Original Articles

Differential expression of CD40 and CD95 in ovarian carcinoma

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Summary

Purpose: The role of CD95 (Fas) as a mediator of apoptosis has been well documented. CD40 ligation has been recently shown to initiate apoptosis and modulate CD95 mediated apoptosis in normal and some neoplastic tissues. Here we report the expression of CD95 and CD40 in cryopreserved cell suspensions from ovarian cancer associated ascites, fresh primary and recurrent ovarian carcinoma (OVCA) specimens, and ten established ovarian cancer cell lines. The effect of CD95 and CD40 receptor binding on apoptosis is described in two cell lines.

Experimental Design: Ascites specimens, fresh primary and recurrent OVCA specimens were dissociated to single cell suspensions. Expression of CD95 and CD40 was analyzed using flow cytometry. Apoptosis was determined via annexin uptake by flow cytometry following incubation with anti-CD95 antibody, CH11 and trimeric CD40L.

Results: Ascites showed the highest expression of both CD95 and CD40. Recurrent OVCA, in contrast, expressed low levels of CD95 and CD40. Primary OVCA showed moderate expression of both receptors. CD40 expression in ascites was significantly greater when compared to solid specimens ($p < 0.05$). Both CD40 and CD95 were strongly expressed in eight of ten cell lines studied. Binding of CD40L did not influence CD95 mediated apoptosis.

Conclusions: CD40 is ubiquitously expressed in ovarian carcinomas and expression differs between ascites and solid tumor. There may be differential expression of both CD40 and CD95 in recurrent vs primary ovarian carcinoma, which may contribute to increased clinical malignancy of recurrent disease. In contrast to other epithelial malignancies, CD40 ligation does not appear to modulate CD95 mediated apoptosis.

Key words: Apoptosis; Epithelial malignancy.

Introduction

Gynecologic cancers are a significant cause of morbidity and mortality in women. Despite improvements in diagnosis and treatment, the annual incidence of ovarian cancer remains high. During the year 2000, in the United States, 23,100 ovarian cancers were diagnosed. Sixty percent of ovarian cancers present at an advanced stage and have a five-year survival rate of 28%. One woman in 58 can expect to develop ovarian carcinoma over her lifetime and, in spite of progress in surgical staging, radical debulking and platinum-based chemotherapy, five-year survival, across all stages, has remained at 39-47% [1-3]. Continued effort to establish new treatment modalities is imperative as the average age of the United States population increases.

The mechanisms through which signaling pathways regulate the growth and viability of transformed cells are critical to the pathogenesis of cancer. In particular, cellular pathways that trigger programmed cell death (apoptosis) may be important in therapeutic intervention of human malignancies [4-6]. Apoptosis can be induced through a number of well characterized receptor/ligand interactions involving paired proteins belonging to the TNF-receptor and TNF families, respectively [7, 8]. The TNF-receptor family now consists of 29 members, of type I transmembrane proteins, containing homologous extracellular domains with three to six cysteine rich 40 amino acid long pseudo-repeats, and cytoplasmic domains of variable length without significant sequence homology [7, 9].

Both CD40 and CD95 are members of the TNF receptor family. The role of CD95 has been extensively studied in the context of developmental biology and lymphocyte regulation [10, 11]. Initially, CD95 and CD95L expression was found in hematopoietic cells such as T cells, natural killer cells, B cells and macrophages. It plays an important regulatory role in development of an immune response, since mice with loss of function mutations for CD95/CD95L develop autoimmune disease [12, 13]. Subsequent work has revealed CD95 and CD95L expression in various non-hamatopoietic normal and neoplastic tissues, including immuno-privileged sites such as the anterior chamber of the eye [14]. In the normal ovary, CD95 is involved in the apoptosis of thecal and interstitial cells of atretic ovarian follicles [15, 16]. Studies on ovarian carcinoma cell lines have shown them susceptible to CD95 mediated apoptosis [17]. Further, cytokine induced upregulation of CD95 receptors has been shown to potentiate apoptosis in ovarian cancers [18]. CD95 mediated apoptosis has also been implicated in tumor response to chemotherapy in ovarian tumor cells [19, 20].

