Evaluation of Cell Surface Expression of Phosphatidylserine in Ovarian Carcinoma Effusions Using the Annexin-V/7-AAD Assay

Clinical Relevance and Comparison With Other Apoptosis Parameters

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Abstract

Phosphatidylserine cell surface exposure during apoptosis can be detected by its binding to the protein annexin-V. We investigated annexin-V expression in 76 ovarian carcinoma effusions using flow cytometry. Results were analyzed for association with clinicopathologic parameters and survival. Annexin-V expression was additionally compared with the previously studied apoptotic markers cleaved caspase-3, cleaved caspase-8, and deoxyuridine triphosphate (dUTP) incorporation into DNA fragments. Annexin-V was expressed in all specimens and was more frequently detected compared with cleaved caspases and dUTP incorporation (P < .001). Annexin-V expression was higher in grade 3 vs grades 1 and 2 tumors (P = .014). A higher percentage of annexin-V–expressing cells in postchemotherapy specimens was associated with poor overall (P = .005) and progression-free (P = .013) survival. We present the first evidence of annexin-V expression in ovarian carcinoma effusions. The higher annexin-V expression compared with other apoptosis parameters and its association with high-grade disease and poor survival in postchemotherapy patients suggest a role in cell survival rather than apoptosis in effusions.

Apoptosis is a well-regulated process of cell death and has an important role in the development and maintenance of cellular homeostasis. This process is characterized by specific morphologic changes, including cell shrinkage, chromatin condensation, and nuclear fragmentation. Finally, engulfment of apoptotic cells by macrophages, dendritic cells, or neighboring cells prevents an inflammatory response in the surrounding tissue. Apoptosis is tightly balanced and regulated in a physiologic context, and failure of this mechanism may result in pathologic conditions such as developmental defects, autoimmune diseases, neurodegeneration, and cancer.1-4

Different flow cytometric (FCM) techniques have been developed for characterization and quantification of the various cellular events during apoptosis. FCM techniques have been used to study apoptotic events and their temporal course in different cell types in response to various triggers.5-14 The studied parameters include changes in distribution of phospholipids across the cell membrane,5-9 morphologic changes and chromatin condensation,10 increased membrane permeability,11 dissipation of the mitochondrial transmembrane potential,9,12 activation of caspases,12,13 and DNA fragmentation.13,14

Membrane phospholipids are asymmetrically distributed between the 2 leaflets of the plasma membrane. Phosphatidylserine (PS) is an anionic phospholipid normally localized at the cytoplasmic side of the plasma membrane. The preservation of PS in the cytosolic leaflet of cells has an important role in cell physiology because it facilitates binding...
of proteins at the inner membrane surface, serves as a cofactor for several membrane-bound enzymes (eg, protein kinase C), and promotes membrane fusion during exocytosis and similar processes. However, exposure of PS in the outer leaflet of the plasma membrane has been detected in erythrocytes and activated platelets, as well as in undifferentiated tumor cells. The externalization of PS in activated platelets serves as a procoagulant surface, whereas in erythrocytes and undifferentiated tumor cells, PS exposure mediates cell recognition and phagocytosis by macrophages and other cells. Several reports have shown increased exposure of PS on the outer leaflet of the plasma membrane of different cell types undergoing apoptosis, including lymphocytes, thymocytes, and tumor cell lines of lymphoid and of neural origin.

Annexin-V, a 35-kDa Ca²⁺-binding protein, was first described by Reutelingsperger et al as a vasculature-derived protein with strong anticoagulant properties. Annexin-V binds with high affinity to PS, and commercially available annexin-V conjugated to fluorochromes is used in an apoptotic detection assay by FCM analysis. The affinity and specificity of this binding have been previously described. By using conjugated annexin-V in combination with a membrane-impermeable DNA dye such as propidium iodide or 7-aminoactinomycin D (7-AAD), one can discriminate among viable, apoptotic, and secondary necrotic cells. To date, the annexin-V assay has been mostly used to measure apoptosis in WBCs and cell lines. Its application to malignant effusions has not been studied to date. In the present report, we describe an annexin-V–based assay for the quantitative measurement of PS exposure on ovarian carcinoma (OC) cells in effusions using 4-color FCM analysis. Results were compared with our recently published data regarding apoptosis measurement by cleaved caspase levels and the degree of DNA fragmentation in the same material. Finally, the clinical significance of annexin-V expression was studied.

Materials and Methods

Patients and Material

The 76 studied effusions were submitted to the Division of Pathology, Norwegian Radium Hospital, Rikshospitalet Medical Center, Oslo, for routine diagnostic purposes in the 1999-2004 period. Fresh, nonfixed malignant peritoneal (n = 63) and pleural (n = 13) effusions were obtained from 56 patients diagnosed with OC, predominantly of the serous type (68 effusions), 4 patients with primary peritoneal serous carcinoma (4 effusions), and 3 patients with tubal serous carcinoma (4 effusions; total, 63 patients). Owing to their closely linked histogenesis and phenotype, henceforth, these tumors are all referred to as OC. Effusions were processed immediately after tapping with centrifugation for 10 minutes at 2,000 rpm. The resulting pellet was used for routine cytologic diagnosis and evaluation of specimen adequacy. The remaining material was divided for freezing at −70°C in RPMI 1640 medium with 50% fetal calf serum and 20% dimethyl sulfoxide at a ratio of 1:1 and for cell block preparation using the thrombin clot method. Diagnoses were established by using morphology and immunohistochemical analysis.

