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Human ovarian carcinomas express the CA-125, HER2/neu, and MUC1 tumor-associated Ags as potential targets for the induction of active specific immunotherapy. In the present studies, human ovarian cancer cells were fused to human dendritic cells (DC) as an alternative strategy to induce immunity against known and unidentified tumor Ags. Fusions of ovarian cancer cells to autologous DC resulted in the formation of heterokaryons that express the CA-125 Ag and DC-derived costimulatory and adhesion molecules. Similar findings were obtained with ovarian cancer cells fused to allogeneic DC. The fusion cells were functional in stimulating the proliferation of autologous T cells. The results also demonstrate that fusions of ovarian cancer cells to autologous or allogeneic DC induce cytolytic T cell activity and lysis of autologous tumor cells by a MHC class I-restricted mechanism. These findings demonstrate that fusions of ovarian carcinoma cells and DC activate T cell responses against autologous tumor and that the fusions are functional when generated with either autologous or allogeneic DC. The Journal of Immunology, 2000, 165: 1705–1711.

Epithelial ovarian cancer is the leading cause of death for patients with gynecologic malignancies (1, 2). The majority of women with ovarian cancer present with advanced stage disease. Moreover, most patients with advanced disease recur after standard treatment with surgical debulking and combination chemotherapy (3). Immune-based therapies for ovarian cancer have been proposed as alternative approaches to improve survival (2, 4). In this context, ovarian cancer cells express mutated forms of the p53 (5–7) and/or BRCA-1 (8, 9) tumor suppressor genes. Ovarian tumors also overexpress the CA-125 (10–12) and DF3/MUC1 (13) carcinoma-associated Ags. In addition, these tumors overexpress the HER2/neu (c-erbB2) and epidermal growth factor receptors (14–17). Thus, certain targets for immunotherapy of ovarian cancer are already known, and others, although they remain undefined, presumably exist.

Dendritic cells (DC) are potent APC that can elicit primary immune responses (18). DC express MHC class I and II, costimulatory and adhesion molecules that provide secondary signals for stimulation of naive T cell populations (19, 20). In animal models, antitumor vaccines have been developed by pulsing DC with peptides derived from tumor Ags (21, 22). With regard to ovarian cancer, human DC loaded with HER2/neu peptides have been shown to stimulate proliferation of autologous T cells that induce lysis of peptide-pulsed targets (23, 24). Other studies have demonstrated that transduction of DC with viral vectors, which encode tumor Ags, confers presentation of tumor peptides to T cells (25–27). Using this strategy, DC transduced to express the MUC1 Ag have been shown to induce anti-MUC1 immune responses (25, 28). However, immunotherapeutic approaches that are dependent on the response to a single Ag are potentially subject to resistance by down-regulation of that Ag.

Other DC-based strategies have been developed to induce a polyclonal immune response against multiple tumor Ags. DC have been pulsed with tumor cell lysates (29–31), loaded with peptides acid-eluted from tumor cells (32, 33), and transfected with tumor cell RNA (34, 35). Fusions of DC and tumor cells have also been developed to induce a polyclonal antitumor immune response (36). In this approach, multiple tumor Ags, including those yet unidentified, are endogenously processed and presented by MHC class I pathways (36). Vaccination with fusions of murine tumor cells and syngeneic DC have been shown to eliminate established metastatic disease (36–39). Moreover, immunization of MUC1-transgenic mice with MUC1-positive fusion cells reversed immunologic unresponsiveness to MUC1 and induced immunity against MUC1-expressing tumors (40).

The present studies demonstrate the fusion of human ovarian carcinoma cells with autologous and allogeneic DC. We show that the human ovarian/DC fusions express both ovarian carcinoma-associated Ags and DC-derived MHC class II and costimulatory molecules. The fusion cells stimulate autologous T cells and induce CTL activity against autologous ovarian tumor cells.

