Treatment of Melanoma with 5-Fluorouracil or Dacarbazine In Vitro Sensitizes Cells to Antigen-Specific CTL Lysis through Perforin/Granzyme- and Fas-Mediated Pathways

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Several factors may influence sensitivity of melanoma cells to CTL lysis. One is the avidity of the CTL TCR. A second is that certain cytotoxic drugs have been reported to sensitize cancer cells to CTL lysis through Fas-mediated apoptosis. In this study, we examined whether antineoplastic agents 5-fluorouracil (5-FU) and dacarbazine (DTIC) sensitize melanoma cells to lysis of G209 peptide-specific CTL. Our results show that CTL generated from PBMC are HLA-A2 restricted and gp100 specific. Treatment with 5-FU or DTIC sensitized melanoma cells to lysis of G209-specific CTL. Most importantly, 5-FU- or DTIC-treated melanoma cells also became sensitive to low-avidity CTL, which per se are less cytolytic to melanomas. We sought to identify apoptotic pathways mediating this effect. The enhanced cytolysis was mediated through the perforin/granzyme pathway. Although 5-FU up-regulated FasR expression on melanoma cells, sensitization was not blocked by anti-Fas Ab, and the G209-specific CTL was Fas ligand (FasL) negative. However, when G209-specific CTL were stimulated to express FasL, FasL signaling also contributed to enhanced cytolyis. DTIC treatment, which did not increase FasR expression, also sensitized FasL-mediated killing induced by neutralizing anti-Fas Ab. For CD95L-positive G209-specific CTL, the sensitization was primarily mediated through the perforin/granzyme pathway regardless of up-regulation of FasR. The findings demonstrate that cytotoxic drug-mediated sensitization primes both perforin/granzyme and Fas-mediated killing by melanoma-specific CTL. Considering that most of autoreactive antitumor CTL are low avidity, the findings provide experimental basis for understanding cytotoxic and immunologic therapeutic synergy in melanoma. The Journal of Immunology, 2004, 172: 4599 – 4608.

I dentification and characterization of human melanoma-associated Ag (MAA) and antigenic epitope peptides recognized by T lymphocytes on cancer cells have led to efforts aimed at designing effective and widely applicable protocols of immunotherapy of cancer (1, 2). Most human MAA recognized by T lymphocytes, such as melanoma Ag recognized by T cells-1 (MART-1) (3), gp100 (4), tyrosinase (5), and the melanoma Ag gene (6, 7) family, are also expressed by some normal tissues, and thus can be viewed as self Ags. Numerous clinical trials have been devised to use antigenic epitope peptides derived from gp100, MART-1, melanoma Ag gene, and tyrosinase as immunogens to treat advanced melanomas, and the results have been promising (8–11). However, in many situations, immunization has failed to eliminate melanomas or to slow disease progression, despite the presence of MAA-specific CTL (9, 12). Thus, tumors are resistant to immunotherapy, despite our ability to engender active CTL.

Loss or down-regulation of HLA and/or MAA on melanoma cells, down-regulation of downstream apoptotic signaling components, up-regulation of protective antiapoptotic factors, or resistant melanoma variants to CTL-mediated cytotoxic pathways may all contribute to the observations of failed immunity and low disease response. An important factor is the capacity of host T cells for CTL-mediated killing. But due to negative selection of high-avidity autoreactive T cell subsets, self-reactive T cell precursors in the host are low avidity. The activated low-avidity Ag-specific CTL elicited following immunization may not mediate efficient killing of melanomas, and low level expression of self MAA on melanoma cells may be insufficient to trigger CTL-mediated killing (13, 14). It has been shown that melanomas with endogenously presented antigenic peptides are less sensitive to lysis by peptide-specific CTL than peptide-pulsed targets (15–18). Our most recent studies (19) further demonstrated that antimalanoma activity of peptide-specific CTL was directly correlated to their TCR avidity, and only high-avidity peptide-specific CTL lyse melanoma cells.

Ag-specific T lymphocytes lyse target cells including tumor cells by inducing apoptotic cell death through two main pathways: granule (mainly perforin and granzyme) and Fas-mediated pathways (20, 21). Although the Fas-mediated mechanism requires the transient expression of Fas ligand (FasL) by activated lymphocytes, granule-mediated lysis is due to the vectorial exocytosis of granule contents, perforin and granzymes. Although both pathways induce apoptotic cell death, activation of caspases ensuing FasR-FasL engagement follow a somewhat linear pathway, while granzyme B may activate both caspase-dependent and -independent pathways (22, 23).

Recent evidence supports the hypothesis that chemotherapeutic drugs and radiation induce an apoptotic pathway that requires the active participation of the cells (24). Many studies indicate that antineoplastic drugs induce up-regulation of FasR expression on tumor cells and/or activation of components of the Fas-mediated...
apoptotic death pathway (25–30). However, a number of melanomas expressing FasR are resistant to Fas-induced apoptotic target cell death (31–33). Treatment of malignant melanoma with chemotherapeutic drugs has generally yielded little success. Among many mechanisms of resistance to therapy, one may be that the threshold for Fas-induced apoptosis in melanomas is high and/or Fas signaling in melanomas is altered through unknown mechanisms.

