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Review

The effects of chemotherapeutics on cellular metabolism and consequent immune recognition

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Abstract

A widely held view is that oncolytic agents induce death of tumor cells directly. In this report we review and discuss the apoptosis-inducing effects of chemotherapeutics, the effects of chemotherapeutics on metabolic function, and the consequent effects of metabolic function on immune recognition. Finally, we propose that effective chemotherapeutic and/or apoptosis-inducing agents, at concentrations that can be achieved physiologically, do *not* kill tumor cells directly. Rather, we suggest that effective oncolytic agents sensitize immunologically altered tumor cells to immune recognition and immune-directed cell death.

Review

Do drugs kill tumor cells directly?

Our laboratories have been investigating the consequences of chemotherapeutic agents on cell surface expression of immunologically important molecules, including Major Histocompatibility Complex (MHC) encoded molecules (both MHC class I and II), B7.1 (CD80), B7.2 (CD86), Fas (CD95), and Fas Ligand (CD95L) [1]. T cell activation requires recognition of antigens associated with MHC molecules [2] and a second signal provided by co-stimulation [3] provided by the interaction of molecules including B7.1 or B7.2 or Fas (CD95) on the cell being recognized and CD28 or CTLA-4 or Fas Ligand on the T cell. We, and others, have reported that changes in the cell surface occur in drug-treated cells [4-10]. First, we observe changes and increases in cell surface expression of the B7 family members, CD80 and CD86, on drug-treated (adriamycin, 5-fluorouracil, or methotrexate-treataed) tumor cells. These cell surface molecules have been extensively studied and are now widely accepted as important in promoting the immunogenicity of tumor cells by providing costimulation for T cells [5]. Second, we, and others, have observed that most of the drugs we have used increase cell surface expression of Fas (CD95) and sensitize the Fas-bearing tumor to Fas-induced death [1,7,9]. In the present report,

we discuss our working model that the concert of metabolic interference with the ability of the tumor to be more readily "seen" by the immune system may be the basis for effectiveness of many currently effective strategies or the basis for developing novel therapeutic approaches to treating cancers.

We first explore one of the relevant immunological cell surface receptors, Fas (CD95). Fas is a member of the tumor necrosis receptor (TNFR) family. The cytoplasmic tail of Fas contains a death domain able to trigger intracellular caspase cascades that culminate in apoptotic cell death [11-13]. Fas can induce apoptosis when ligated by its cognate ligand (FasL, CD95L) in Fas sensitive cells [11,12]. Paradoxically, Fas, like other members of its family, can transduce growth-enhancing signals as well as death signals [14-18]. In chemo-sensitive leukemia and solid tumors, anti-cancer drugs have been shown to induce apoptosis and for many tumors the pathways involved include, but are not limited to, Fas and FasL [19-21].

In an attempt to reflect *in vitro* the concentrations of drugs that can be achieved physiologically *in vivo*, we were surprised to observe that tumor cells from many tissue origins were not dead at such concentrations. However, we found (and continue to find with a broad spectrum of agents) that the drugs have several important consequences. Our results have shown that chemotherapeutic agents sensitize Fas-bearing, Fas-insensitive tumors to Fas-susceptibility and Fas-induced death [1]. Consistent with these observations, cross-resistance to Fas/FasL and oncolytic agents has been reported by our group and others [1,8,10,22]. While much of our work has involved Fas and FasL, other members of "death inducing" receptor-ligand pairs likely perform similarly in the presence of effective oncolytic agents [23].

Together these data indicated that an important mechanism of chemotherapeutic agents may be to sensitize tumor cells to immune-directed death. Implied by these results is the importance of identifying and preserving (from death by high dose chemotherapy) the FasL (or other ligand)-bearing cells to facilitate immunological destruction of drug-treated tumor cells.

How do chemotherapeutic agents sensitize the tumor cells to immune-mediated death?