Materials and Methods

Isolation of PBMC

Mononuclear cells were isolated from the peripheral blood of patients with ovarian cancer and normal donors by Ficoll-Hypaque density gradient centrifugation. The PBMC were cultured in RPMI 1640 medium containing 1% autologous serum for 1 h. The nonadherent cells were removed, and the T cells were purified by nylon wool separation. The adherent cells were cultured for 1 wk in RPMI 1640 medium containing 1% autologous serum, 1000 U/ml GM-CSF (Genzyme, Cambridge, MA), and 500 U/ml IL-4 (Genzyme). DC were harvested from the nonadherent and loosely adherent cells. The firmly adherent monocytes were cultured in RPMI 1640 medium containing 20% FCS, 1% penicillin/streptomycin, 50 U/ml IL-1, and 10% autologous serum. The monocytes were harvested from the nonadherent and adherent cell populations 7 days later.
containing 10% autologous serum without GM-CSF/IL-4 and harvested after treatment with trypsin.

**Preparation and fusion of ovarian carcinoma cells**

Ovarian carcinoma (OVCA) cells obtained from primary tumors and malignant ascites were separated in HBSS (Ca²⁺/Mg²⁺ free) containing 1 mg/ml collagenase, 0.1 mg/ml hyaluronidase, and 1 mg/ml DNase. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated autologous human serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin until fusion. Autologous or allogeneic DC were incubated with the OVCA cells for 5 min at a ratio of 10:1 in serum-free RPMI 1640 medium containing 50% polyethylene glycol. RPMI 1640 medium was then added slowly to dilute the polyethylene glycol. After washing, the cells were resuspended in RPMI 1640 medium supplemented with 10% autologous serum and 500 U/ml GM-CSF for 7–14 days.

**Phenotype analysis**

Cells were incubated with mouse anti-human Abs directed against DF3/MUC1 (mAb DF3) (13), CA-125 (mAb OC-125) (41), MHC class I (W6/32), MHC class II (HLA-DR), B7-1 (CD80), B7-2 (CD86), ICAM (CD54; PharMingen, San Diego, CA), and CD83 (PharMingen) for 1 h each. After washing with PBS, the cells were incubated with fluorescein-conjugated goat anti-mouse IgG for 30 min. For dual expression analysis, cells were incubated with mAb OC-125, washed, and then incubated with phycoerythrin-conjugated anti-MHC class II, anti-B7-2, or anti-CD83 for 1 h at 4°C. Samples were washed, fixed in 2% paraformaldehyde, and analyzed by FACSScan (Becton Dickinson, Mountain View, CA).

**Immunohistochemical staining**

Cytospin cell preparations were fixed in acetone and incubated with mAb OC-125 for 30 min at room temperature. The slides were washed and incubated with biotinylated horse anti-mouse IgG for an additional 30 min. Staining (red color) was generated with ABC solution (Vector Laboratories, Burlingame, CA). The slides were then incubated with murine anti-human MHC class II for 30 min and then with alkaline phosphatase-labeled anti-mouse IgG. AP-ABC solution (Vector Laboratories) was used to generate a blue counterstain.

**T cell proliferation assays**

Cells were exposed to 30 Gy ionizing radiation and added to T cells in 96-well flat-bottom plates for 5 days. Uptake of [³H]thymidine by the T cells was measured after incubation in the presence of 1 μCi/well for 12 h.

**Cytotoxicity assays**

T cells were stimulated with the indicated cell preparations for 1 wk in the presence of 20 U/ml human IL-2 (hIL-2). The T cells were harvested by nylon wool separation and used as effector cells in CTL assays. Autologous OVCA cells, allogeneic OVCA cells, autologous monocytes, MCF-7 breast carcinoma cells, and K562 cells were labeled with ⁵¹Cr for 60 min at 37°C. After washing, targets (2 × 10⁴) were cultured with the T cells for 5 h at 37°C. In certain experiments, the labeled target cells were incubated with mAb W6/32 (anti-MHC class I) for 30 min at 37°C before addition of the effector cells. Supernatants were assayed for ⁵¹Cr release in a gamma counter. Spontaneous release of ⁵¹Cr was assessed by incubation of the targets in the absence of effectors. Maximum or total release of ⁵¹Cr was determined by incubation of the targets in 0.1% Triton X-100. Percentage
of specific $^{51}$Cr release was determined by the following equation: percent specific release = $\frac{(\text{experimental} - \text{spontaneous})}{(\text{maximum} - \text{spontaneous})} \times 100$.