Although chemotherapy has had limited success in the treatment of melanoma, it is clear that combining cytotoxic therapy and immunotherapy can enhance rates of tumor response. Randomized studies of so-called biochemotherapy have demonstrated that clinical response rates can be substantially increased (34). More recently, approaches combining dose-escalated preparative chemother-apy regimens with adoptive immunotherapy have demonstrated promise (35). Although the usual explanation for such successes involves differing and noncross-reactive mechanisms of tumor cell resistance to chemo- and immunotherapy, the possibility of synergistic mechanisms of sensitivity has not been much explored. This is the subject of this work.

Several studies have demonstrated that treatment of tumor cells with chemotherapeutic drugs induces or increases FasR expression, and thus enhances the sensitivity to CTL lysis in a Fas-de-pendent manner (36–39). Melanoma cells are resistant to both chemotherapy and FasL-mediated cytoly-sis. Resistance to immuno-therapy may occur partly because many CTL elicited by peptide immunization are low avidity and are ineffective in mediating mel-anoma killing. Thus, we are particularly interested in investigating whether cytotoxic drugs sensitize melanoma cells to MAA-specific CTL killing through specific pathways (either Fas, perforin/gran-zyeme pathway, or both) and whether treatment with cytotoxic drugs enhances the efficacy of low-avidity peptide-specific CTL.

Materials and Methods

Tumor cell lines

Human melanoma line DM13 (HLA-A2, -A31; gp100+) was a gift from T. Darrow and H. Seigler (Duke University Medical Center, Durham, NC) (40), and melanomas A375 (HLA-A2, -A31; gp100+) and UACC903 (HLA-A2, -A31; gp100+) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Tumor cells were cultured in DMEM supplemented with 5% FCS (Life Technologies, Carlsbad, CA), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 50 μg/ml gentamicin), 450 μg/ml L-glutamine, and 2.5 mg/ml sodium bicar-bonate in 75-cm² T flasks. Nonadherent cells were then gently washed off and saved as responders for CTL generation. The remaining adherent cells were cultured with 100 ng/ml GM-CSF (Immunex, Seattle, WA) and 20 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) in RPMI 1640 me-dium containing 1% human male AB serum. On day 6, the cells were harvested, washed, and replated in ultra-low adherence six-well culture trays (Costar; Ultralow 3471), and matured with CD40L-trimeric (1 μg/ml; Immunex) for 24 h.

Purification of PBMC and CD8+ cells

PBMC from HLA-A2+ normal donors were purified by centrifugation in Ficoll-Paque from buffy coat or leukapheresis products. CD8+ T cells were isolated by negative selection of PBMC or nonadherent PBMC using a panel of mAb and magnetic beads. Briefly, PBMC were incubated with anti-CD4 (OKT4; ATCC), anti-HLA-DR (L243; ATCC), anti-CD200 (1FS; ATCC), anti-CD14 (3C10; ATCC), and anti-CD56 (B159; BD PharMingen) at saturating concentrations for 60 min at 4°C. Cells were washed twice and then allowed to incubate with magnetic particles coated with goat anti-mouse IgG (PerSeptive Biosystems, Framingham, MA) for 60 min with rocking at 4°C. Cell separation was performed with a strong magnet. Cells were collected, washed twice, and resuspended in RPMI 1640 medium containing 10% human male AB+ serum. Purified CD8+ T cells were >85% positive for expression of CD3/CD8 and less than 10% positive for CD3/CD4.

Generation of dendritic cells (DC) from PBMC

DC were generated from PBMC, as described by Romani et al. (41), with some modifications (42). Briefly, PBMC isolated from normal donors’ buffy coat or leukapheresis products were cultured for 2 h in RPMI 1640 medium (Life Technologies) supplemented with 1% of human AB serum at 5 × 10^5 cells/ml in triple flasks. Nonadherent cells were then gently washed off and saved as responders for CTL generation. The remaining adherent cells were cultured with 100 ng/ml GM-CSF (Immunex, Seattle, WA) and 20 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) in RPMI 1640 me-dium containing 1% human male AB+ serum. On day 6, the cells were harvested, washed, and replated in ultra-low adherence six-well culture trays (Costar; Ultralow 3471), and matured with CD40L-trimeric (1 μg/ml; Immunex) for 24 h.

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Reagents

The 5-fluorouracil (5-FU), dacarbazine (DTIC), EGTA, and purified mouse IgG1 and IgM were purchased from Sigma-Aldrich (St. Louis, MO). Both Fas-neutralizing Ab clone ZB4 (IgG1) and agonist Fas Ab clone CH-11 (IgM) were purchased from Immunotech (Beckman Coulter, Fullerton, CA).

Synthetic peptides

HLA-A*0201-restricted peptides G209 (ITDQVYPPSV) and its anchor residue-modified counterpart G209-2M (IMDQVYPPFSV) were synthesized at the Massachusetts General Hospital Biopolymer Core Facility (Charlestown, MA). Peptides were purified to >90% by reverse-phase HPLC, as confirmed by mass spectrometry. Peptides were dissolved in DMSO and then diluted with PBS at 2 mg/ml (the final concentration of DMSO in stock solution is <5%, v/v), filtered through a 0.2 μM membrane, and stored at −80°C.

