Increased Sensitivity of Adriamycin-selected Tumor Lines to CTL-mediated Lysis
Results in Enhanced Drug Sensitivity

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Abstract

The emergence of drug resistance to chemotherapeutic agents is a major cause of treatment failure in cancer therapy. Therefore, much effort has been aimed at circumventing or reversing this undesired effect. Recently, we found that tumor cell lines selected for their multidrug-resistant phenotype can also exhibit increased levels of TAP mRNA and MHC class I proteins. This raised the question of whether drug-resistant tumors are more readily recognized by MHC-restricted CTLs. In this report, we show that five of five MHC class I+ tumor cell lines grown in medium containing Adriamycin developed into variants that expressed higher levels of MHC class I than did their corresponding parental cell lines. This was not observed with a MHC class I- cell line. No similar association was noted for changes in the expression of either HER-2 or intercellular adhesion molecule 1 protein. We also found that MHC class I+ drug-selected variants were more readily lysed by MHC-restricted, tumor-associated CTLs than were the drug-sensitive parental cell lines. When the drug-selected variants were cocultured with the same CTLs to eliminate tumor cells expressing higher levels of MHC-I (MHC-Ib), the CTL-resistant tumor cells exhibited a drug sensitivity profile similar to that of the parental cell lines that were not exposed to Adriamycin. These findings suggest that certain chemotherapeutic drugs may increase the immunogenicity of some tumors, and that CTL immunotherapy may help reverse drug resistance.

Introduction

Prolonged exposure of tumor cells to cytotoxic chemotherapeutic drugs such as ADR, etoposides, and Vinca alkaloids leads to the development of the MDR phenotype, which results in resistance to various types of drugs (1). The development of multidrug resistance plays a major role in the failure of treatment of many types of cancers. Consequently, much effort has been directed at both understanding its development and deriving the means to reverse or circumvent its effects. Still, this problem represents a major obstacle to progress in cancer therapy.

Two different proteins are known to mediate multidrug resistance activity: (a) P-glycoprotein, the product of the MDR1 gene (2); and (b) MRP (3). These proteins are thought to act as energy (ATP)-dependent efflux pumps that prevent the intracellular accumulation of cytotoxic compounds. Both proteins belong to the ABC superfamily of transmembrane transporters, the family that also includes the TAP proteins (4–6). TAP is a heterodimer that transports peptides from the cytosol into the endoplasmic reticulum, where they are available for binding to the MHC class I heavy chain (7). Such presentation of antigenic peptides is a prerequisite for the recognition and lysis of infected or transformed cells by CTLs.

Because of the structural and functional similarities between the genes associated with MDR and TAP, we recently investigated whether MDR tumor cells also have altered peptide transport systems (8). We found that the development of the MDR phenotype was paralleled by an increased accumulation of TAP mRNA, resulting in a higher level of MHC class I expression relative to that of the parental cell lines. These findings were recently confirmed by Izquierdo et al. (9), who also found both TAP and MHC class I to be overexpressed in several MDR tumors.

The findings of enhanced antigen-presenting capabilities among MDR tumors raised questions about the immune recognition of drug-resistant cells in comparison to their drug-sensitive counterparts. It has been demonstrated in experimental models that anticancer drugs, although often thought of as immunosuppressive, can actually potentiate a variety of immune responses (e.g., delayed-type hypersensitivity and abrogation of tolerance; Ref. 10). One of the most widely studied chemotherapeutic agents in this regard is cyclophosphamide (reviewed in Ref. 10). The immunopotentiating observed with cyclophosphamide is thought to result from the inhibition/depletion of suppressor T cells and may be observed with the administration of cyclophosphamide before tumor challenge (10, 11). It has also been shown that the administration of chemotherapeutic agents such as melphalan can result in increased tumor infiltration by CD8+ T lymphocytes with potent, antigen-specific cytotoxic activity in vitro (12). ADR was found to result in a dose-dependent increase in tumor-specific CTL activity in mice receiving tumor cell vaccines, particularly when it was administered 1 week after vaccination as opposed to administration before vaccination (13). Furthermore, the development of regimens that alternate cytotoxic therapy with immunotherapy (sequential chemoinmunotherapy) has demonstrated a synergistic effect of the two modalities in clinical trials (14, 15). The exact mechanism by which chemotherapy induces this immunopotentiating remains to be elucidated. We hypothesized that: (a) the increased expression of TAP and MHC class I proteins associated with the MDR phenotype renders such tumor cells more susceptible to recognition and lysis by MHC class I-restricted, tumor-specific CTLs; and (b) the elimination of the MHChi cells within a population of MDR cells results in increased sensitivity of the remaining population of cells to the cytotoxic effects of chemotherapeutic drugs by also eliminating the MDR- or MRP-overexpressing cells.