**Results**

**Characterization of OVCA cells fused with autologous and allogeneic DC**

DC were generated from patients with metastatic ovarian cancer and from normal volunteers. Adherent cells were isolated from PBMC and cultured in the presence of GM-CSF and IL-4 for 1 wk. The resulting population was subjected to FACS analysis. The DC displayed a characteristic phenotype with expression of MHC class I and class II, costimulatory molecules, and ICAM, but not of the DF3/MUC1 or CA-125 carcinoma-associated Ags (Fig. 1A). By contrast, OVCA cells isolated from a patient with metastatic ovarian cancer expressed MUC1, CA-125, MHC class I, and ICAM, but not MHC class II, B7-1, or B7-2 (Fig. 1A). Similar findings were obtained with OVCA cells obtained from primary ovarian tumors and from malignant ascites (data not shown). Fusion of the OVCA cells to autologous DC (OVCA/FC) resulted in the generation of heterokaryons that express the CA-125 and MUC1 Ags, MHC class II, B7-1, and B7-2 (Fig. 1A). Moreover, the pattern of Ag expression was similar when the OVCA cells were fused to allogeneic DC (Fig. 1B). Whereas cytokines produced by fusing OVCA cells and DC could alter expression of DC-derived molecules, the OVCA/FC exhibited similar levels of MHC class II and costimulatory molecules as found on unfused DC. As a control, monocytes (MC) were fused with autologous OVCA cells. Fusions of MC to OVCA cells also expressed CA-125 and MHC class I. However, compared with OVCA/FC, the OVCA/MC exhibited lower levels of MHC class II and little if any B7-1 (Fig. 1C).

Double immunofluorescence was used to assess efficiency of the fusions. In contrast to DC, the OVCA cells expressed CA-125, but not MHC class II, B7-2, or CD83 (Fig. 2A). Analysis of OVCA cells fused with autologous DC demonstrated that 32.6% of the population expressed both CA-125 and MHC class II (Fig. 2A). Assessment of CA-125 and B7-2 demonstrated that 30% of the autologous OVCA/FC expressed both Ags (Fig. 2A). Moreover, 10.8% of the autologous OVCA/FC population expressed both CA-125 and CD83 (Fig. 2A). Notably, expression of CD83 varied on DC preparations from different individuals. Immunostaining confirmed that the DC expressed MHC class II and not CA-125 (Fig. 2B). Conversely, the OVCA cells expressed CA-125 and not MHC class II (Fig. 2B). Analysis of the fusion cells (OVCA/FC) demonstrated expression of both Ags (Fig. 2B). These findings demonstrate the formation of heterokaryons by fusing OVCA cells to DC.

**Stimulation of antitumor CTL by autologous OVCA/FC**

To assess the function of OVCA/FC, the fusion cells were cocultured with autologous PBMC. As a control, the PBMC were also cultured with autologous OVCA cells. The fusion cells, but not the tumor cells, stimulated the formation of T cell clusters (Fig. 3A).
After 10 days of stimulation, the T cells were isolated for assessment of cytolytic activity. Using autologous OVCA cells as targets, there was a low level of lysis when assaying T cells that had been incubated with autologous DC, autologous tumor, or a mixture of unfused DC and tumor (Fig. 3B). By contrast, T cells stimulated with the OVCA/FC were effective in inducing lysis of autologous tumor targets (Fig. 3B). Similar results were obtained with T cells from three patients with ovarian cancer (Fig. 3B). As a control, T cells stimulated with OVCA cells fused to autologous monocytes or DC fused to monocytes had little effect on stimulation of antitumor CTL activity (Fig. 3C).

