Review

Immunogenic Chemotherapy: Discovery of a Critical Protein through Proteomic Analyses of Tumor Cells

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Abstract. *The aim of chemotherapy and radiotherapy is to eliminate tumor cells. While the outcomes of these cytotoxic treatments have previously been assigned to their direct effects on tumor cells, recent findings have shown that the host's immune system also contributes to the success of chemotherapeutic and radiotherapeutic regimens. The finding that some cytotoxic antitumor coumpounds such as anthracyclines were capable of triggering a potent T-cell-dependent antitumor response has prompted the search for molecular determinants responsible for the immunogenicity of anthracyclines. Proteomic analyses of anthracycline-treated tumor cells have recently revealed the critical involvement of calreticulin in mediating the immunogenicity of dying tumor cells. Here, we focused on the molecular study of immunogenic chemotherapy which led to the characterization of calreticulin as a critical protein in immunogenic cancer cell death.*

Genetic mutations that lead to altered protein signaling pathways within cells may trigger their transformation, eventually leading to tumor formation. However, immunosurveillance of tumor cells might allow, in some cases, the detection and the destruction of these pre-malignant cells by the immune system before clinical detection. Factors involved in the early elimination of tumor cells include innate effectors and innate molecules, such as gamma delta T-cells (1),

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natural killer cells (NK) (2), dendritic cells (DCs) (3) and natural killer T-cells (NKT) (4), TRAIL (5), perforin/ granzyme (6) as well as members of adaptive immune responses (B- and T-cells) (7). It has been shown that mice lacking recombination-activating gene 2 (RAG2) and the signaling pathway of IFNγR or STAT-1 are more likely to develop spontaneous and chemically induced tumors, suggesting the critical involvement of the immune system in antitumor responses, a concept called "tumor immunoediting" (7). Nonetheless, tumors evoke several mechanisms to escape immunosurveillance. Immunosubversion by tumors of the immune system could be considered as the seventh hallmark of cancer (8).

One of the goals of cytotoxic treatments could be to induce a potent antitumor immunity to apoptotic or dying tumors and restore an effective immune memory to tumor antigens. Radiotherapeutic and chemotherapeutic regimens both have the ability to induce potent tumor cell death. Although the efficacy of these cytotoxic treatments on tumor growth is generally attributed to their direct killing efficacy, the release of cellular components from dying cells such as "damage-associated molecular patterns" (9) has previously been shown to enhance immune responses (10-12). However, to elicit an efficient cytotoxic T-lymphocyte (CTL) response, several immunological steps are required to trigger links between innate and cognate immunity (13). First, the phagocytosis/capture of exogenous antigens by dendritic cells is critical in enabling the second phase *i.e.* antigen processing/presentation of the phagocytic cargo in late endosomes by DC. DCs then need to receive an "activation stimulus" which will promote their migratory capacity from tumor beds to draining lymph nodes and their maturation to the state of antigen-presenting cells (14). Cross-presentation of antigenic peptides on DC major histocompatibility (MHC) class I molecules to antigen

specific T-cells will promote T-cell activation (signal 1). The recognition of co-stimulatory molecules (signal 2) and the production of cytokines (signal 3) leads to the polarization and full activation of T-cells by DCs (15, 16). Finally, DC imprinting of T-cell chemokine receptor expression (signal 4) (17, 18) associated with inflammatory processes located at the effector phase (tumor beds) will induce T-cell migratory patterns leading to tumor eradication (19, 20).

The pioneering work of Casares *et al.* (21) first showed that anthracyclines, a subclass of chemotherapeutic cytotoxic agents, can induce an immune response depending on caspases controlling antigen uptake and not DC maturation.

The exact molecular mechanisms underlying the immunogenicity were recently unraveled by Obeid *et al.* (22) who identified the role of surface calreticulin (ecto-calreticulin) in the immunogenicity of anthracycline-treated tumor cells using proteomic analyses. This finding delineates novel approaches for the generation of cancer vaccines and therapeutic intervention.