Our efforts at understanding the molecular mechanisms by which chemotherapeutic agents affect metabolism and immune recognition have been focused primarily on the expression and function of Fas on the cell surface of tumor cells. Fas is expressed on most rapidly dividing cells, including tumor cells, hepatocytes, epithelial cells, and lymphocytes [24-26]. Interestingly, tissues that express Fas and yet remain insensitive to Fas-induced death (including most dividing, regenerating, and self-renewing cells) exhibit a metabolic phenotype characterized by high rate, cytosolic glycolysis. This "respiratory deficiency" is the result of a metabolic change in tumor cells that was first observed by Warburg in 1926 [27]. The coincidence of increased cytosolic glycolysis and increased Fas expression on tumor cells (and other dividing cells) provided the basis for examining a causal link between Fas expression and the use of glucose as a primary, glycolytic source of fuel.

Our experiments have demonstrated that the distribution and levels of expression of Fas are altered in response to changing concentrations of glucose in many cell lines and in freshly isolated cells from a variety of tissues. Limited glucose supplementation is known to enhance proliferation of tumor cells and has been used for topical applications to accelerate wound healing *in vivo* [28,29]. Some of our recent results suggest that glucose availability and consequent production of intracellular reactive oxygen species may regulate the striking change in the results from Fas engagement that promotes proliferation to Fas engagement that promotes death. Supporting this observation is the recent report that increasing glucose concentrations can induce increased free radical production [30] and increases in reactive oxygen or free radicals are known to cause Fas engagement to result in cell death [31-33]. In addition, we have observed and reported that drug resistant cells appear to readily utilize the carbons derived from beta oxidation of fatty acids and exhibit a consequent loss of cell surface Fas. Taken together these observations support the notion that Fas expression and function are intertwined with glucose metabolism and the potential for changes in reactive intermediates in tissues or cells exhibiting changes in glucose metabolism. The fact that selection in drugs results in loss of Fas and in metabolic changes that may protect the cells from free radical damage will be important in designing novel cancer therapies.

We have performed experiments to examine the correlation between cell surface Fas expression and glucose metabolism. As a prototype for the Fas positive and Fas negative cells we have used the L1210 cell and the L1210DDP as Fas positive and Fas negative, respectively, Figure 1. In these experiments, we directly measured the rates of glucose utilization and oxidation of L1210 and L1210DDP [34].

L1210 DDP cells express no cell surface Fas [1]. To address the possibility that Fas is expressed, but has been targeted to a subcellular organelle, we permeabilized and stained L1210 and L1210DDP cells with fluorochrome conjugated anti-Fas antibody (J02.2, Pharmingen). The cells were examined by flow cytometry. Our data indicate that