**Generation of antitumor CTL by OVCA cells fused to allogeneic DC**

To assess OVCA/FC function when the fusion is performed with allogeneic DC, we stimulated autologous PBMC with OVCA cells fused to autologous or allogeneic DC. As controls, the autologous PBMC were also stimulated with unfused DC or OVCA cells. Incubation of the T cells with allogeneic DC was associated with greater stimulation than that obtained with autologous DC (Fig. 4A). The results also demonstrate that T cell proliferation is stimulated to a greater extent by OVCA fused to allogeneic, as compared with autologous, DC (Fig. 4A). Similar findings were obtained with T cells obtained from two patients (Fig. 4A). After stimulation for 10 days, the T cells were isolated and assessed for lysis of autologous tumor. Stimulation with unfused allogeneic or autologous DC had little if any effect on lytic function compared with that obtained with OVCA cells (Fig. 4B). Moreover, for both patients, T cells stimulated with OVCA cells fused to autologous or allogeneic DC exhibited induction of CTL activity (Fig. 4B and Table I). These findings demonstrate that the antitumor activity of autologous CTLs is stimulated by fusions of tumor cells to autologous or allogeneic DC.

**Specificity of OVCA/FC-stimulated CTL**

To assess the specificity of CTL induced by fusion cells, T cells stimulated with OVCA cells fused to autologous DC were incubated with autologous tumor, autologous monocytes, MCF-7...
breast carcinoma cells, allogeneic OVCA cells, and NK-sensitive K562 cells. Incubation of the OVCA/FC-stimulated T cells with autologous tumor or monocytes demonstrated selective lysis of the tumor (Fig. 5A). In addition, there was no significant lysis of the MCF-7, allogeneic OVCA, or K562 cells by these CTL (Fig. 5A).

Preincubation of the targets with an anti-MHC class I Ab blocked lysis of the autologous OVCA cells and had little effect on that obtained for the other cell types in the absence of Ab (Fig. 5A). The targets were also preincubated with control IgG (■) or anti-MHC class I Ab (W6/32; 1:100 dilution; ◊) and then assayed for lysis. CTL activity was determined by 51Cr release. The results are expressed as the mean ± SD of three replicates.

Table I. Comparison of CTL activity against autologous OVCA cells after incubation of T cells with OVCA, DC, autologous OVCA/FC, or allogeneic OVCA/FC

<table>
<thead>
<tr>
<th>Patient</th>
<th>E:T Ratio</th>
<th>Autologous</th>
<th>Allogeneic</th>
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<tr>
<td></td>
<td></td>
<td>OVCA</td>
<td>DC</td>
</tr>
<tr>
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<td>9.2</td>
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<td></td>
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<td>8.47</td>
<td>4.21</td>
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*The results represent the percentage lysis (mean of three replicates) of autologous OVCA cells after incubation of autologous T cells with the indicated stimulators.
²ND, not done.
DC also demonstrated selective lysis of the autologous tumor (Fig. 5B). Moreover, lysis of the autologous tumor was abrogated by preincubation of the targets with anti-MHC class I (Fig. 5B).

Discussion
Ovarian carcinoma cells overexpress the CA-125, HER2/neu, and/or MUC1 tumor-associated Ags (11, 13, 15, 17, 42, 43). Little is known about the peptide structure of the CA-125 Ag and, consequently, there is no information available regarding expression of CA-125 peptides that bind to MHC molecules (44, 45). In studies of HER2/neu, multiple HLA-A2.1-binding peptides have been identified that can elicit CTL responses (24, 46, 47). DC from patients with OVCA have been pulsed with synthetic HER2/neu-derived peptides identified by screening the HER2/neu amino acid sequence for HLA-A2.1 anchor residues (24, 47). This approach has defined HER2/neu-derived peptides that induce CTL activity against autologous and allogeneic HLA-A2+ OVCA cells (24).