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CD40, initially described as a B cell activation antigen, is a 48KD membrane protein, activated by CD154 (CD40L), its cognate ligand. Similar to CD95, CD40 and its ligand have been extensively studied for their role in immune regulation, in particular, survival, proliferation, and differentiation of B cells [7, 11, 21-23]. The CD40-CD40L interaction is also vital for T-cell dependent humoral immune response, isotype switching and affinity maturation [24, 25]. Besides an essential role in the immune system, CD40 receptor-ligand interaction is also crucial in other organ systems [26, 27]. Ubiquitous expression of these two proteins is found in epithelial cells and solid epithelial cancers [28-38]. More recently, again similar to CD95, CD40 ligation has been reported to result in growth inhibition of solid epithelial cancers [39-42].

More interestingly, accumulating evidence points to a novel cooperation between these two proteins in regard to survival and death in various cell types. CD40, generally thought of as a B cell growth promoter, has been shown to rescue B cells from CD95 mediated apoptosis [43, 44]. However, CD40 has also been shown to potentiate CD95 mediated apoptosis in many B lymphocyte associated studies [45-48]. It thus seems that in B cells CD40 can inhibit or augment CD95 mediated cell death depending on the inherent signal cascade in the original cell type.

Studies in various epithelial carcinomas have also revealed a dual influence of CD40 on CD95 mediated apoptosis. CD40, expressed on majority of bladder carcinomas [49], protects cells from Fas mediated apoptosis [50]. Similarly, CD40 positive prostate carcinoma cell lines are resistant to CD95 apoptosis, whereas their CD40 negative counterparts are not. Transfection of CD40 cDNA into CD40 negative cell lines transmitted apoptosis resistance, thereby confirming a link to CD40 [51]. CD40 binding also diminishes CD95 induced apoptosis in nasopharyngeal carcinoma [52]. In contrast, CD40 binding increased apoptosis in breast carcinoma cell lines and inhibited growth of squamous carcinoma cells of the head and neck [53, 54]. Consequently, it appears that even in non-hematopoietic tissues, the two proteins can act in concert or in opposition depending on the overall cell signaling status.

In this study, we report the expression of CD95 and CD40 in fresh primary and recurrent ovarian carcinoma specimens. CD95 and CD40 expression in cryopreserved cell suspensions from ovarian cancer associated ascites and ten established ovarian cancer cell lines is also reported. The effect of CD95 and CD40 receptor binding on apoptosis is described in two cell lines. Because of the synergistic and anti-synergistic capabilities between these two proteins the joint evaluation will lead to a better understanding of the signaling pathways operational in malignant ovarian tissues.

Materials and Methods

Fresh ovarian carcinoma

Ovarian carcinoma samples were obtained from 15 patients undergoing surgery as part of their cancer treatment. Tissue samples were obtained as part of a study approved by Stanford University's Internal Review Board and Committee for the Protection of Human Subjects. Preoperative informed consent was obtained from all participants. Tumor samples were obtained from nine patients with primary disease who underwent initial surgical staging and from six patients with recurrent disease who underwent secondary debulking. Pathologic diagnosis of ovarian carcinoma was made by our Department of Pathology, with each patient's histology reviewed by a single pathologist to confirm the diagnosis. All tissue samples were obtained fresh at the time of surgery and processed immediately after retrieval. Tumor specimens were mechanically dissociated into a cell suspension. Dissociation into a cell suspension was confirmed using microscopy.

Ascites

Cryo-preserved cells, from the malignant ascites of four patients, were obtained from our laboratory's tissue bank. Specimens were initially placed in liquid nitrogen between April 1986 and June 1988. At the time of initial storage, cells were separated from ascites fluid by centrifugation and placed in a freezing medium as cell suspensions. They were maintained in liquid nitrogen until the time of thawing. Upon thawing for these experiments, cells were washed twice in PBS and analyzed immediately as described below.