Flow cytometry

For cell surface molecule staining, 2–5 × 10⁵ cells were incubated with optimal concentration of FITC-conjugated CD95 (clone DX2; BD Phar-Mingen, San Diego, CA), FITC-conjugated CD95L (clone NOK-1; BD PharmMingen), or anti-HLA-A2 (clone BB7.2; ATCC) for 30 min on ice. Cells stained with unconjugated primary Ab were followed by staining with FITC-conjugated anti-mouse IgG on ice for 30 min. Following staining, cells were washed three times with PBS containing 1% BSA and 0.1% azide, and then fixed with 2% formaldehyde. For intracellular staining, 1–2 × 10⁶ cells were permeabilized first with Cytofix/Cytoperm (BD PharmMingen) and then stained with PE-conjugated anti-bcl-2 (clone 6C8; BD PharMingen) Ab, as instructed by the manufacturer. Expression of molecules of interest was monitored by FACScan and analyzed using CellQuest software.

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CTL generation

DC were pulsed with 20 μg/ml peptide (G209-2M) at a concentration of 1–2 × 10⁵/m Determine the optimal concentration of peptide and dilute with PBS at 2 mg/ml. Spontaneous release was always spontaneous release was calculated as: ((mean experimental cpm spontaneous release) - (mean spontaneous release in the absence of neutralizing Fas Ab clone ZB4 or purified mouse IgG1)) / (mean spontaneous release in the absence of neutralizing Fas Ab clone ZB4 or purified mouse IgG1)) × 100%, in which spontaneous release represents cpm in supernatants from wells containing target cells with medium only, and maximum release represents cpm in supernatants from wells containing target cells in medium with 2% Triton X-100. Spontaneous release was always <20% of maximum release. The SD of duplicate wells was less than 10%.
Apoptosis assay
UACC903 (UACC) cells treated with or without 5-FU (25 μg/ml) or DTIC (20 μM) for 48 h were incubated with agonist anti-Fas Ab, CH-11 (500 ng/ml), for 5 and 16 h, respectively. To distinguish whether death/apoptosis was mediated by FasR, blocking anti-Fas Ab ZB4 (2 mg/ml) was added in the corresponding groups. At the end of incubation, cells were harvested and stained with propidium iodide and FITC-annexin V (BD PharMingen) for detecting apoptosis according to the instruction provided by the company. Ten thousand cells from each group were collected, and dead and apoptotic cells were analyzed without gating using CellQuest software.

Cloning and expansion of peptide-specific CTL
After testing for peptide recognition (day 21 of culture), CTL cultures were plated at 0.5 cell/well in 96-well round-bottom plates with 5 × 10^4 transfected B cells (EBV-B) in 200 μl of medium supplemented with 30 ng/ml OKT3. After 24 h and every 3 days thereafter, 300 U/ml IL-2 was added for ~20 days. Wells positive for clonal growth were identified 15–20 days after plating and were tested for peptide reactivity. Peptide-specific clones were transferred to 24-well plates (2 ml/well) and restimulated with OKT3, and irradiated autologous PBMC (2 × 10^5/well) and EBV-B (5 × 10^5/well) were added as feeder cells. The expanded clones were tested for their avidities and cytolytic activity to melanoma cells.

Induction of FasL expression on CTL
Peptide-specific CTL (1 × 10^5/well) were stimulated for 2 h at 37°C in 24-well plates coated with anti-CD3 (1 μg/well, clone H1T3a; BD PharMingen). At the end of stimulation, CTL were collected and used for CTL assay, and expression of FasL (FITC-conjugated clone NOK-1; BD PharMingen) was examined by flow cytometry.

Results
Treatment of melanoma cells with 5-FU and DTIC sensitizes melanomas to lysis of peptide-specific CTL
Chemotherapeutic drugs have been reported to sensitize tumor cells to T cell killing (CTL and lymphokine-activated killer) through FasR-FasL interaction between tumor cells and lymphocytes and through up-regulation of class I expression on tumor cells (36–38, 43). To investigate whether anticancer drugs sensitize melanoma cells, which are relatively resistant to chemotherapy and Fas-mediated killing, to lysis by Ag-specific CTL, we observed HLA class I, FasR, and FasL expression on melanoma cells UACC903 following treatment with 5-FU or DTIC. The 5-FU is a widely used chemotherapeutic drug and is documented to up-regulate FasR on many kinds of tumor cells. DTIC is an antimelanoma drug used in the clinic. Therefore, we chose these two agents to investigate whether they sensitize melanoma cells to CTL lysis. The doses of the drugs were titrated, and subtoxic doses were used in this study. Exposure of UACC903 to 5-FU (25 μg/ml) or DTIC (20 μM) for 24–72 h had no significant cytotoxic effects, as determined by trypan blue assay. At a dose of 25 μg/ml, 5-FU showed significant inhibition on the growth of a melanoma cell line UACC903, while DTIC at a dose of 20 μM did not show significant inhibitory effect on UACC903 growth (Table I). The viability of cells in untreated and 5-FU- and DTIC-treated groups was all over 85% (Table I).