Similar studies have identified HLA-A2-binding peptides from the MUC1 protein (48, 49). DC obtained from normal donors and pulsed with the MUC1 peptides induced CTL, which lyse HLA-A2 tumor cells expressing MUC1 (48).

The present studies describe an alternative approach to a DC-based vaccine for ovarian carcinoma. Previous reports in mouse tumor models have demonstrated that vaccination with heterokaryons prepared by fusing tumor cells and DC induces the regression of established carcinomas, lymphomas, and melanomas (36–39). Fusion cell vaccines have also been shown to be effective in reversing immunologic unresponsiveness to MUC1 in MUC1-transgenic mice (40). In contrast to the identification of tumor-associated peptides that associate with HLA molecules, fusion cells provide a strategy for inducing immunity against both known and unknown tumor Ags (36). In addition, as fusion cells present peptides in the context of MHC class I molecules expressed by the tumor cell, this vaccine is not restricted to certain HLA types. In this regard, fusion of human OVCA cells to DC is associated with expression of the OVCA-associated Ags, CA-125 and MUC1, and DC-derived costimulatory and adhesion molecules necessary for the activation of T cells. The results also demonstrate that the fusion cells are functional in inducing MHC class I-restricted CTL activity.

The demonstration that human OVCA cells can be fused to DC provides the experimental basis for using these fusions as vaccines in the treatment of ovarian cancer. OVCA cell fusions have been generated with both autologous and allogeneic DC. The phenotype of the OVCA cells fused to autologous DC was similar to that obtained with allogeneic DC. In addition, although tumor cells were ineffective in stimulating autologous T cells, fusions of OVCA cells to both types of DC were effective in inducing a T cell proliferative response. As expected, incubation of autologous T cells with OVCA fused to allogeneic DC induced a more pronounced proliferative response than that obtained with OVCA fused to autologous DC. By contrast, induction of CTL activity was similar with OVCA fused to autologous or allogeneic DC. These findings indicate that, although T cell stimulation is greater with OVCA fusions expressing DC-derived allogeneic Ags, the induction of CTL activity against autologous OVCA cell Ags is not enhanced by fusions to allogeneic, as compared with autologous, DC. Thus, the results support the fusion of OVCA cells to either autologous or allogeneic DC in the development of a clinical vaccine.

OVCA cells fused to either autologous or allogeneic DC were effective in inducing antitumor CTL, which lyse autologous OVCA cells by a MHC class I-restricted mechanism. The autologous OVCA/FC can present tumor Ags by OVCA- or DC-derived MHC class I molecules. Moreover, autologous OVCA/FC can present tumor Ags by DC-derived MHC class II molecules and thereby stimulate helper CD4+ cells. By contrast, presentation of tumor Ags by the allogeneic OVCA/FC cells is dependent on OVCA-derived MHC molecules. The allogeneic OVCA/FC cells can also stimulate alloreactive T cells and thereby release of cytokines, which contribute to the activation of tumor-specific CTL (50). The induction of antitumor CTL provides an opportunity to define the epitopes, and thereby the tumor-associated Ags, that are targets of the immune response. Thus, although screening of known Ags, such as HER2/neu or MUC1, for epitopes that bind to HLA-A2 represents one approach, the induction of antitumor CTL with fusion cells represents another strategy for defining peptides that function in the immune recognition of tumor cells. The present findings demonstrating human OVCA/DC fusions thus could have potential applicability to the field of antitumor immunotherapy as vaccines and in the identification of novel OVCA-associated Ags.

Note added in proof. Recent studies have demonstrated that fusions of human renal carcinoma cells and allogeneic DC are effective in the treatment of metastatic renal cell cancer (51).

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