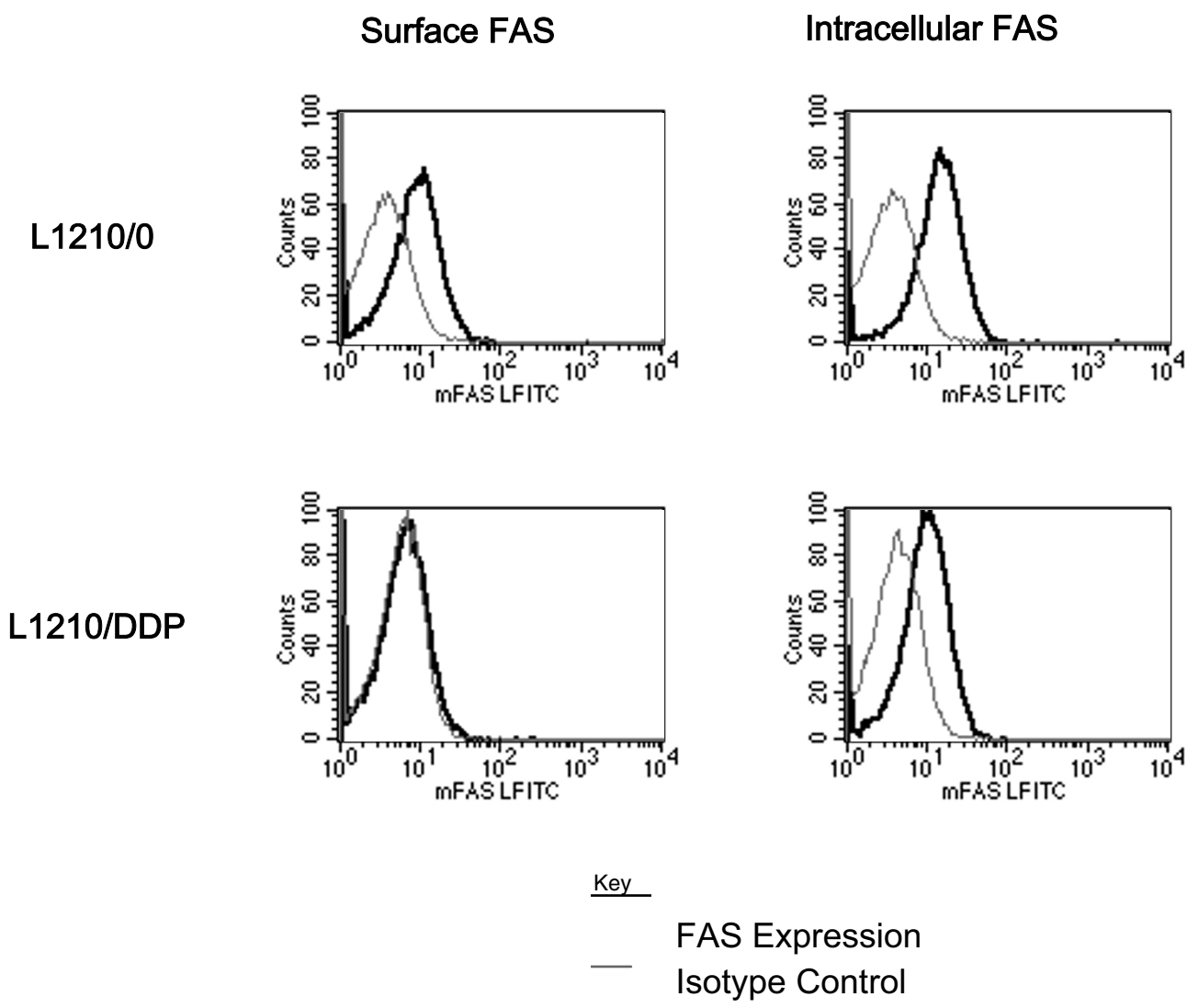

Figure 1
Distribution and Level of Fas in L1210/0 and L1210/DDP Cells. Expression of cell-surface Fas, leftmost panels, and intracellular Fas, right most panels in L1210/0, upper two panels, and L1210/DDP cells, lower two panels. The levels of cell surface Fas (dark lines) were determined using fluorochrome conjugated anti-Fas antibodies (Pharmingen Inc.) and flow cytometry. The levels of intracellular Fas were determined subsequent to cellular permeabilization and fixation. The Fas levels are measured relative to staining for fluorochrome-conjugated isotype control (grey lines).

L1210 DDP cells express no cell surface Fas; however, the cells do express intracellular Fas. Fluorochrome-conjugated isotype matched antibody was used as control, and specific antibody stains were confirmed as specific. These data demonstrate that the Fas negative, apoptosis resistant cells, express *intracellular* Fas, Figure 1 below. The relevance of internal Fas in drug-selected, drug resistant tumor cells is that the cell is rendered Fas-insensitive to cell death unless the intracellular pool can be redistributed to the cell surface and potentially re-wired to "death-inducing" machinery.

It is known that T cells require two signals for activation [3]. One of these signals involves the binding of the proteins CD28 or CTLA-4, which are constitutively expressed on most resting T cells, with the proteins B7.1 (CD80) or

B7.2 (CD86). T cell activation through CD28 binding, results in a proliferative T cell response, enhanced T cell survival and cytokine release [35]. Conversely, CTLA-4 engagement induces powerful inhibitory signals in T cell activation resulting in the negative regulation of T cell responses [36]. Collins et al. recently showed that B7.1 favors CTLA-4 over CD28 engagement [37]. This is still controversial, nonetheless is raises the possibility that co-stimulatory receptor/ligand pairs are multifunctional.

