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Permalink
https://escholarship.org/uc/item/8xk7q9qq

Journal
International Journal of Cancer, 65(5)

ISSN
0020-7136

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Publication Date
1996-03-01

DOI
10.1002/(SICI)1097-0215(19960301)65:5<688::AID-IJC21>3.0.CO;2-2

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EFFECTS OF CYTOKINES COMBINED WITH HIGH-DOSE GAMMA IRRADIATION ON THE EXPRESSION OF MAJOR HISTOCOMPATIBILITY COMPLEX MOLECULES AND INTERCELLULAR ADHESION MOLECULE-1 IN HUMAN OVARIAN CANCERS

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In this study, we examined the effects of cytokine exposure as well as high doses of irradiation on the surface expression of MHC and ICAM-1 molecules in 2 established ovarian carcinoma cell lines and in several freshly isolated tumor-cell lines of ovarian cancer origin. We found that irradiation given immediately after a brief exposure to TNF-α plus IFN-γ induces a highly significant and long-lasting expression of each surface antigen which is either up-regulated or induced by the cytokines. Moreover, the combination of the 2 procedures appeared to have an additive effect on the expression of these surface molecules. These findings provide new information for use in the future development of tumor vaccines.

MATERIAL AND METHODS

Fresh tumors and tumor-cell lines

Single-cell suspensions were prepared from 7 ovarian adenocarcinomas of primary or metastatic origin obtained at the time of surgery. Human serous papillary ovarian carcinoma cell lines UCI-101 and UCI-107 were kindly provided by Dr. A. MANETTA, University of California, Irvine (UCI), UCI-101 and

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Abbreviations: PPC, percentage of positive cells; MFI, mean channel fluorescence intensity; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; MHC, major histocompatibility complex; ICAM-1, intercellular adhesion molecule-1; MAb, monoclonal antibody.

Received: September 14, 1995 and in revised form November 16, 1995.
UCI-107 cell lines were maintained at 37°C, 5% CO₂ in complete medium (CM) containing RPMI 1640 (GIBCO Life Technologies, Grand Island, NY) and 10% fetal bovine serum (FBS, Gemini, Calabasas, CA), while fresh single-cell suspensions and short-term cultures were maintained at 37°C, 5% CO₂ in complete medium (CM) containing RPMI 1640, 5% FBS (Gemini). All the fresh tumors were of serous papillary histologic origin. OVA-1, OVA-9, OVA-12 and UCI-107 were obtained from patients who had not been previously treated with chemotherapy, while OVA-6, OVA-7, OVA 13, OVA 14 and UCI-101 were obtained from patients treated with different chemotherapy regimens. Briefly, single-cell suspensions were obtained by processing solid tumor samples under sterile conditions at room temperature. Viable tumor tissue was mechanically minced in RPMI 1640 to portions no larger than 1–3 mm³ and washed twice with RPMI 1640. The portions of minced tumor were then placed into 250-ml trypsinizing flasks containing 30 ml of enzyme solution [0.14% collagenase Type I (Sigma, St. Louis, MO) and 0.01% DNase (Sigma, 2000 KU/ml)] in RPMI 1640, and incubated on a magnetic stirring apparatus overnight at 4°C. Enzymatically dissociated tumor was then filtered through 150-mm nylon mesh to give a single-cell suspension. The resultant cell suspension was then washed twice in RPMI 1640. Experiments were performed only with suspensions which had at least 90% viability as determined by trypan-blue exclusion. The percentage of tumor cells was determined by cytoxiffer expression using immuno-histochemical techniques. In this regard, all the fresh tumor samples evaluated contained > 99% tumor cells.

**IFN-γ and TNF-α preincubation**

Single-cell suspensions of freshly isolated ovarian tumors and the 2 established cell lines were incubated in CM or in CM containing a combination of IFN-γ and TNF-α for 3 days (500 units/ml each) and subsequently evaluated for antigen expression by FACS analysis. Recombinant human IFN-γ (specific activity; 2.5 × 10⁷ U/mg) was purchased from Genzyme (Cambridge, MA) and recombinant human TNF-α (specific activity; 1.0 × 10⁷ U/mg) was obtained from Genentech (S. San Francisco, CA).