Effects of the two drugs on cell surface molecule expression were monitored by flow cytometry. Untreated UACC903 cells expressed HLA-A2 and CD95, but not CD95L. Exposure of UACC903 to 5-FU for 48 h increased both HLA-A2 and FasR expression, but could not induce FasL expression (Fig. 1). Under identical conditions, DTIC had no significant effect on expression of HLA-A2, FasR, and FasL (Fig. 1). Increasing DTIC concentrations (up to 400 μM) did not change expression patterns of the surface markers studied, although DTIC at higher doses inhibited UACC903 growth in a dose-dependent manner (data not shown).

Sensitivity of drug-treated melanoma cells to CTL lysis was tested using gp100-derived G209 peptide-specific CTL. As seen from Fig. 2A, the CTL recognized G209 peptide, but irrelevant HLA-A2-restricted influenza matrix-derived peptide Flu M1, presented on T2 targets (p = 0.00167, ANOVA). In addition, the CTL also demonstrated killing to HLA-A2+ gp100+ melanoma DM13 (p = 0.0265, vs A375, ANOVA) and UACC903 (p = 0.0163, vs A375, ANOVA), but not to HLA-A2+ gp100+ melanoma A375, indicating that the CTL was HLA-A2 restricted and gp100 specific. Treatment of HLA-A2+/gp100+ melanomas UACC903 (p = 0.0308, medium vs 5-FU; p = 0.0240, medium vs DTIC; ANOVA) and DM13 (p = 0.0402, medium vs 5-FU; p = 0.0341, medium vs DTIC; ANOVA) with either 5-FU or DTIC enhanced sensitivity to lysis of G209 peptide-specific CTL (Fig. 2B). Increased sensitivity was peptide specific because treatment of A375 (HLA-A2+/gp100+) did not enhance their sensitivity to lysis by G209-specific CTL (Fig. 2C). However, loading G209 peptide (1 nM) on drug-treated A375 cells increased specific killing by G209 peptide-specific CTL, as compared with untreated G209-pulsed A375 melanoma cells (p = 0.0199, medium vs 5-FU; p = 0.0227, medium vs DTIC; ANOVA; Fig. 2D).

Sensitization of melanoma cells treated with 5-FU or DTIC to melanoma-specific CTL was mediated through Fas-independent pathway
Because both 5-FU and DTIC treatment equally increased tumor cell lysis by G209-specific CTL, and DTIC had no effect on FasR expression, we then investigated whether FasR played any role in the enhanced sensitivity. Both DTIC- and 5-FU-treated UACC903 cells were used as targets of CTL in the presence or the absence of Fas-neutralizing Ab or EGTA (to chelate Ca^{2+}). Cytotoxicity toward UACC903 treated with either 5-FU (p = 0.803, IgG1 vs ZB4; ANOVA; Fig. 3A) or DTIC (p = 0.898, IgG1 vs ZB4; ANOVA; Fig. 3B) was not inhibited by neutralizing anti-Fas Ab ZB4 (Fig. 3). However, specific lysis was almost completely

Table 1. Cell yield and viability following treatment with 5-FU (25 μg/ml) or DTIC (20 μM) for 48 h

<table>
<thead>
<tr>
<th></th>
<th>Yield</th>
<th>Viability</th>
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<tr>
<td>24 h</td>
<td></td>
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<tr>
<td>Medium</td>
<td>0.9 ± 0.17</td>
<td>95 ± 4</td>
</tr>
<tr>
<td>DTIC</td>
<td>0.7 ± 0.15</td>
<td>92 ± 2</td>
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<tr>
<td>5-FU</td>
<td>0.4 ± 0.10</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>2.6 ± 0.19</td>
<td>93 ± 3</td>
</tr>
<tr>
<td>DTIC</td>
<td>2.1 ± 0.19</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>5-FU</td>
<td>0.9 ± 0.11</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>72 h</td>
<td></td>
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</tr>
<tr>
<td>Medium</td>
<td>5.4 ± 0.22</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>DTIC</td>
<td>4.1 ± 0.21</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>5-FU</td>
<td>1.6 ± 0.15</td>
<td>88 ± 6</td>
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*Cells were distributed at 3 × 10^5/well, three wells in each group, in the presence or absence of 5-FU or DTIC. Twenty-four, 48, and 72 h later, cells were harvested by trypanizing. Cell number (× 10^6 cells/well ± SD) and viability (%) ± SD) were determined by trypan blue assay.

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blocked by EGTA (medium vs EGTA for 5-FU, \( p = 0.0298 \); medium vs EGTA for DTIC, \( p = 0.0195 \); ANOVA; Fig. 3), indicating that lysis was primarily dependent on the perforin/granzyme pathway, but not the Fas pathway.

Next we looked at whether bcl-2, an important antiapoptotic factor, is involved in drug-induced sensitization of melanoma cells to G209-specific CTL. To determine this, we examined bcl-2 expression following exposure of UACC903 to 5-FU or DTIC. Twenty-four to seventy-two hours after exposure, UACC903 cells were stained intracellularly with PE anti-bcl-2 and monitored by flow cytometry. Treatment of UACC903 with either 5-FU or DTIC slightly increased bcl-2 expression as expressed in mean fluorescence intensity 24–72 h after exposure. Fig. 1 shows the data of bcl-2 expression 48 h after exposure in untreated UACC903 cells as well as drug-treated cells. The data suggested that alteration of bcl-2 expression might not contribute to the enhanced cytolysis.