We propose that co-stimulatory interactions between B7 family members and CD28 or CTLA4-bearing T cells and the resulting cytokines directly impact the subcellular distribution of Fas and the ultimate outcome of Fas engagement on tumor cells.

In Figure 2 we show that B7.2 levels in HL60 (human leukemic cells) also increase after treatment with 10^{-8} M of Adriamycin. We note that HL60 is a human cell line and that the drug is different than that in previous figures. This figure is representative of many experiments with other cell lines and additional drugs that include methotrexate, adriamycin, and 5-fluorouracil. While we have not tested the ability of all drugs to promote immunogenicity, these resuts *may imply* that the increase in the co-stimulatory signal as a result of drug treatment is a general phenomenon.

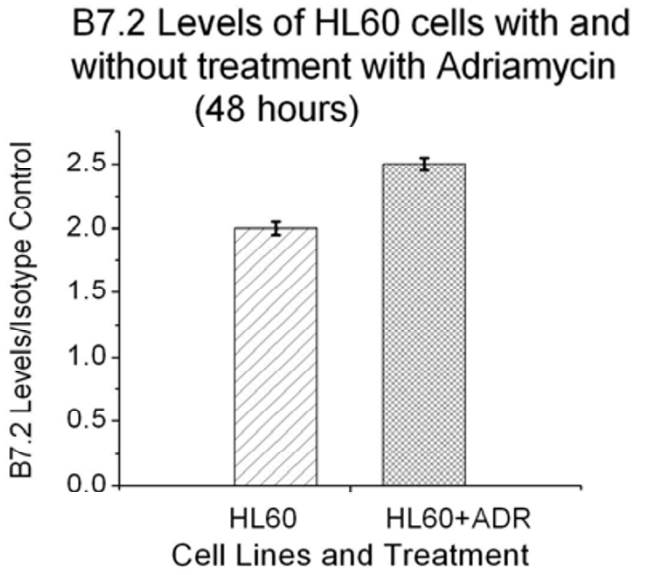

Figure 2
Adriamycin Induced Increase in B7.2 Expression. Expression of the cell-surface co-stimulatory molecule B7.2 as a function of treatment with adriamycin. The level of cell-surface B7.2 was determined using fluorochrome conjugated anti-B7.2 antibodies and flow cytometry. The B7.2 levels are measured relative to staining for fluorochrome-conjugated isotype control.

Which immune cell can kill the tumor cell?

The first attempts at cancer immunotherapy were made over 100 years ago on the assumption that tumor antigens might be recognized as foreign [38]. These studies gave rise to animal tumor models using syngeneic tumors, spontaneously arising tumors, and xenografts into immunodeficient hosts. The collective of these studies resulted in a variety of immunotherapeutic protocols including adjuvant therapy, cytokines, NK cell activation, macrophages, and attempts to stimulate tumor antigen specific B and/or T cell responses against tumor antigens. Some approaches have had partial success, but what has become clear is that tumor cells are, by definition, "immunologically privileged" and successfully evade effective tumoricidal immune recognition [38]. An alternate possibility is suggested by the premise which Prehn has postulated that effective chemotherapies may result from suppressing a particular type of immune response that supports tumor cell growth [39]. An example of this notion would be T cell-produced cytokines which have been reported to support neural regeneration [40].