Cell lines

Ten ovarian carcinoma cell lines were assessed for CD95 and CD40 expression using flow cytometry. Cell lines, 2774, SKOV-3, and ES-2 were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cell lines, CAOV3, OV433, OV429, HEY, IGROV, A2789, and OVCAR-3 were a generous gift from Dr. Branamir Sikic (Stanford University, Stanford, CA). Cell lines were maintained in log phase growth as an adherent monolayer using Iscove's medium with 10% FCS in a humidified atmosphere with 5% CO_2 at 37°C without antibiotics. Prior to antibody labeling, cells were removed from the culture flasks using 1mM EDTA-Trypsin in PBS.

Flow cytometry

Cell suspensions (5 x 10^5 cells) from fresh tumor specimens and cell lines were prepared in a final volume of 50 µl staining medium (PBS with 3% FCS and 0.01% NaAz). Cells were incubated with primary antibody, mouse anti-human CD95 or mouse anti-human CD40 (Pharmingen, San Diego, CA) according to the manufacturer's instructions. Cells were then incubated with the secondary antibody, biotin-conjugated anti-mouse Ig (Caltag Laboratories, Burlingame, CA) and phycoerythrin (PE)-conjugated anti-human CD2 (Pharmingen, San Diego, CA), followed by fluorescein-conjugated Avidin (Caltag Laboratories, Burlingame, CA). All incubations were for 20 minutes at 4°C. Cells were washed twice with staining medium between successive incubations. After the final incubation and wash, cells were fixed with 1% paraformaldehyde and stored at 4°C in the dark. Cells were analyzed on the FACScan (Becton-Dickinson, Mountain View, CA), interfaced with a Vax 6300 computer (Digital Equipment, Maynard, MA) running FACS/Desk software, within four hours of fixing. In order to ensure reproducibility the sorter was calibrated with standard polystrene microspheres (Pandex, Mundelein, IL). Data was collected on 100,000 cells. PE-conjugated anti-human CD2 (Pharmingen, San Diego, CA) was included to exclude T lymphocytes, the major contaminant originating from patient blood. PE-conjugated CD2 was not used during the staining of cell lines.

Apoptosis assay

Exponentially growing cell lines (5 X 10^5 cells), Hey and OVCA433 were treated overnight with anti-CD95 antibody, CH11 (Upstate Biotechnology Inc., Lake Placid, NY) at a concentration of 2 μg/ml. Jurkats (ATCC), a T cell line, was treated simultaneously as a positive control susceptible to CH11 mediated apoptosis. Apoptosis was measured by annexin uptake by flow cytometry. Briefly, following overnight exposure to CH11, cells were washed twice with PBS, incubated with FITC-conjugated Annexin (Pharmingen, San Diego, CA) and analyzed on FACscan.

Statistics

Statistical analysis was performed using GraphPad InStat version 4.10, 1998, San Diego, CA.

Results

Expression of CD40 and CD95 in ascites, ovarian carcinoma tissue and OVCA cell lines

CD95 and CD40 expression on OVCA cell lines and on OVCA tissue specimens was quantified by flow cytometry. The mean channel fluorescence for CD40 and CD95 stained specimens was divided by the mean channel fluorescence of the control sample consisting of the same cell population incubated only with the secondary antibodies described above. This mean fluorescence ratio (MFR) allowed quantitative comparison of CD95 and CD40 expression across primary carcinoma, recurrent carcinoma, ascites, and OVCA cell lines. A representative histogram depicting expression of CD40 and CD95 in an ascites specimen and the generation of the MCF ratio is shown in Figure 1.

Among tumor specimens, ascites (n = 4) showed the highest level of both CD95 and CD40 expression (Figure 2). The mean MFR for CD95 expression in this group was 7.5 (range 3.2 to 16) and 8.7 (range 3.0 to 17.7) for CD40. Specimens from primary (n = 9) and recurrent (n = 6) carcinoma revealed differential expression of cell surface receptors. Mean MFR for primary specimens was 5.8 (range 2.1 to 13.1) and 3.5 (range 1.6 to 6.1) for CD95 and CD40 expression, respectively. For recurrent tumor specimens mean MFR for CD95 was 3.6 (range 1.8 to 5.7) and mean MFR for CD40 was 1.9 (range 0.9 to 3.9). All cell lines studied, except A2789, showed strong expression of CD95. Similarly, strong expression of CD40 was seen in all cell lines studied except ES2 (Figure 3).