Treatment with 5-FU and DTIC sensitizes melanoma cells to agonist anti-Fas-mediated lysis

Previous studies by other investigators showed that melanoma cells were resistant to Fas-mediated apoptosis, even though they expressed FasR (31–33), suggesting that FasR on melanoma might lose function, or Fas-FasL interaction on melanoma cells did not reach the threshold to mediate apoptosis. To examine whether the FasR on the melanoma cells we used was functional, we exposed untreated UACC903 and DTIC- or 5-FU-treated UACC903 to various concentrations of Fas-apoptotic Ab CH-11 for 16 h in the presence or absence of Fas-neutralizing Ab ZB4. As seen in Fig. 4, CH-11 induced only marginal lysis of untreated UACC903, suggesting that the melanoma cell line UACC903 is resistant to Fas-mediated apoptotic cell death, even though UACC903 expresses FasR. In contrast, CH-11 induced significant lysis of 5-FU-treated

FIGURE 1. Phenotypic changes of UACC903 following exposure to 5-FU (25 \( \mu \)g/ml) or DTIC (20 \( \mu \)M) for 48 h, as determined by flow cytometer. Cells were treated with or without cytotoxic drugs, either 5-FU (upper panel) or DTIC (lower panel) for 48 h. Following treatment, cells were stained and monitored by flow cytometry, as described in Materials and Methods. Histograms were plotted by overlapping drug-treated groups (thick curves) with isotype control (dotted curves) and medium (untreated) groups (thin curves).

FIGURE 2. Cytotoxic drugs, 5-FU and DTIC, sensitize melanoma cells to killing by gp100 peptide G209-specific CTL. G209 peptide-specific CTL were generated from normal donors, as described in Materials and Methods. Target cells treated with or without either 5-FU (25 \( \mu \)g/ml) or DTIC (20 \( \mu \)M) for 48 h were tested for their sensitivity to G209-specific CTL using 6-h \( ^{3} \)HCr release assay. A, CTL generated by G209 peptide recognized both exogenous G209 peptide presented on T2 targets and endogenously processed and presented G209 peptide on HLA-A2\(^{+}\) melanomas DM13 and UACC903 (UACC). B, DM13 and UACC903 (both HLA-A2\(^{+}\)/gp100\(^{+}\)) melanoma cells treated with either 5-FU or DTIC were sensitive to lysis of G209-specific CTL. C, 5-FU or DTIC treatment did not sensitize melanoma A375 (HLA-A2\(^{+}\)/gp100\(^{+}\)) to lysis of G209-specific CTL. A375 was either left untreated or treated with 5-FU or DTIC for 48 h. Cells were harvested and labeled with \( {^{51}} \)Cr for 2 h as targets of G209-specific CTL. D, Loading G209 peptide on drug-treated A375 cells rendered them sensitive to G209-specific cell death, even though UACC903 expresses FasR. In contrast, CH-11 induced significant lysis of 5-FU-treated
UACC903 cells in a dose-dependent manner, and lysis was almost completely blocked by neutralizing anti-Fas ZB4 (IgG1 vs ZB4, \( p = 0.0036; \) ANOVA; Fig. 4C), indicating that sensitization of UACC903 cells to CH-11 was Fas specific. Interestingly, although DTIC treatment did not affect FasR expression, exposure of DTIC-treated UACC903 to CH-11 did slightly induce enhanced Fas-dependent lysis, as compared with the untreated UCAA group (\( p = 0.0193; \) ANOVA; Fig. 4B), and the enhanced lysis could be blocked by neutralizing anti-Fas Ab, ZB4 (IgG1 vs ZB4, \( p = 0.0359; \) ANOVA; Fig. 4B).

Fas-mediated apoptotic cell death was also investigated by staining cells with propidium iodide, which stains dead cells, and annexin V, which stains both apoptotic and dead cells. Untreated UACC903 and 5-FU- or DTIC-treated UACC903 cells were exposed to agonist anti-Fas Ab CH-11 (500 ng/ml) for 5 and 16 h in the presence or absence of neutralizing anti-Fas Ab, ZB4 (IgG1 vs ZB4, \( p = 0.0359; \) ANOVA; Fig. 4B).

Melanoma cells treated with 5-FU or DTIC are also sensitive to CD95L+ CTL.