Immunogenic Chemotherapy

In addition to its direct cytotoxic antitumor effect, chemotherapy would ideally be able to harness the host's immune system to combat the tumor. However, the type of cell death mainly induced by chemotherapy is apoptosis, which is often defined as a non-immunogenic or even tolerogenic cell death modality (12). By contrast, some studies have suggested that rather than the type of cell death, the tumor cells themselves or the nature of the death-inducing stimulus would influence the immunogenicity of dying cells (23, 24). The latter hypothesis drove Casares *et al.* (21) to discover that drugs inducing endoplasmic reticulum (thapsigargin, tunicamycin, brefeldin), lysosome (bafilomycine A1) or mitochondrial stress (arsenite, betulinic acid, C2 ceramide), or drugs inhibiting the proteasome (MG132, lactacystin, ALLN), NF-KB (Bay 11-7082), or components causing DNA damage (Hoechst 33342, camptothecin, etoposide, mitomycin C) did not promote antitumor immunity. Indeed, a mouse model of vaccination was set up to assess the immunogenicity of dying tumor cells (Figure 1A). In this model, BALB/c mice were injected in one flank with CT26 colon adenocarcinoma tumor cells induced to die with various pharmacological drugs and were then rechallenged one week later with live syngeneic tumor cells. A screening of components commonly used in the clinic of the oncological armamentarium was performed and X-ray- or anthracycline-treated tumor cells were found to selectively elicit a protection against a tumor challenge. In contrast, mitomycin C- or etoposide-treated cells failed to do so, although both treatments exhibited comparable direct cytotoxic activity. This antitumor response could be attributed to T-cells, as the effects of vaccination with anthracycline- or X-ray-treated tumor cells were completely lost in athymic

nude mice lacking T-lymphocytes. Interestingly, antitumor responses in immunocompetent mice were long lasting as mice had mounted a memory response against CT26 tumor cells. Anthracycline treatment appeared to be an immunogenic chemotherapy, *i.e.* a cytotoxic therapy triggering the activation of the host immune system. These immune responses elicited by anthracyclines treated cells could be blunted in the presence of a pan caspase inhibitor Z-VAD-fmk, underlining an important role of caspases in this process. As CD11c+ DC are pivotal in the activation of T-cells, their implication in the immunogenicity of anthracycline treated tumor cells was investigated. Taking advantage of a diphtheria toxin receptor (DTR) transgenic mice expressing DTR in CD11c+ cells (25), it was shown that DCs were indeed required to mediate the immunological responses to dying tumor cells by mediating their phagocytosis. Strikingly, while both mitomycin C-, etoposide- or doxorubicin-treated tumor cells elicited comparable levels of DC maturation, only doxorubicin treated cells were efficiently phagocytosed by DC. This observation formally demonstrated a tight link between phagocytosis of apoptotic bodies and immunogenicity. The molecular mechanisms responsible for the phagocytosis of tumor cells nevertheless remained unexplored until the identification of calreticulin as the key protein in mediating phagocytosis and therefore the immunogenicity of dying cancer cells by Obeid *et al.* [(22), discussed below].

Background of Calreticulin

Calreticulin was first identified as a calcium-binding protein of the muscle sarcoplasmic reticulum (26). This protein was later found to be present in every cell of higher organisms. Under classical conditions, calreticulin is found in the lumen of the reticulum participating in calcium homeostasis and acting as a protein chaperone (27, 28). However, a few reports showed that calreticulin could also be detected in many intracellular compartments and at the plasma membrane (29-31). Indeed, it was recently demonstrated by Henson *et al.* (32) that calreticulin is a marker for phagocytosis on the surface of apoptotic cells. They claimed that membrane calreticulin expression was the second signal (in addition to phosphatidylserine) for apoptotic cells to be phagocytosed (32). Calreticulin was also identified as a marker for the phagocytosis of apoptotic cells in Drosophila (33) and was recently shown to be required for the adiponectin-mediated clearance of early apoptotic bodies (34).