**IFN-γ and TNF-α preincubation followed by high-dose γ-irradiation**

Fresh ovarian tumors that could be grown as short-term cultures (OVA-6, OVA-7, OVA-9, OVA-12, OVA-13, OVA-14) and the 2 established cell lines were irradiated with a total dose of 10,000 rads with or without pre-incubation with IFN-γ and TNF-α for 3 days (500 units/ml each). One portion of the pre-incubated cells from 2 short-term cultures (OVA-13, OVA-14) and UCI-101 and UCI-107 cell lines was seeded in CM without cytokines just before irradiation. This allowed us to follow the natural course of surface antigen expression with time but without the influence of irradiation. The remaining portion of the same cell cultures was irradiated in 15-ml conical tubes in CM at room temperature with gamma rays (Cesium 137) at a dose rate of 200 rads/min. Immediately after irradiation, cells were seeded into T 75 tissue-culture flasks (Corning, Corning, NY) in CM devoid of cytokines and cultured at 37°C in a 5% CO₂ atmosphere. Spent medium was changed every other day. Irradiated cells preincubated with cytokines, and irradiated control cells were harvested at days 2 and 6 for FACS analysis. Cells from OVA-13, OVA-14, UCI-101 and UCI-107 cell lines pre-incubated with cytokines, not irradiated but put into normal medium just after the completion of pre-incubation (72 hr) were evaluated by FACS after 2 weeks of culture following 10,000 rads of irradiation.

**Indirect immunofluorescence and flow cytometry**

Cells obtained from single-cell suspensions or after short-term cultures, as well as the established ovarian cell lines, were harvested with 0.25% Trypsin in HBBS (GIBCO), and washed once in CM. Cell suspensions were counted and distributed into 12 × 75-mm tubes at 5 × 10⁵ cells/tube. Mouse MAbs [anti-HLA class I (MAB W6/32; Accurate Chemical and Scientific Corp., Westbury, NY); anti-HLA class II (MAB CR3-43; Accurate Chemical); anti-ICAM-1 (MAB LB-2; Becton Dickinson, Mountain View, CA)] were diluted in cold assay buffer (PBS, pH 7.2, supplemented with 0.1% FCS) and added in a 50-ml volume. A mouse IgG preparation (MAB IgG₀α, Becton Dickinson) was used as negative control. The fluorescence induction index was determined as the ratio between the mean fluorescence intensity of experimental cells compared to the mean fluorescence intensity of untreated control cells:

 indsuction index = experimental mean channel fluorescence intensity - control mean channel fluorescence intensity

Statistical analysis

Significance analysis was performed using a paired Student’s t test. Only p values < 0.05 were considered significant.

**RESULTS**

**Expression of surface antigens on freshly isolated ovarian tumors and UCI-101 and UCI-107 continuous cell lines**

Flow cytometric analysis of MHC class-I and -II antigens as well as ICAM-1 antigen was performed on 7 freshly isolated ovarian tumors and 2 continuous cell lines. The results shown in Table I indicate that MHC class-I antigens were expressed at high levels by all 7 of the freshly isolated ovarian tumors [mean channel fluorescence intensity (MFI) ranged from 855 to 2658]. Class-I antigens were also expressed on the 2 established cell lines, although at a much lower level (MFI evaluated in 3 different experiments ranged from 78 to 211 for UCI-101 and from 155 to 358 for UCI-107). Class-II antigens were expressed at low levels in 4 out of 7 freshly isolated ovarian tumors and then only in a subset of cells, while they were not detected at all on the continuous ovarian cell lines. ICAM-1 was expressed in all the cells evaluated with the exception of UCI-107.