Our data in Fig. 3 clearly showed that sensitization of drug-treated UACC903 cells to peptide-specific CTL was Fas independent,
while the data in Figs. 4 and 5 demonstrated that FasR on melanoma cells was functional. Two explanations for the observations are possible. First, the G209-specific CTL we used do not express FasL. Second, agonist Fas Ab and natural FasL stimulate different signaling pathways, as indicated by Thilenius et al. (44) and Zipp et al. (45). We thus examined whether the CTL we used expressed CD95L using FITC-conjugated CD95L Ab. No detectable CD95L was shown on the unstimulated G209-specific CTL as determined by flow cytometry (Fig. 6A, left panel). The CTL stimulated with immobilized CD3 Ab for 2 h expressed a significant level of CD95L (Fig. 6, A (right panel) and E). The CD95L+ G209-specific CTL were immediately tested for reactivity to cytotoxic drug-treated melanomas. Although CD95L+ CTL-mediated killing toward 5-FU-treated melanoma cells occurred primarily through the perforin/granzyme pathway as evidenced by EGTA blocking ~60–80% specific killing (EGTA vs medium, p = 0.0081; ANOVA), the inclusion of Fas-neutralizing Ab ZB4 in the CTL assay system did significantly block lysis accounting for ~30% (ZB4 vs control IgG1, p = 0.0388; ANOVA; Fig. 6B). Enhanced sensitivity of DTIC-treated UACC903 cells to CD95L+ G209-specific CTL was almost completely blocked by EGTA (EGTA vs medium, p = 0.0280; ANOVA; Fig. 6C). Although we consistently observed that including anti-Fas Ab ZB4 reduced specific lysis, there was no significant difference between ZB4 and control IgG1 (p = 0.0635, Fig. 6C). Consistent with other reports (31–33), untreated UACC903 cells were resistant to CD95L-mediated cytolysis (Fig. 6D).

The role of CD95L in enhancing sensitivity of 5-FU-pretreated cells to lysis by another G209-specific, CD95L+ CTL (Fig. 6E) was also investigated using another melanoma cell line, DM13. As seen in Fig. 6F, lysis of DM13 by the peptide-specific CD95L+ CTL was blocked by EGTA (DM13 vs DM13 + EGTA, p = 0.0323; ANOVA), but not by ZB4. Treatment of DM13 with 5-FU increased its sensitivity to the peptide-specific CD95L+ CTL (DM13 vs DM13/5-FU, p = 0.0278; ANOVA). The enhanced lysis was blocked by EGTA (DM13/5-FU vs DM13/5-FU + EGTA, p = 0.0239; ANOVA) and partially blocked by ZB4 (DM13/5-FU vs DM13/5-FU + ZB4, p = 0.0330; ANOVA).

**Treatment of melanoma cells with anticancer drugs renders low-avidity G209 peptide-specific CTL to melanoma reactive**

It has been demonstrated that many peptide-specific CTL generated by peptide immunization fail to recognize tumor cells with endogenously presented peptide, although the CTL demonstrate highly specific killing to target cells exogenously pulsed with peptide (46, 47). We (19) have shown that failure of peptide-specific CTL to recognize endogenously processed peptide on tumor cells is due to their low avidity of TCR, and that TCR avidity of CTL is well correlated to the cytotoxicity toward tumor. We thus asked whether chemotherapeutic drug-treated melanoma cells are sensitive to low-avidity, G209-specific CTL.

We therefore chose a low-avidity G209 peptide-specific CTL 2B4 (19), which per se was less cytolytic to melanoma cells, and tested whether it killed anticancer drug-treated melanoma. As shown in Fig. 7A, 2B4 required much more peptide presented on T2 target cells to mediate significant killing as compared with high-avidity CTL 1F1. Consistent with our previous study (19), high-avidity CTL 1F1 demonstrated significant melanoma killing (Fig. 7B), while low-avidity CTL 2B4 did not kill melanoma UACC903 (Fig. 7C). However, when UACC903 cells were pretreated with either 5-FU or DTIC, they became much more sensitive to both high- and low-avidity CTL, 1F1 (Fig. 7B) and 2B4.
FIGURE 6. CD95L signaling also contributed to enhanced lysis of drug-treated melanoma cells by CD95L<sup>−</sup> G209-specific CTL. A, Phenotype analysis of CD95L expression of G209-specific CTL before (unstimulated) and after stimulation with anti-CD3 Ab. CTL were stained with FITC-conjugated isotype mouse Ig or FITC-CD95L (clone NOK-1). The solid curve histogram is isotope FITC-Ig staining, and the shaded histogram is FITC-CD95L staining. B, 5-FU (25 μg/ml)-treated UACC903 cells were used as targets of CD95L<sup>−</sup> G209-specific CTL in a 6-h <sup>51</sup>Cr release assay in the presence of either neutralizing anti-Fas ZB4 (10 μg/ml) or EGTA (4 mM)/MgCl<sub>2</sub> (2 mM). Mouse IgG1 (10 μg/ml) was used as a control of ZB4. C, DTIC (20 μM)-treated UACC903, and D, untreated UACC903 cells were examined for their sensitivity to CD95L<sup>−</sup> G209-specific CTL in the presence or the absence of anti-Fas Ab ZB4 or EGTA (4 mM)/MgCl<sub>2</sub> (2 mM). E, CD95L expression on another G209 peptide-specific CTL, and F, its cytolytic activity against untreated and 5-FU-treated DM13 in the presence or absence of ZB4 or EGTA. Both DM13 and DM13/5-FU groups contain isotype IgG1 and were used as control for both isotype IgG and medium control because IgG1 has no effect on CTL-mediated lysis. Concentration of Abs and EGTA was the same as that in B–D.
(Fig. 7C), respectively. Importantly, the capacity of low-avidity G209-specific CTL to lyse drug-treated UACC903 is comparable to that of high-avidity G209-specific CTL (Fig. 7, B and C).