Materials and Methods

Fluorescence-activated Cell-sorting Analysis. Tumor surface antigens were detected as described previously (16), using an EPICS V Profile Analyzer (Coulter Corp., Hialeah, FL). Antibodies to HLA ABC (W6/32; DAKO, Glostrup, Denmark), HER-2/neu (Ab2; Oncogene Science, Manhasset, NY),
and ICAM-1 (Calbiochem, San Diego, CA) were not conjugated. Cells to be examined were incubated with the appropriate antibody at 4°C for 30 min, washed, and further incubated with goat antimouse IgG (Boehringer Mannheim, Indianapolis, IN). Cells were washed again after 30 min and then analyzed.

**CTL Cytotoxicity Assays.** Cytotoxic activity of tumor-associated lymphocytes/CTLs was determined using the in vitro 51Cr release assay (16). CTLs used as effectors were generated as described previously (16, 17). For the cytotoxicity assay, 1 × 10^6 target cells were labeled with 100 μCi of 51Cr (Amersham, Arlington Heights, IL) at 37°C for 90 min, washed three times, and plated in triplicate at a final concentration of 5 × 10^4 cells/well in 96-well V-bottomed microtiter plates (Costar, Cambridge, MA) containing the appropriate number of effector cells. For MHC class I inhibition, 5 μl of W6/32 were added to the appropriate wells. Maximum release was obtained by adding 0.1N HCL. The percentage of specific target cell lysis was determined by the following formula:

\[
\text{Experimental } 51\text{Cr release} - \text{Spontaneous release} \times 100
\]

**Drug Selection.** Drug-selected variants were derived from breast (SKBR3, MCF-7, and MDA MB453) and ovarian (SKOV3, MDA 2774, and CaOV3) tumor cell lines by exposure to gradually increasing concentrations of ADR. In brief, 1 × 10^6 cells were seeded in T-25 flasks with 12 ml of RPMI-FCS [RPMI 1640 (Life Technologies, Inc.) + 10% FCS + 40 μg/ml gentamicin]. ADR (Sigma) was added at a final concentration of 1 ng/ml. Cultures were split every 3–4 days, at which time the ADR concentration was increased. Concentrations were increased from 1 ng/ml to 2, 4, 10, 15, 20, . . . 100 ng/ml over a 1-month period. ADR-selected tumor cells were 100% viable in 125 μM ADR (Sigma). Increased expression of the proto-oncogene HER-2 has also been described as being associated with MDR1-overexpressing breast and ovarian tumors (19). Because increased HER-2 expression results in CTL recognition (17), we also determined the levels of HER-2 expression on the ADR-selected variants. As shown in Table 1, HER-2 expression was slightly increased in one cell line, unchanged in three cell lines, and decreased in two other cell lines. We also examined for differences in ICAM-1 expression between drug-selected and nonselected tumor cells, because this adhesion molecule can facilitate tumor recognition by cellular immune effectors. Two of the six tumor cell lines were negative for ICAM-1 expression, as were the corresponding drug-selected variants. Of the four ICAM-1-positive cell lines, two showed an increase in ICAM-1 expression in the drug-selected variants, one showed no change, and one showed a slight decrease. Thus, whereas MHC class I expression was clearly increased in all ADR-selected variants, HER-2 and ICAM-1 expression showed no such association.

