histocompatible mice. One week later, the animals were rechallenged with live CT26 or MCA205 cells, and the absence of tumor growth was scored as an indication of successful anticancer vaccination. Using this protocol, we found that most cell death inducers including agents that damage DNA, mitochondria, lysosomes, or the endoplasmic reticulum (ER) failed to elicit antitumor immune responses. However, a few lethal agents turned out to be highly efficient in triggering immunogenic cell death. This applied to anthracyclines (doxorubicin, idarubicin, mitoxanthrone) as well as to γ-irradiation (8, 11, 15). Importantly, caspase inhibition by Z-VAD or by the baculoviral caspase inhibitor, p35, which suppress the late manifestations of apoptosis (yet do not prevent cell death as such), completely abolished the immunogenicity of cell death induced by a long treatment (24 h) with doxorubicin (15).

Systematic comparisons of CT26 cells succumbing to immunogenic and nonimmunogenic cell death failed to reveal clear-cut ultrastructural differences. Both immunogenic cell death inducers (anthracyclines) and nonimmunogenic death stimuli (such as etoposide and mitomycin C) were indistinguishable in triggering the hallmarks of apoptosis including cellular and nuclear shrinkage with chromatin condensation, outer mitochondrial membrane permeabilization, and phosphatidylserine exposure on the outer leaflet of the plasma membrane before viability is lost. Both immunogenic and nonimmunogenic cell death led to caspase activation, indicating that activation of this class of cysteine proteases was required but was not sufficient for immunogenic cell death (15). We therefore set out to determine subtle biochemical changes in the plasma membrane surface of cells undergoing immunogenic versus nonimmunogenic cell death. Two-dimensional gel electrophoreses coupled to mass spectroscopy revealed that anthracyclines were uniquely capable of inducing the exposure of calreticulin on the outer face of the plasma membrane (11).

Calreticulin is a Ca^{2+}-binding lectin chaperone that is mostly present in the ER lumen. Indeed, calreticulin is frequently used as

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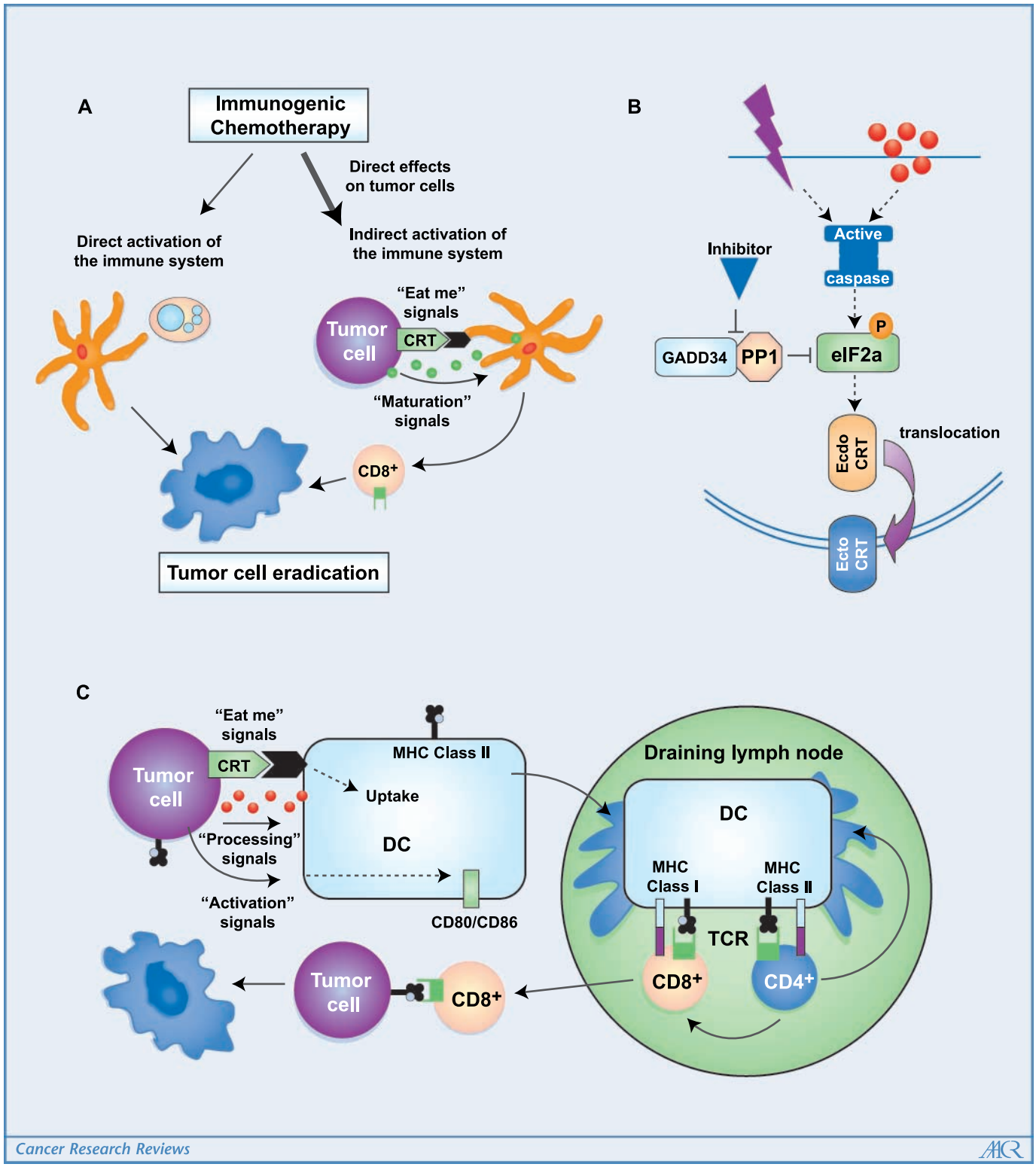