**Effect of IFN-γ plus TNF-α on surface antigen expression**

The effect of IFN-γ plus TNF-α on the expression of MHC and ICAM-1 antigens on freshly isolated and continuous ovarian tumors was determined by FACS analysis. In all of the ovarian carcinoma cells studied, IFN-γ plus TNF-α markedly up-regulated the level of MHC class-I antigen expression (Table I). The increase in MHC class-I expression by the freshly isolated ovarian tumors ranged from 2.28- to 4.27-fold (induction index), while the increase in expression by UCI-101 and UCI-107 ranged from 11.1 to 29.9 and from 9.1 to 20.5, respectively. IFN-γ plus TNF-α up-regulated the expression of MHC class-II antigens on all freshly isolated ovarian tumors as well as the UCI-107 cell line. In particular, for those tumors which did not express MHC class-II antigens prior to cytokine exposure, IFN-γ plus TNF-α induced its expression, albeit at low levels. In all of the ovarian carcinoma preparations studied, IFN-γ and TNF-α either induced (UCI-107) or up-regulated the expression of ICAM-1 antigens. In this regard, the induction index ranged from 1.64 to 19.2 fold (Table I).

**Persistence of surface antigen expression after cytokine withdrawal**

The time course of MHC and ICAM-1 surface antigen expression following withdrawal of TNF-α plus IFN-γ
studied in 2 freshly isolated ovarian tumors (OVA-13, OVA-14) and the 2 continuous ovarian cell lines. The results of these studies are shown in Table II. For the 2 continuous cell lines, a significant up-regulation of MHC class I molecules was present 2 days after removal of the cytokines. However, by 6 days after cytokine withdrawal, no significant difference was noted between treated and untreated cells. When MHC class I and ICAM-1 were evaluated in freshly isolated OVA-13 and OVA-14 tumors, a slower return to the basal (i.e. untreated) level of surface antigen expression was noted. Indeed, MHC class I molecules were still significantly up-regulated after 6 days following cytokine withdrawal ($p \leq 0.05$).

Expression of surface molecules after high-dose $\gamma$-irradiation

Cell-surface antigen expression on 2 fresh ovarian tumors (OVA-13, OVA-14) and the 2 established cell lines was evaluated 2 and 6 days after irradiation and the results were compared to the level of expression of unirradiated control cells. As shown in Table III, for the freshly isolated tumors, irradiation caused a modest but not significant up-regulation of both MHC class I and ICAM-1 surface antigens which remained at this level for up to 6 days. In contrast, for the continuous cell lines, irradiation induced a marked increase in the expression of class-I and ICAM-1 antigens which persisted for at least 2 days. The marked sensitivity to high-dose irradiation of the established lines prevented the evaluation of antigen expression after 6 days. It is interesting that irradiation did not induce neoexpression of antigens previously not present on these cells, such as MHC class II. It is also worth noting that, while irradiation could cause an increased expression of all antigens tested expressed by the cells, the levels reached were much lower than those attained by exposure to IFN-$\gamma$ and plus TNF-$\alpha$.

Expression of surface molecules after irradiation with or without previous exposure to IFN-$\gamma$ plus TNF-$\alpha$

Cell-surface antigens were evaluated 2 and 6 days after irradiation in 6 freshly isolated ovarian tumors (OVA-6, OVA-9, OVA-12, UCI-101, UCI-107, MFI) and the two continuous ovarian cell lines. The results of these studies are shown in Table II. For the 2 continuous cell lines, a significant up-regulation of MHC class I molecules was still present 2 days after removal of the cytokines. However, by 6 days after cytokine withdrawal, no significant difference was noted between treated and untreated cells. When MHC class I and ICAM-1 were evaluated in freshly isolated OVA-13 and OVA-14 tumors, a slower return to the basal (i.e. untreated) level of surface antigen expression was noted. Indeed, MHC class I molecules were still significantly up-regulated after 6 days following cytokine withdrawal ($p \leq 0.05$).
OVA-7, OVA-9, OVA-12, OVA-13, OVA-14) and after 2 days in the established cell lines with or without prior exposure to IFN-γ plus TNF-α. In all the tumor cells studied, prior exposure to cytokines followed by irradiation induced a highly significant and long-lasting expression of all the surface antigens either up-regulated or induced by the cytokines (Table IV and Fig. 1). Indeed, in the freshly isolated tumors, the levels of antigen expression following cytokine exposure and irradiation remained significantly elevated for up to 6 days. This was in marked contrast to the effects of cytokine exposure only, in which surface antigen expression rapidly and consistently decreased after treatment. In addition, when the level of antigen up-regulation reached with cytokine exposure at day 0 and day 2 was directly compared to the level of antigen expression after the combination of the 2 procedures (day 2), an additive and persistent increase in antigen expression was noted both for the freshly isolated tumors and for the continuous cell lines (Fig. 2).