Identification of Calreticulin as a Protein Involved in Immunogenic Cell Death

As previous data obtained from Casares *et al.* (21) pointed to a major role of phagocytosis of apoptotic cells in mediating an immune response, the assumption that some
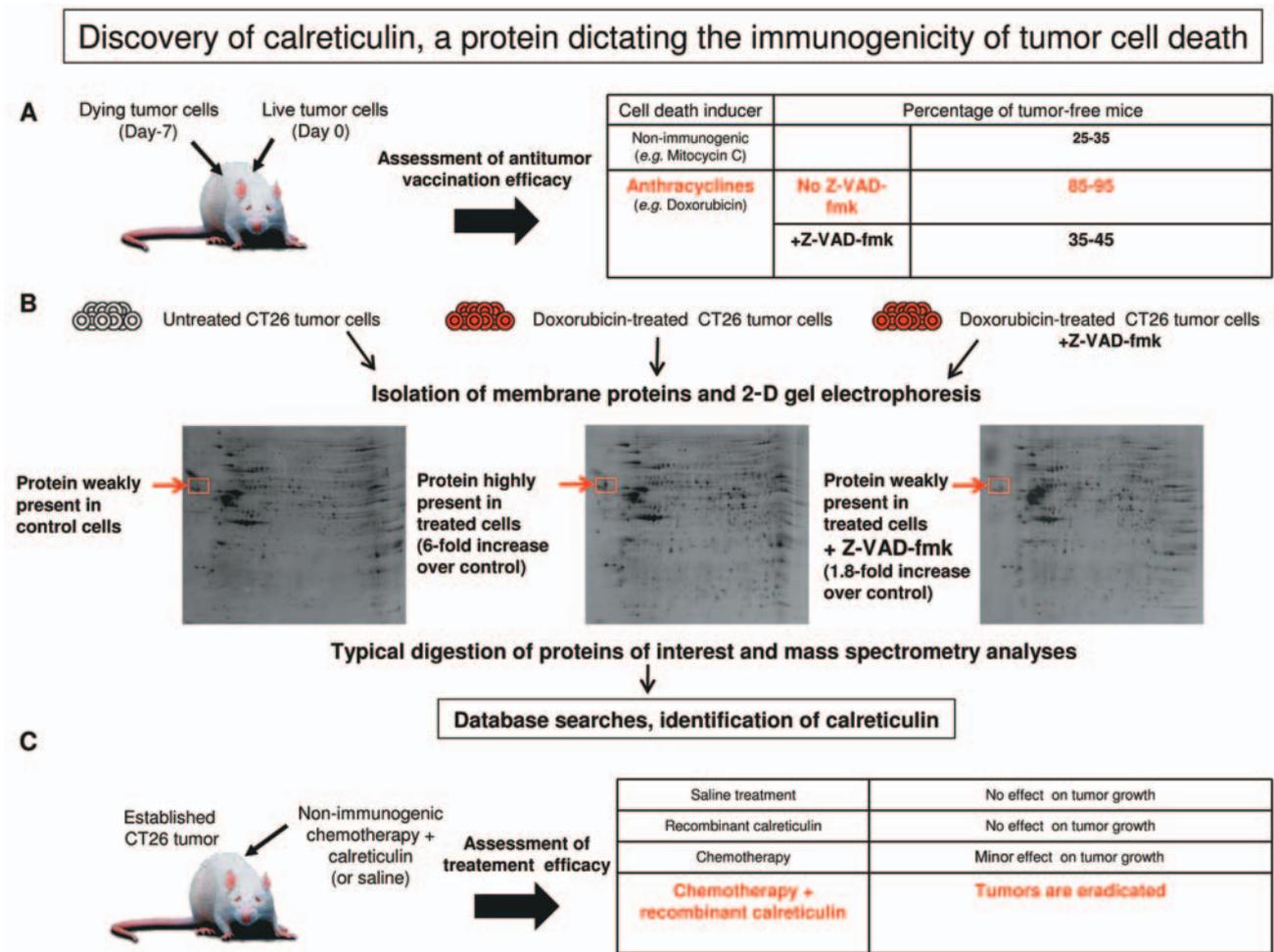
Figure 1. *The discovery of the concept of immunogenicity of cell death. The sequential steps which led Obeid et al. (22) to the discovery of ecto-calreticulin as a predictive factor for the immunogenicity of chemotherapy are detailed. The proteomic analyses of anthracycline-treated CT26 tumors in the presence of Z-VAD-fmk versus live CT26 are shown. The synergistic antitumor effects between non-immunogenic cell death inducers and recombinant calreticulin or inhibitors of PP1/GADD34 are depicted.*

induced (or repressed) membrane proteins might account for the immunogenicity of doxorubicin treatment was made. As phagocytosis was shown to be a rapid process occurring within one hour following anthracycline exposure, the differential immunogenicity obtained with various regimens was unlikely to result from transcription regulatory processes. Thus, rather than examining the transcriptome expression induced by treatment, Obeid *et al.* (22) focused on the potential proteomic changes of the external membranes of tumor cells. A proteomic comparison of membranes coming from doxorubicin-treated cells and control cells led to the discovery of calreticulin as shown in Figure 1B. CT26 cells were either left untreated or treated with doxorubicin or treated with doxorubicin and Z-VAD-fmk, a pan caspase inhibitor that reduces the uptake and immunogenicity of doxorubicin-elicited cell death (21).

Membrane proteins were then biotinylated, affinity purified and finally subjected to 2-dimensional electrophoresis. Calreticulin was eventually identified as a protein that was dramatically upregulated by CT26 cells upon treatment with doxorubicin (by a factor of 6), but less (by a factor of 1.8) upon treatment with doxorubicin and Z-VAD-fmk. Another protein identified was ERP57, a calreticulin-interacting chaperone (30).