MHC encoded molecules were defined by Peter Gorer and George Snell as surface molecules responsible for the rejection of tumor cells between genetically distinct members of the same species [41]. These molecules are also responsible for graft rejection and T cell activation. The mechanism for both phenomenons has been attributed to T cell receptor recognition and effector functions that occur only when MHC molecules and antigen are recognized by the T cell receptor for antigen. Cells implicated in tumor cell death include $CD4^+$ T cells, $CD8^+$ T cells, natural killer (NK) cells, or more recently, gamma delta ($\gamma\delta$) T cells [38]. Immune recognition and destruction of allogeneic tumor cells likely results from increased expression of MHC antigens on the tumor cell surface, processing and presentation of tumor antigens, and expression of costimulatory molecules on the tumor cell. Rejection of tumor cells following drug treatment, therefore, may be directly related to "recognition" of a cell which has changed in cell surface expression of immunologically important cell surface receptors and that has been metabolically "rewired" by chemotherapeutic agents.

Conclusion

Thus, we suggest that a drug-treated tumor cell is made susceptible *by* drugs or radiation to "death-inducing" receptor/ligand pairs, including, but not limited to, Fas and FasL expressed on candidate immune cells, such as CD4+ T cells, CD8+ T cells, gamma delta T cells, and NK cells. We propose that selective identification of the

immunocytes proliferating in the tumor-bearing lymph node as a key element in personalizing and selectively sensitizing an individual's tumor cells to chemo-, radio-, and immunotherapy.

While the potential of immune-directed cytotoxicity of drug-treated tumor cells may provide an important new perspective, the question arises as to how to reconcile this idea with the accepted notion that chemotherapy can be immunosuppressive. The key factor in resolving this seeming paradox may be the dose of the agent or the nature of a given chemotherapeutic agent. Clearly, there are cases where drugs at high doses have immunosuppressive effects (perhaps by direct cytotoxicity of the immune cells). In contrast, decreased doses have recently been shown to be more effective in the clinic. Taken together, both views suggest that "less may be more" effective for chemotherapy [45,46]. We propose that an in depth evaluation of the effects of popular chemotherapeutic agents on induction of immunologically relevant molecules on the tumor be rigorously evaluated.

Considering the potential importance of cells of the immune system in controlling cancer growth, with or without chemotherapy, an important question is raised. Should lymph nodes, the local "home" too many immune cells, be removed as therapy? Although axillary node removal is still a standard regime for treatment of invasive breast cancer, it is clear that regional lymph nodes have biological significance for being more than just anatomical filters. The regional lymph node is the heart of our immunologic defense system and the present routine practice of partial resection of the regional nodes where they are easiest to remove undoubtedly has an effect on immunological and physiological function.

Macroscopically involved lymph nodes should possibly be removed for prognosis [42] and for the identification of the immune cells involved in tumor recognition, but the routine removal of lymph nodes is questioned as noted above by our group and others [43]. It is becoming clear that many patients can be spared axillary node dissection without adversely affecting outcome [44]. As we begin to better understand the inter-relationships of surgery, tumor cell kinetics, chemotherapy, and the host immune response, new paradigms are developing. These include the notion that routine surgical removal of axillary nodes provides no additional benefit and could be omitted to spare the patient unnecessary axillary node removal [43].

In summary, we suggest a novel perspective be applied to the clinical diagnosis and treatment of tumors. Maximally, we suggest that each tumor be screened for the effects of potential chemotherapeutics on immunogenicity. We suggest identifying cells responding to the tumor in the node (unsuccessfully or not) so that drug-sensitized tumor cells can be killed rather than supported by the identified immune cells. Minimally we suggest that a re-evaluation of the mechanism of tumor cell death and therapeutic approaches be experimentally and clinically considered.

Declaration of Competing Interests

None declared.

Authors' Contributions

This paper is distinct because it is an opinion paper. However, each author contributed uniquely to the manuscript. Author 1, MKN, provided the conceptual framework for the model presented in this paper. Author 2, RM, participated in discussions and drafts of the manuscript. Author 3, EVM, performed the flow cytometric data provided in this manuscript. Author 4, DS, provided discussion about a supportive role for T-Cells in the growth of a tumor. Author 5, RT, participated in discussions and drafts of the manuscript. Author 6, WC, contributed the effects of surgery on tumor growth. Author 7, RC, participated in discussions and drafts of the manuscript.

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