**DISCUSSION**

This study investigated the expression of MHC and ICAM-1 surface antigens on freshly isolated and continuous cell lines of human ovarian epithelial tumors. The effects of high-dose γ-irradiation, the exposure to cytokines TNF-α plus IFN-γ and the combination of the 2 procedures on the expression of these antigens were carefully studied. The results indicate that our series of freshly isolated ovarian epithelial tumors express high levels of MHC class-I and ICAM-1 antigens compared to the 2 continuous cell lines, and that 4 out of these 7 tumors also expressed detectable levels of MHC class-II antigens. Following exposure to TNF-α plus IFN-γ, all tumors studied rapidly up-regulated their levels of expression of MHC class I and ICAM-1. Furthermore, in those tumors which already expressed MHC class-II antigens (4 out of 7), cytokine exposure also markedly increased its surface expression. In those tumors which did not express MHC class-II antigens, exposure to cytokines induced their expression in all cases. In the continuous ovarian cell lines, MHC and ICAM-1 antigens were expressed at a much lower level than in the freshly isolated tumors, but exposure to cytokines also markedly up-regulated their level of expression to values comparable to those seen for the fresh tumors. It is worth noting that the striking difference in the up-regulation of surface molecules seen in the 2 groups (induction index for MHC I from 2.28 to 4.27 in the fresh tumors, compared to an induction index of 9.1 to 29.9 in UCI-101 and UCI-107 cell lines) appears to be due to different levels of expression in the resting basal state and shows that cells which express low levels of these surface molecules are capable of up-regulating them to very high levels after exposure to the appropriate cytokines. It is noteworthy that exposure to TNF-α plus IFN-γ did not cause any observable toxic effects to any of the freshly isolated tumor samples but appeared to decrease the growth rate of the established ovarian cell lines.

The effect of γ-irradiation on surface antigen expression was also investigated. Our results indicate that, in all tumors evaluated, an increase in the expression of MHC class-I and ICAM-1 antigens consistently occurred, although this increase was statistically significant only in the 2 continuous cell lines and was only modest when compared to the up-regulation induced by the cytokines. Our previous studies showed that irradiation up-regulated other antigens in addition to MHC and ICAM-1 (i.e., Her-2/Neu) and that this was dose-dependent, higher expression being associated with higher doses of irradiation (data not shown). In the present study, in agreement with other reports (Hauser et al., 1993; Hareyama et al., 1991), we were able to confirm that an increase in expression of surface antigens is a general feature induced by high doses of γ-irradiation. However, such up-regulation, unlike that caused by TNFα plus IFN-γ, did not induce expression of antigens not previously expressed in the cell lines prior to irradiation.

The increased level of antigen expression observed following exposure to TNF-α plus IFN-γ was not persistent and dropped rapidly after removal of the cytokines. However, a somewhat slower return to the basal surface expression was noted in the fresh tumors.

Our study design differed from that of previous reports evaluating the effects of irradiation on antigen expression (Hauser et al., 1993; Hareyama et al., 1991) inasmuch as the cells were preincubated with cytokines prior to irradiation. We found that, following exposure to cytokines, irradiation caused a highly significant and long-lasting expression of all the surface antigen, either up-regulated or induced by the cytokines. Moreover, a further additive effect was noted upon increasing the expression of the surface molecules, either up-regulated or induced by the cytokines. Finally, the combination of such procedures was clearly shown to be superior to either modality alone.