Use of Calreticulin in Anticancer Therapy

Although proteomic studies of tumor cells provided putative interesting candidates for the improvement of the current management of cancer patients, the functional validation of these candidates is unfortunately missing from most studies. However, Obeid *et al.* (22) provided extensive data to confirm

and extend their discovery of calreticulin as a mediator of immunogenic cell death. Using fluorescence-activated cell sorter and immunofluorescence analyses, they have indeed shown that surface calreticulin (ecto-calreticulin) expression on tumor cells upon treatment with cytotoxic agents was strongly correlated with the ability of dying tumor cells to elicit tumor antigen uptake by DC and subsequent antitumor immunity. By preventing calreticulin expression using a specific small interference RNA, they showed that ecto-calreticulin is responsible for triggering an immune response to idarubicin/doxorubicin/mitoxantrone-treated tumor cells and by adding back recombinant calreticulin to mitomycin C-etoposide-treated tumor cells, they restored antitumor immune responses. Obeid *et al.* (22) finally unraveled the molecular pathway dictating ecto-calreticulin exposure to cell surface. They identified that anthracyclines induced the phosphorylation of eIF2α, a protein which is hyperphosphorylated in ER stress. Interestingly, the use of compounds which inhibit the PP1/GADD34 complex (involved in eIF2α dephosphorylation) was sufficient to induce calreticulin exposure. Furthermore, when these inhibitors were injected into CT26 tumors along with a non-immunogenic cell death inducer (etoposide or mitomycin C), synergistic antitumor effects were observed (Figure 1C). These latter results delineate an immunogenic chemotherapy strategy to combat cancer.

Importance of Proteomic Studies in Immunogenic Chemotherapy

Proteomic analyses have traditionally been used to study correlations between proteins expressed in healthy *versus* tumor tissues. These investigations have revealed cellular processes and biochemical networks at the protein level that are responsible for the development of a particular disease. At the clinical level, the characterization of proteins directly involved in the disease process can lead to the design of drugs capable of targeting selective oncogenic pathways in tumor cells. One example of a paradigmatic drug designed to specifically interfere with c-kit/bcr-abl or PDGFRα tyrosine kinases is imatinib mesylate (35-37). The ability of proteomics to detect variations of cell protein expression therefore allows the use of this method to study the molecular events occurring during disease processes (38), identify biomarkers of disease (39, 40), or explore the mechanisms of action of anticancer drugs at the molecular level (41, 42). Nevertheless, because of variations in protein expression, post-translational modifications and compartmentalization, results from proteomic studies should be analysed cautiously in the context of the biological processes involved. Results obtained from whole tumor cell extracts may lead to misinterpretation of results as they give an "average picture" of proteome variations within a cell, while the study of subcellular fractions

would have provided a more accurate picture of cellular alterations within tumor cells upon treatment (43, 44).

The recent paper of Obeid *et al.* (22) is in this regard very interesting as the background information on the importance of phagocytosis in mediating immunogenic chemotherapy has led to the study of membrane fractions rather than whole tumor cell lysates. This strategy allowed the identification of the membrane translocation of calreticulin upon immunogenic cytotoxic treatment of tumor cells. This finding could have clinical implications if the authors could show that calreticulin exposure on tumor cells following patient therapy with defined/selected chemotherapeutic regimen is correlated with long term disease-free survival and immune responses. Calreticulin exposure could easily and routinely be monitored on plasma membranes of tumor cells and could become a predictive factor of an immunogenic chemotherapy (45). The identification of immunogenic proteins expressed by tumor cells could be regarded as a new application of proteomics. Besides its potential use as a predictive factor, ecto-calreticulin could be systematically induced by inhibitors of PP1/GADD34 during non-immunogenic chemotherapies. Moreover, proteomic technology could be extremely useful in determining other proteins within tumor cells that account for immunogenic chemotherapy. For instance, while calreticulin exposure on tumors is now characterized as the first checkpoint to elicit an immune response, the absence of immune response against live cells that express ecto-calreticulin prompts the search for additional proteins responsible for the activity of dying tumor cells. The knowledge of the different actors involved in the immunogenicity of dying tumor cells could aid in the design of future anticancer therapies.

Conclusion

The initial demonstration of immunogenic chemotherapy regimens has challenged the common view that chemotherapy mediates its effects only through cell-autonomous effects. The work of Obeid *et al.* (22) illustrates a utilization of proteomic tools for a new objective: the identification of proteins which are critically involved in the immunogenicity of dying tumor cells. Not only does this work confirm the previous studies of Casares *et al.* (21), but it also provides clinical relevance to these findings by showing that non-immunogenic treatments could be rendered immunogenic using compounds leading to overexpression of calreticulin by tumor cells. These data have indeed arisen great interest among scientists as chemoresistance of tumors could be reversed by the modulation of the PP1/GADD34 complex. In addition, calreticulin could be used to predict the lack of efficacy of a chemotherapeutic regimen *in vitro* or *in vivo*. We further plan (i) to investigate the effects of

calreticulin in humans and (ii) to study the efficacy in humans of inhibitors of PP1/GADD34 as potential enhancers of antitumor responses.

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