Figure 1. Calreticulin (*CRT*) exposure in immunogenic cell death. *A,* the principle of immunogenic chemotherapy. The chemotherapeutic agent can have an immunostimulatory effect, either by a direct action on the immune system or by inducing a type of tumor cell death that elicits an immune response. The immune system then contributes to the eradication of residual tumor cells. *B,* signal transduction pathway leading to calreticulin exposure. Anthracyclines or γ-irradiation lead to the activation of one or several unknown caspases and then to the phosphorylation of eIF2α, followed by the translocation of calreticulin to the cell surface. Anthracyclines or γ-irradiation also cause cell death. Inhibitors of the PP1/GADD34 complex stimulate the phosphorylation of eIF2α without caspase activation and without cell death. The hyperphosphorylation of eIF2α is then followed by the translocation of preformed calreticulin from the ER to the plasma membrane surface. *C,* effects of immunogenic cell death on DC. Early calreticulin exposure is required for the uptake of antigen by DC. Additional signals emanating from the dying tumor cell are required for the maturation of DC, and hence, for optimal MHC class I–restricted presentation of tumor-derived peptides to CTLs.

an ER-specific marker in subcellular localization studies (16, 17). However, calreticulin can also appear on the surface of dying cells, serving as an "eat-me" signal for neighboring phagocytes (14, 18). Contrasting with the standard kinetics of calreticulin exposure, which parallels the presence of phosphatidylserine on the cell surface (18), we found that anthracyclines elicited calreticulin exposure with very rapid kinetics. Thus, calreticulin exposure occurred within minutes after the addition of anthracyclines, whereas phosphatidylserine exposure occurred only after several hours of treatment. Additional experiments revealed that calreticulin exposed at the cell surface of anthracyclin-stressed (or γ-irradiated) tumor cells translocated as a preformed (rather than neo-synthesized) protein to the cell surface, correlating with the phosphorylation of the eukaryotic translation initiation factor eIF2α. Inhibition of the phosphatase that dephosphorylates eIF2α (which is composed of a catalytic subunit, protein phosphatase 1 or PP1, and an adaptor called GADD34) also led to the hyperphosphorylation of eIF2α and efficiently induced the surface exposure of calreticulin. Chemical inhibitors of PP1 (such as tautomycin or calyculin A) or of the PP1/GADD34 complex (such as salubrinal; ref. 19) induced calreticulin exposure on the cell surface without any major cytotoxic effects (Fig. 1*B*).