Using one-way analysis of variance (ANOVA) with Tukey-Kramer correction for multiple comparisons, CD40 expression in ascites was found to be significantly greater ($p < 0.05$) than in fresh specimens. This continued to be true when ascites was compared to the primary and recurrent subgroups. The difference between CD40 and CD95 expression in recurrent versus primary specimens reached near significance ($p < 0.08$ and $p < 0.15$, respectively).

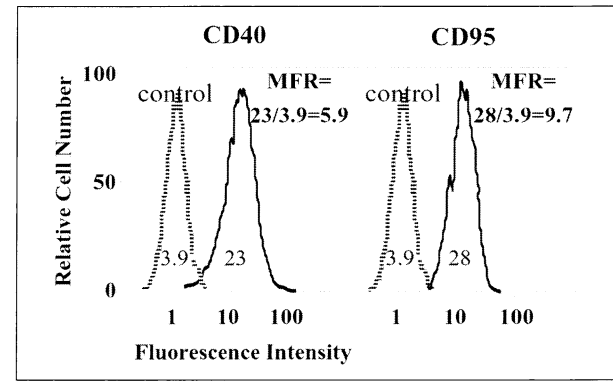

Figure 1. — Generation of mean fluorescence ratio (MFR). Tumor specimens and cell lines were stained with anti-CD95 or anti-CD40 Abs, followed by biotin-conjugated anti-mouse Ig, followed by avidin-FITC. Simultaneous control samples were stained with two secondary reagents only. The histogram depicts the fluorescence intensity of the test and control samples for a representative tissue specimen (primary OVCA). Specimens with larger cell size (*e.g.*, ascites) exhibit greater background fluorescence. The generation of the MFR balances for the size differences and allows for quantitative comparisons of fluorescence among specimens.

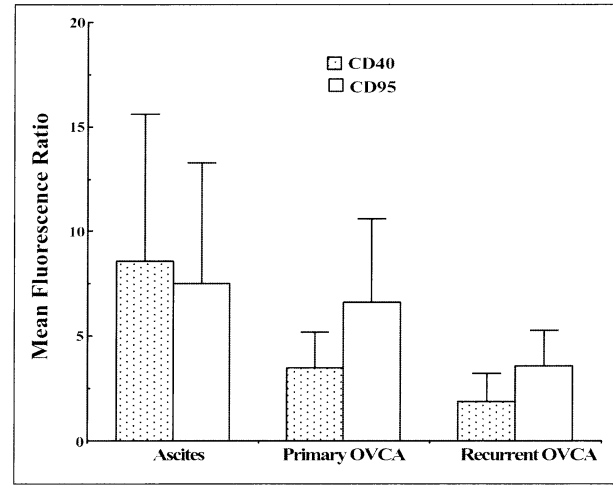

Figure 2. — Mean fluorescence ratio of CD95 and CD40 expression on ascites (n = 4), primary (n = 9) and recurrent (n = 6) OVCA.

Effect of CD40L and anti-fas antibodies on OVCA cell lines

CD40L has been previously shown to influence the signaling cascade induced by Fas ligation. Here we studied the effect of the two members of the TNF-receptor family on OVCA cell lines. Treatment of Hey and OVCA433 with anti-CD95 mAb, CH11 induced apoptosis as measured by Annexin uptake (Figure 4). Next, the effect of CD40 signaling on Fas mediated apoptosis was measured. The CD40 signal was delivered either by trimeric CD40L (Pharmingen, San Diego, CA), at a concentration of 1 μg/ml, or by murine anti-CD40 (Ancell Corp., N. Bayport, MN), at a concentration of 2 μg/ml. This was