The importance of these findings is underscored by previous reports on the influence of surface antigen expression and immune recognition. Several studies on human tumor cells of different origins exposed to TNF-α plus IFN-γ have shown that the induction of cytotoxic effector cells requires a higher level of antigen expression than cytotoxic interaction (Vanky et al., 1989; Anichini et al., 1993). In fact, tumors not previously considered to be immunogenic but so induced to express higher levels of MHC antigens appear to recruit high-avidity T cells, which can then recognize target cells which express virtually undetectable levels of MHC antigens (Vanky et al., 1989). Moreover, in studies on melanoma (Anichini et al., 1993), the expression of ICAM-1 in different melanoma clones, correlated with the susceptibility of the tumor cells to lysis by specific and non-specific T-cell clones and, more importantly, up-regulation of ICAM-1, by treatment with IFN-γ in low-expressor clones, could boost tumor lysis only by specific TCR-dependent and HLA-restricted effector cells. In such studies, the relative surface expression of ICAM-1 was a key factor for the outcome of the interaction with T cells and only those clones that expressed these molecules at levels high enough for an efficient interaction with antigen-specific T cells were able to trigger lysis.
FIGURE 1 – Effects of irradiation versus cytokine plus irradiation after 2 and 6 days on the expression of MHC class-I, class-II and ICAM-1 surface antigens on 6 freshly isolated ovarian tumors. Data from one representative experiment are shown. a, b, c, Results after 2 days. d, e, f, Results after 6 days. Open columns: irradiation only. Hatched columns: cytokine exposure followed by irradiation. The p-values after 2 and 6 days were 0.006 and 0.002, for MHC class I, 0.001 and 0.05 for class II and 0.004 and 0.04 for ICAM-1, respectively.
The importance of the direct expression of MHC class-II antigens on tumor cells has been highlighted in recent studies of transduction of class-II genes in murine tumor models (Collins et al., 1992). Such studies have shown that the expression of class-II molecules can make such tumor cells function as antigen-presenting cells provided that the invariant chain (II) is not expressed (Clements et al., 1992). Indeed, because most non-lymphoid and non-myeloid-derived murine tumor cells do not express II (Koch and Harris, 1984) and the expression of MHC class-II molecules can occur in human cells in the absence of II (Sekaly et al., 1986), the possibility of increasing or of inducing expression of such molecules after cytokine treatment could also augment the possibility of direct activation of CD4 helper-inducer T cells. Finally, in a prospective randomized clinical trial of colon carcinoma, Hanna et al. (1993) demonstrated that immunization with autologous tumor cells mixed with BCG led to a significant increase in disease-free survival only in those patients whose tumor cells expressed high levels of MHC class-II and ICAM-I surface antigens.

Until common tumor antigens are identified at the genetic level, and the prevalence and biorelevance of these antigens are assessed, whole tumor cells remain the most reliable source of specific tumor antigens for cancer immunotherapy. The possibility of increasing or inducing de novo expression of MHC molecules and ICAM-1 or other important surface antigens in tumor cells after exposure to TNF-α plus IFN-γ has already been recognized as an efficient method of increasing in vitro induction of T-cell responses against these tumors (Vanky et al., 1989; Pflzenmaier et al., 1985). However, one of the problems to consider when such up-regulated cells are used as tumor vaccines is the relatively short half-life of the up-regulation following removal of the cytokines. Moreover, when using vaccines derived from single-cell suspensions or from allogeneic-autologous established tumor-cell lines, it is necessary to inhibit cell replication in order to prevent tumor formation at the immunization site. Such procedures should not compromise the immunogenicity of the vaccine; rather, they should maintain or even enhance the intrinsic immunogenic potential. High doses of γ-irradiation represent one of the most commonly used methods of inhibiting cell replication for human tumor immunotherapy. Taken together, the observations reported here support the use of a combination of such procedures when preparing tumor cells for use as tumor vaccines. These important findings provide new and additional strategies for active specific immunization in patients with cancer.

ACKNOWLEDGEMENTS

This work was supported in part by grants from Memorial Health Services of Long Beach Memorial Hospital (Long Beach, CA), Oncotech, Inc., Irvine, CA, and the Camillo Golgi Foundation, Brescia, Italy.

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