**Increased CTL-mediated Lysis of ADR-selected Tumor Cell Lines.** CTL cytotoxicity assays were performed to determine whether the increased levels of MHC class I expression in ADR-selected variants resulted in increased target sensitivity to lysis. To ensure that the results were relevant for several HLA types, we used three ovarian CTL lines that express at least one HLA in common with the MDA 2774 cell line (CTL-B, HLA-A3; CTL-E and CTL-R, HLA-A24) as effectors. These CTL cell lines have been previously shown to preferentially lyse autologous tumors (15). All three CTL cell lines exhibited between a 40 and 80% increase in MHC class I expression. One cell line, MDA 2774, consisted of two distinct populations expressing high and low levels of MHC class I. Both populations in the ADR-selected variant, 2774-DR, exhibited increased levels of MHC class I.

One parental tumor cell line, MDA MB453, which was negative for MHC class I expression, was used as a control. The corresponding ADR-selected variant, MB453-DR, was the only drug-selected cell line that did not show any changes in MHC class I expression. Hence, the loss of MHC class I expression is not corrected by selection with ADR or the development of drug resistance.

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Drug sensitive</th>
<th>Drug resistant</th>
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<tr>
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<td>MDA MB453</td>
<td>116</td>
<td>119b</td>
<td>1.0</td>
</tr>
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</table>

* MCF-R, mean channel fluorescence ratio was obtained by dividing each value in the second column by the corresponding value in the first column (i.e., 215/122 = 1.39).
* Negative samples (antibody-stained population showed no difference in comparison to the negative control).
the parental MDA 2774 cells, but a higher lysis of the ADR-selected 2774-DR variant than of the parental cell line was observed (Fig. 1, A and B). Lysis was inhibited by the addition of the anti-MHC class I antibody W6/32 (Fig. 1B). As expected, neither the MHC class I– MDA MB453 nor the MB453-DR tumors were lysed, indicating that the increased sensitivity to lysis is dependent on MHC class I expression, and that tumor lysis by these effectors is not likely to be the result of a natural killer-lymphokine-activated killer cell activity.

A fourth CTL cell line, CTL-V (HLA-A11, B60, 62), was tested against SKBR3 (HLA-A11, B18, 40) and its ADR-selected variant, SKBR3-DR. Neither SKBR3 nor SKBR3-DR was lysed by CTL-V (data not shown). Because both targets shared HLA-A11 but expressed lower levels of MHC class I than did the other tumor cell lines tested (Table 1), we retested CTL-V–mediated lysis after pretreating the tumors with 300 units/ml IFN-γ for 24 h. However, the IFN-γ–treated tumors were still resistant to lysis (data not shown). These results suggest that CTLs that lack antigen recognition of the ADR-sensitive tumor will not recognize the ADR-selected tumor. Drug selection did not appear to alter the antigen profile of the tumor as recognized by these effectors but merely increased the antigen presentation.

Immunoselection with CTLs of ADR-selected Variants Increases Drug Sensitivity. On the basis of findings of increased sensitivity of ADR-selected variants to CTL-mediated lysis, we hypothesized that selection by the CTLs may result in the elimination of those tumor cells with greater drug resistance potential. If this hypothesis is correct, then the resulting population of CTL escape tumor variants would then be more susceptible to the cytotoxic activity of ADR. To test this hypothesis, we derived CTL escape variants by coculturing the drug-selected cell lines with CTLs. We then compared the ADR sensitivity of the CTL escape variants with that of both the non-CTL-selected drug-resistant variants (cultured for the same interval in the absence of ADR) and the drug-sensitive parental cell lines in MTT assays. As shown in Fig. 2A, the non-CTL-selected 2774-DR cell line was resistant to the cytotoxic activity of ADR up to concentrations of 125 ng/ml. The parental MDA 2774 cell line exhibited sensitivity at ADR concentrations as low as 8 ng/ml. Interestingly, the CTL-resistant variant derived from 2774-DR by selection with CTL-B exhibited an ADR sensitivity profile that was indistinguishable from that of the parental MDA 2774 cell line.