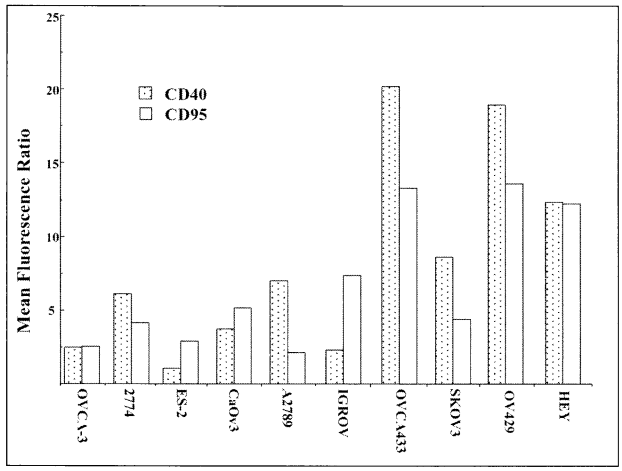

Figure 3. — Mean fluorescence ratio of CD95 and CD40 expression on ten OVCA cell lines.

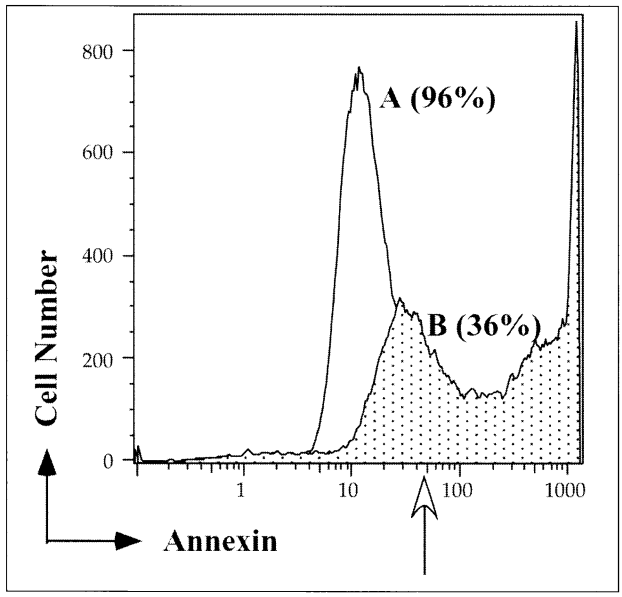

Figure 4. — Apoptosis measured as annexin uptake by flow cytometry. OVCA cell line Hey, was incubated overnight with or without the presence of anti-CD95 Ab, CH11. Annexin uptake by cells treated with CH11 (B) and by control cells (A) is shown as histograms. The arrow shows the electronic gate used to calculate annexin+ve cells. The percentage of viable cells (annexin-ve) is shown in parenthesis. Annexin binds to negatively charged phospholipid surfaces with a higher specificity for phosphatidylserine (PS). Since PS exposure is an early event in apoptosis, annexin-conjugated to FITC serves a sensitive marker for identification of apoptotic cells.

followed by anti-mouse IgG (Caltag Laboratories, Burlingame, CA), at a concentration of 5 μg/ml, to cross-link the primary antibody. In five independent experiments, Fas-mediated apoptosis of Hey and OVCA433 was not altered regardless of the source of CD40 signal (Figure 5).

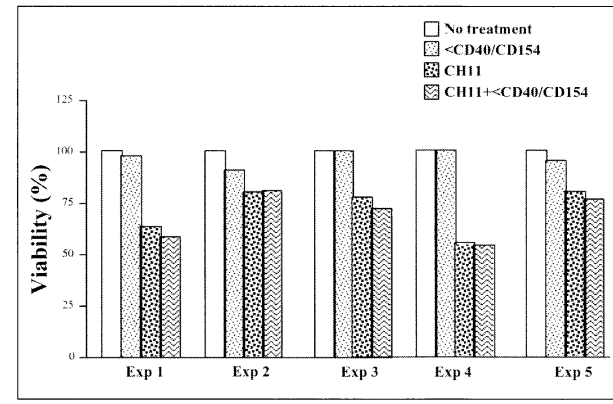

Figure 5. — Ligation of CD40 did not modulate CD95 mediated apoptosis. Exponentially growing cells lines, Hey (Exp 1, 2, 4) and OVCA433 (Exp 3, 5) were exposed to CH11 as described in materials and methods. Apoptotic cells were measured by annexin uptake. Annexin-negative cells were considered viable and were plotted as a percentage of viable cells compared to the cells with no treatment (medium alone). Simultaneous cultures were setup for co-exposure with CH11 and either trimeric CD40L (Exp 1, 3, 5) or murine anti-CD40 and anti-mouse IgG (Exp 2, 3).