Preapoptotic calreticulin exposure strongly correlated with the immunogenicity of cell death induced by distinct lethal compounds. More convincingly, we found that the knockdown of calreticulin by means of a small interfering RNA sufficed to abolish the immunogenicity of cell death elicited by anthracyclines (11) or γ-irradiation (8), as determined by injecting dying cells into immunocompetent mice and rechallenging the same animals with live tumor cells. When calreticulin expression was knocked down with a small interfering RNA, and recombinant calreticulin protein was absorbed to the surface of the cells, recombinant calreticulin restored the immunogenicity of cells subjected to calreticulin knockdown plus anthracyclin treatment. In addition, recombinant calreticulin could reestablish the immunogenicity of cells treated with etoposide or mitomycin C—two agents that induce per se nonimmunogenic cell death (and fail to expose calreticulin on the surface; refs. 11, 12). These findings indicate that early calreticulin exposure is both necessary and sufficient to render cell death immunogenic. The fact that exogenous recombinant calreticulin (which should be free from tumor-derived peptides) could substitute for endogenous calreticulin in this setting, also suggests that it is not the presentation of tumor peptide by calreticulin that accounts for its immunogenic effect (20).

These results were obtained in prophylactic antitumor vaccination experiments and could be recapitulated in the treatment of established tumors. Local injection of established CT26 colon carcinomas (in BALB/c mice) or MC205 fibrosarcomas (in C57Bl/6 mice) with anthracyclines led to the permanent regression of neoplasias. The treatment of such tumors could only be obtained in animals bearing an intact immune system and became impossible when dendritic cells, $CD4^+$, or $CD8^+$ T cells were eliminated. Conversely, local injection of mitomycin C and etoposide (which induce tumor cell death but not calreticulin exposure), PP1/GADD34 inhibitors (which induce calreticulin exposure but not cell death), or recombinant calreticulin alone had no curative effects. However, the combination of cytotoxic agents plus PP1/GADD34 inhibitors or recombinant calreticulin cured most, if not all, tumors established in immunocompetent hosts. No such curative effect was ever seen in athymic *nu/nu* mice (11, 12). These results indicate that cell death coupled to calreticulin exposure could elicit an immune response that contributes to the cure of cancer. Indeed, animals that had been cured from tumors exhibited long-term immunity against specific tumor antigens.

What then, are the mechanisms through which calreticulin exposure renders cell death immunogenic? One of the rate-limiting steps in the stimulation of cytotoxic T cell responses is the uptake of antigenic material by immature DC, which can present antigenic peptides bound to MHC class I molecules after a critical maturation step. Hence, cell death must trigger at least two essential steps to be immunogenic: (*a*) uptake of antigen by DC and (*b*) DC maturation (21). Blockade of calreticulin with an avian antibody or prevention of calreticulin exposure (either by caspase inhibition of by calreticulin knockdown) abolished the recognition of dying tumor cells by DC *in vitro* and *in vivo*. However, inhibition of calreticulin exposure did not affect the capacity of dying tumor cells to stimulate DC maturation, and recombinant calreticulin failed to elicit the activation or maturation of DC (11). This suggests that calreticulin exposure is essential for the phagocytic recognition of apoptotic cells by DC, yet is dispensable for DC maturation and activation (Fig. 1*C*). Other molecules liberated from dying cells must account for DC maturation (22).

The aforementioned results may, potentially, have profound implications for anticancer therapy (23, 24). Provided that these data can be extrapolated to the human system, we can formulate the following predictions:

1. Some, but not all, chemotherapeutic regimens can induce immunogenic cell death with early calreticulin exposure, thus eliciting a therapy-associated anticancer immune response that determines disease outcome. It will be important to determine which anticancer regimens induce calreticulin exposure and immunogenic cell death. Such regimens may be particularly efficient in treating cancer if applied in a context in which patients do not suffer from therapy-induced immunosuppression.
2. Tumors that fail to expose calreticulin in response to radiotherapy or immunogenic chemotherapy (e.g., with anthracyclines) should escape from the immunogenic component of the antitumor response, and hence, should have a particularly negative prognosis. As a result, it may be interesting to monitor calreticulin exposure or the presence of all components of the signal transduction machinery leading to calreticulin exposure in tumor cells, with the hope of establishing new prognostic or predictive biomarkers.
3. Therapeutic regimes designed to reestablish calreticulin exposure (for instance by inhibiting PP1/GADD34) should enhance the immunogenicity of cell death, and hence, boost the therapeutic efficacy of per se nonimmunogenic regimens. Thus, it may be expected that combinations of tumor-cytotoxic and immunostimulatory agents would synergize in therapeutic efficacy.

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