We repeated the experiment with CTL-E and the SKOV3-DR cell line, which share HLA-B35. Based on the MTT assay, the parental SKOV3 cell line appears to be more inherently resistant to ADR than MDA 2774, exhibiting sensitivity only at high concentrations of ADR (≥250 ng/ml; results not shown), similar to the profile of the drug-selected SKOV3-DR cell line. Thus, the pattern of sensitivity to ADR was in the range of 4–125 ng/ml (Fig. 2B). This may be a reflection of prior in vivo selection with chemotherapeutic drugs, which is supported by the observation that among the MHC class I+ cell lines, SKVO3 exhibited the lowest increase in MHC class I expression with ADR exposure (Table 1). Of interest, the CTL-resistant variant of SKOV3-DR was sensitive to much lower concentrations of ADR than were the SKOV3 and SKOV3-DR cell lines. Thus, our findings suggest that CTL-mediated lysis could eliminate those cells within a tumor population that are more resistant to ADR, leaving a more susceptible population.

Discussion

In this report, we present novel evidence that the development of resistance to chemotherapeutic agents such as ADR is associated with the increased susceptibility of tumors to CTL lysis. This is paralleled by an increase in MHC class I expression. Increased levels of MHC class I associated with drug selection resulted in an increased sensitivity to CTL-mediated lysis. Lysis was MHC restricted and required MHC expression. Lysis also required the tumor to present peptide antigen to be recognized by TCR. This was suggested by the finding that CTL-V, which could not lyse the MHC class I+ SKBR3 parental cell line, was also unable to lyse the drug-resistant SKBR3-DR cell line, even after pretreatment with IFN-γ. Therefore, the increased sensitivity to lysis of drug-resistant variants seems to require both an intact antigen presentation pathway and the presence of antigen recognized by effectors.

Immunoselection by coculture of drug-selected tumors with CTLs resulted in the reversion of the surviving tumor cells to a more
drug-sensitive status. However, this did not reflect a spontaneous reversion of escaping tumors to a sensitive phenotype, because the same cells cultured in the same conditions without CTLs were far more resistant to ADR. Thus, the induction of reversion of drug sensitivity is likely the result of the elimination of those tumors expressing the MDR phenotype with a concomitantly higher level of MHC class I. If exposure to a chemotherapeutic drug selects for tumor cells with increased expression of both the MDR phenotype and MHC class I, it follows that after the elimination of those cells, the remaining population of cells will have a lower potential for expressing the MDR phenotype. The mechanisms involved in the increased TAP/MHC class I expression are not known but may involve common transcription factors and intermediates (adapter proteins) also used by the proteins encoded by the drug resistance genes MDR and MRP. NFκB has recently been demonstrated to be involved in the transcriptional regulation of genes in the mdr family (19) as well as the regulation of TAP1 expression (20). NFκB activity can be induced by a variety of stimuli, including cytotoxic compounds and other cellular stressors (21). The concomitant induction of stress response NFκB transcription factors in response to cytotoxic stress may therefore play a role in the increased expression of both mdr and TAP. Moreover, the Raf-1 kinase, which activates NFκB, was also found to be involved in mdr expression (21, 22).

It is important to emphasize that MHC class I expression does not appear to be required for the development of the drug-resistant phenotype induced by ADR. The absence of a requirement for MHC class I is demonstrated by the ability to select the MHC class I− cell line MDA MB453 in ADR. The drug-selected variant was MHC class I− as well. Thus, genetic changes resulting in the development of drug resistance do not seem to result in changes that can compensate for existing genetic defects in MHC class I expression. We also did not find increased expression of HER-2 and ICAM-1, making it unlikely that these play a role in the increased lysis of drug-selected tumors by CTLs.

Together, our findings suggest a possible mechanism for synergy between chemotherapeutic agents and immunotherapy. Chemotherapy may decrease the tumor burden, but the remaining tumor cells, as shown here, will express increased levels of MHC class I. These remaining cells are then more likely to be recognized and eliminated by tumor-specific CTLs. Furthermore, CTLs may then eliminate those tumor cells with a higher potential for chemotherapeutic resistance, thus sensitizing the tumor population for a subsequent round of chemotherapeutic drug exposure. Repeated rounds of sequential chemoinmunotherapy may thus result in enhanced responses through greater reductions in tumor burdens.

References
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