Discussion

Discovery of human MAA has allowed the rational design of anti-melanoma CTL strategies, which recognize melanoma cells expressing the appropriate Ag in the context of HLA class I (1, 2). Epitope peptides derived from identified MAA have demonstrated immunogenicity to generate peptide-specific CTL that also recognize melanoma cells. However, CTL are generally less cytotoxic to melanoma cells compared with peptide-pulsed targets (15–18). Killing of tumor cells by cytotoxic T cells is mediated through two pathways: perforin/granzyme- and Fas-mediated pathways (20, 21). Cytotoxic drugs also induce tumor cell apoptosis (24). The mechanisms of apoptosis involve up-regulation of CD95, CD95L expression (25–27), and/or activation of apoptotic components, such as caspase-3 and caspase-8 (28–30). Although both CTL and cytotoxic drugs kill target cells by inducing apoptotic cell death, the pathways may be different. For example, CTL-mediated cytolysis is not blocked by bcl-2 expression or by inhibition of protein synthesis (48, 49), while bcl-2 increases resistance to apoptosis induced by anticancer drugs (50–52). Combination of both cytotoxic drugs and tumor-specific CTL may increase their antitumor capacity. Therefore, we propose a strategy to enhance the ability of Ag-specific CTL to lyse melanoma cells by sensitizing melanomas with subtoxic concentrations of chemotherapeutic drugs. We hypothesize that treatment of melanoma cells with cytotoxic drugs combines with CTL to mediate cytolysis. Furthermore, we also hypothesize that cytotoxic drug treatment of melanomas renders them sensitive to low-avidity, MAA peptide-specific CTL.

In the present study, we have demonstrated that treatment of melanoma cells with subtoxic concentrations of 5-FU or DTIC sensitizes melanoma cells to G209 peptide-specific CTL lysis. The sensitization is peptide specific because HLA-A2+/gp100+ melanoma A375 treated with 5-FU or DTIC is not sensitive to CTL lysis unless A375 is loaded with G209 peptide (Fig. 2). Although melanoma cells treated with 5-FU have up-regulated FasR expression, the sensitivity is primarily mediated through the perforin/granzyme pathway, but not the FasR-FasL pathway. However, when CTL was activated to express FasL, the enhanced sensitivity of melanoma treated with 5-FU is also mediated partially through FasR-FasL interaction, although perforin/granzyme plays a primary role. Interestingly, DTIC, which does not increase FasR expression, also primes melanoma cells to Fas-mediated cytolysis to some degree (Figs. 4 and 5). Most importantly, our study showed that treatment of melanoma cells with 5-FU or DTIC rendered melanoma cells sensitive to low-avidity peptide-specific CTL, which per se were not cytolytic to untreated melanoma cells. In addition, the capacity of low-avidity CTL to lyse cytotoxic drug-treated melanomas is comparable to that of high-avidity CTL. Considering that most CTL generated by peptide stimulation are low-avidity CTL, this finding suggests an explanation for chemotherapeutic and immunotherapeutic synergy.

Several groups have reported that anticancer drug treatment sensitizes tumor cells to lysis of CTL and/or lymphokine-activated killer (36–39). The enhanced sensitivity was believed to be mediated by FasL signaling (36, 37, 39). However, numerous studies have shown that melanoma cells express FasR, although most of them are resistant to FasL-mediated killing (31–33). To assay whether FasL-mediated lysis contributes to the enhanced sensitivity of melanomas to Ag-specific CTL, we first examined whether 5-FU or DTIC treatment affected FasR expression. We then investigated whether a combination of sensitizing drug and G209-specific CTL enhanced melanoma cell sensitivity to specific cytolytic pathways (FasR-FasL and perforin/granzyme) by using anti-Fas Ab and EGTA, a Ca2+ chelator, respectively, in CTL assays. We showed that treatment with a subtoxic concentration of 5-FU significantly increased FasR expression as well as HLA-A2 expression on UACC903, while DTIC had no effect on FasR and HLA-A2 expression. Our in vitro CTL assay demonstrated that the sensitivity of both 5-FU- and DTIC-treated melanoma UACC903 is equal to that of G209-specific CTL, and the sensitivity is almost completely blocked by EGTA, but not by anti-Fas Ab ZB4 (Fig. 3). The data suggested that the enhanced sensitivity is mediated by the perforin/granzyme pathway, but not by the FasR-FasL pathway.

Because FasL is expressed only on activated T cells and its expression is transient (53), it is possible that the CTL we used did not express FasL. To test whether melanoma cell UACC903 are really resistant to FasL-mediated cytolysis, we examined the sensitivity of melanomas to agonist anti-Fas Ab CH-11 and FasL+ G209-specific CTL. Using the anti-Fas Ab model, we found that untreated melanoma cells, which expressed FasR, were resistant to Fas Ab CH-11-mediated cytolysis/apoptotic cell death (Figs. 4 and...
5). This result is consistent with previous studies by other investigators (33). Following treatment with 5-FU, melanoma cell line UACC903 became more sensitive to CH-11-mediated cytolysis/apoptotic cell death (Figs. 4 and 5). Unexpectedly, UACC903 treated with DTIC, which had no effect on FasR expression, were also sensitive to CH-11, although to a lesser extent as compared with 5-FU-treated UACC903 (Figs. 4 and 5). The Fas specificity was confirmed by a blocking assay using neutralizing anti-Fas Ab ZB4 in both 5-FU- and DTIC-treated targets (Figs. 4 and 5).