Discussion

To develop strategies for tumor eradication in-depth understanding of the molecular pathways, choosing between proliferation, quiescence, or death, is necessary. The pivotal role of proteins belonging to the TNF-receptor, TNF and caspase families has provided such an entry into the intricate web of signaling that maintains tissue homeostasis. Added to that is the complex mechanism of immune privilege and tumor escape from surveillance [55]. Thus, signaling between proteins sometimes leads to seemingly opposing consequences, depending on the cellular and environmental status [56]. Moreover, where multiple receptor-ligand interactions are involved the hierarchy of signaling events could influence the ultimate fate of the cell.

We have looked at the expression and functioning of two such receptors in ovarian carcinoma. CD95 and CD40 expression was found on all the tumor specimens and cell lines analyzed. Among tissue specimens, the greatest CD95 and CD40 expression was found in cells from ascites. The difference in expression between ascites and solid tumor reached statistical significance for both CD95 and CD40. CD95 and CD40 were also differentially expressed in primary versus recurrent carcinoma. Expression of both was greater in primary carcinoma and reached near significance. We speculate that tumor cells, which lose CD95 and CD40 expression, may become more resistant to apoptosis and thus gain a survival advantage. These cells would then establish the lineage for macroscopically detectable recurrent tumor. One would expect this new tumor would be more resistant to treatment and more difficult to cure. This correlates with clinical data in that recurrent ovarian carcinoma is much less likely to respond to chemotherapy than primary

disease, and is generally considered incurable. The high expression of both CD40 and CD95 in ascites cells may be linked to the increased loss of differentiation, required for an epithelial cell to survive in suspension.

At the functional end, our treatment of Hey and Ovcar433 with anti-CD95 Ab, CH11 resulted in apoptosis as detected by annexin uptake and flow cytometry. This finding is consistent with previous reports and confirmed that our system for detecting apoptosis was reliable. Treatment of the same cell lines with trimeric CD40 ligand or the murine anti-CD40/anti-mouse IgG complex, in the absence of CD95, did not yield any detectable cell death. Co-incubation with of CD40L and CD95L did not increase apoptosis to levels above that induced by CH11 alone. This would seem to indicate that CD40 alone does not induce apoptosis in these cell lines. Further, CD40 binding does not augment or diminish apoptosis due to CD95 binding. This makes ovarian carcinoma different from the other epithelial cell lines where CD40 has been shown to modulate apoptosis [39, 41, 42].

More recently, Gallagher et al. have shown growth inhibition of OVCA cell lines, SKOV-3, A2780, OAW-28, MG-75 and MG-79, following treatment with trimeric CD40L [40]. This discrepancy could be due to the difference in the incubation period (72 hr) in contrast to 24-hr incubation with the CD40L in this study. Alternatively, as the authors suggest adaptation to long-term culture may select cells resistant to CD40 mediated apoptosis. Not surprisingly, an apposite observation regarding CD95 ligation shows resistance of primary OVCA cultures but not cells lines to CD95 mediated apoptosis despite CD95 expression in 96% of tissue specimens [57]. Thus, immortalized cell lines do not accurately reflect the complexity of the apoptotic mechanisms present in ovarian carcinoma.

To accurately characterize the CD40/CD95 interaction, subsequent studies should be conducted using primary culture. Further we have shown that in ovarian cancer these receptors are expressed differently in ascites when compared to solid tumor, and expression likely differs between primary and recurrent disease. As this may have functional consequences, we advocate that investigators take origin of their specimens into account. Additional in-depth studies to define the mechanisms by which tumor proliferation occurs may help to predict which tumors will be responsive to the effects of CD40L and may lead to the understanding of the role of CD40 in the pathogenesis of the these tumors. Dissection of the cell-signaling pathways mediating the effects of CD40 ligation in ovarian carcinoma cells will be relevant to both the understanding of the role of these pathways in the carcinogenic process and to possible development of more effective treatments.

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