We then analyzed whether CD95L^-2 G209-specific CTL induced melanoma lysis through FasL signaling. Flow cytometry analysis showed that the CTL (in Figs. 2 and 3) we used was CD95L negative. To induce CD95L expression, G209-specific CTL were stimulated with immobilized CD3 Ab for 2 h and then used for CD95L expression analysis and CTL assay immediately (Fig. 6). Consistent with the findings of other investigators, untreated UACC903 cells that express FasR were resistant to lysis mediated by CD95L signaling (31–33). However, sensitivity of 5-FU-treated UACC903 cells to CD95L^-2 G209-specific CTL was partially blocked by neutralizing anti-Fas Ab ZB4, indicating that FasR-FasL signaling did contribute to the enhanced cytolysis if CTL expressed CD95L.

Our study clearly demonstrated that sensitivity of drug-treated melanoma cells to Ag-specific CTL was primarily mediated through the perforin/granzyme pathway. FasR-FasL signaling might also contribute to the enhanced sensitivity if CTL express FasL. Considering that FasL is expressed only on activated T cells for a very short time (53), this condition should be considered in designing adoptive immunotherapy strategies.

Other authors have shown similar findings. Most recently, Frost et al. (39) showed that treatment of melanomas and prostate carcinoma cells with cisplatin (CDDP) enhanced their sensitivity to MART-1-specific CTL, and the sensitivity was thought to be mediated solely through FasL signaling. However, they failed to explain why T2 cells that are derived from humans and express a high level of FasR became more sensitive to the CD95L-expressing murine CTL following CDDP treatment (39). In addition, they did not show whether CDTP treatment increased FasR expression on T2, and they did not use anti-Fas Ab blocking to confirm Fas specificity in the experiment (39). Furthermore, cytolysis level of CDDP-treated tumor cells induced by both anti-Fas and FasL^-2 T cells seemed to correlate to CDDP doses used instead of level of FasR expression on target cells (36).

We sought to understand the contributing role of bcl-2 expression to our observations. Bcl-2 is an important antiapoptosis factor. Inhibition of bcl-2 expression using bcl-2 antisense sensitized human melanoma cells to the chemotherapeutic drug DTIC (54, 55). We investigated whether 5-FU and DTIC altered bcl-2 expression. However, treatment of melanoma cells with either 5-FU or DTIC slightly increased the level of bcl-2 expression, indicating that bcl-2 might not be involved in the sensitization of melanoma cells to G209-specific CTL lysis. The observation agreed with the report (49) that bcl-2 expression did not block CTL-mediated cytolysis.

It is still unclear why melanoma cells that express FasR are resistant to Fas-mediated apoptosis. There are also numerous indications that simple expression of surface FasR is not in and of itself sufficient to render a cell entirely Fas sensitive (20). For example, most resting mature T cells express low levels of Fas on their surface, although they are not Fas sensitive (56). In addition, Fasl^- CD4 T cells induce FasR^- CD8 T cells to undergo apoptosis, while Fasl^- CD8 T cells do not induce FasR^- CD4 T cell apoptosis (57). All these studies suggest that something else also regulates FasL-induced death signaling. Because melanoma cells treated with DTIC, which had no effect on FasR expression, were also sensitive to apoptotic cell death mediated through FasR signaling, and drug-treated UACC903 cells were sensitive to CD95L^-2 G209-specific CTL, it is very likely that cytotoxic drug treatment primes melanoma cells to both Fas- and perforin/granzyme-mediated pathways through unknown mechanisms. Based on our findings and the literature cited above, we hypothesize that anticancer drug treatment acts as activation of the apoptosis pathway in target cells, which does not necessarily lead to cell death because of repair mechanisms. Then the "second hit" to target cells occurs through melanoma-specific CTL, either through perforin/granzyme- and/or Fas-mediated pathways. When the two hits work together, they synergistically or additively lead to cytolysis.

Another important finding in the study is that cytotoxic drug treatment also renders melanomas sensitive to low-avidity peptide-specific CTL. It is believed that successful antitumor immunity and clearance of virus in the host depend on the presence of high-avidity Ag-specific CTL (58–62). However, most TAA are normal self proteins expressed in some normal tissues. Due to negative selection of high-avidity autoreactive T cells, most of the detectable TAA-specific T cells have only a low avidity for the TAA and are not capable of efficiently recognizing tumor cells endogenously expressing the TAA (46, 47). Therefore, this limits the capacity of tumor-specific CTL to eliminate tumor cells. Combination of chemotherapy using subtoxic doses of drugs with MAA-specific CTL immunotherapy may lead to more effective immunotherapy strategies for melanomas as well as other cancers.

In summary, our study showed that cytotoxic drug treatment sensitizes melanoma cells to MAA peptide-specific CTL-mediated cytolysis regardless of up-regulation of FasR. The mechanisms are primarily mediated through the perforin/granzyme pathway, although FasR signaling may also be engaged. More importantly, subtoxic concentrations of cytotoxic drugs also sensitize melanoma cells to lysis by low-avidity peptide-specific CTL that per se are melanoma nonreactive. Although further studies are needed to clarify the mechanisms, our study provides experimental basis for understanding adoptive immunotherapy strategies following chemotherapy.

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