Clinicopathologic data are detailed in Table I. Staging and grading was according to the International Federation of Gynecology and Obstetrics (FIGO) classification. The majority of patients (n = 55) received platinum-based therapy. The Regional Committee for Medical Research Ethics in Norway approved the study.

FCM Immunophenotyping

Four-color FCM was undertaken using the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15-mW argon-ion laser (488 nm) and a 12-mW red diode laser (635 nm), as previously described. Briefly, effusions were stained using the following reagents and antibodies: annexin-V binding buffer and allophycocyanin (APC)-conjugated annexin-V ready-to-use solution (BD Biosciences). The 76 studied effusions were submitted to the Division of Gynecology and Obstetrics (FIGO) classification. The Regional Committee for Medical Research Ethics in Norway approved the study.

| Parameter               | No. of Patients |
|-------------------------|-----------------|
| Mean (range) age (y)    | 62 (41-85)      |
| FIGO stage              |                 |
| I                       | 1               |
| II                      | 1               |
| III                     | 36              |
| IV                      | 25              |
| Grade                   |                 |
| I                       | 7               |
| II                      | 12              |
| III                     | 36              |
| NA                      | 8               |
| Residual disease (cm)   |                 |
| ≤1                      | 17              |
| >1                      | 26              |
| NA                      | 20              |
| Chemoresponse at Diagnosis |             |
| Complete                | 32              |
| Incomplete              | 23              |
| Not determined          | 8               |
| at First relapse        |                 |
| Complete                | 9               |
| Incomplete              | 38              |
| Not determined          | 16              |

FIGO, International Federation of Gynecology and Obstetrics; NA, not available.

* Includes 8 patients with resistant disease and 2 patients who died before chemotherapy.
† Includes 12 patients with resistant disease and 8 patients with no record.
‡ Partial response, stable disease, progression, allergic or adverse reaction.
§ Including patients who received no chemotherapy and patients who died before chemoresponse could be assessed.
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Pharmacology, San Diego, CA); 7-AAD staining solution (BD Biosciences Pharmingen); peridinin chlorophyll protein (PerCP)-conjugated anti-CD45, clone 2D1 (BD Biosciences Pharmingen); fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin-conjugated anti-IgG1, clone DAK-G01 (DAKO, Glostrup, Denmark); and FITC-conjugated anti-Ber-EP4, clone Ber-EP4 (DAKO). Antibodies were applied and specimens incubated in the dark at room temperature for 25 minutes. Following washing with phosphate-buffered saline, cells were resuspended in 200 μL of 1× annexin-V binding buffer (except for negative control tubes). Next, 5 μL of 7-AAD and 5 μL of annexin-V–APC were added to the tubes and incubated for 10 minutes at 4°C in the dark. Cells were washed with 500 μL of 1× annexin-V binding buffer and then resuspended in 200 μL of 1× annexin-V binding buffer followed by filtration through a 70-μm nylon filter (BD Biosciences Pharmingen). The samples were placed on ice and analyzed.

Control of instrument performance and time delay calibration were performed using FACSComp software, version 4.1; Calibrite 3 beads; and Calibrite APC beads (all from BD Biosciences Pharmingen), as previously described. The T47-D breast carcinoma cell line was used in each run as the positive control sample. Negative control samples for annexin-V consisted of cells incubated without using the annexin-V binding buffer and tubes run with the isotype control antibody.

Evaluation of FCM Immunophenotyping

Evaluation and scoring of FCM immunophenotyping was undertaken using FlowJo analysis software (version 8.8.4, Tree Star, Ashland, OR). A gating procedure was generated by combining side angle light scatter channel (SSC) vs 7-AAD/CD45 PerCP fluorescence (7-AAD was detected in the FL3 channel), and a region was drawn around clear-cut populations having negative 7-AAD/CD45 PerCP fluorescence. Cells in this region were again viewed by generating a cytogram of SSC vs forward scatter light and gated to exclude cell debris by including only cells with relatively high SSC and forward scatter light values. Quadrant cursors were set by using isotypic negative controls. Quadrants were set so that in negative controls, 99% of the cells were localized in the left lower quadrant. The percentage of carcinoma cells expressing annexin-V was scored. Expression in fewer than 1% of cells was scored as negative. Staining intensity was not scored.

Statistical Analysis

Statistical analysis was performed by using the SPSS-PC package (version 15, SPSS, Chicago, IL). Probability of less than .05 was considered significant. Survival data were available for all 63 patients. Analyses of the association between annexin-V expression and clinicopathologic parameters (effusions site, age, histologic grade, FIGO stage, previous chemotherapy, response to chemotherapy at diagnosis and first disease recurrence) were undertaken using the Mann-Whitney U test. For these analyses and for survival analyses, clinicopathologic parameters were grouped as follows: age, 60 or younger vs older than 60 years; grade, 1 and 2 vs 3; FIGO stage, III vs IV; and response to chemotherapy for primary disease and for disease recurrence, complete vs partial response/stable disease/progression/allergic or adverse reaction. Analysis of differences in the expression level of annexin-V vs the previously studied cleaved caspase-3, cleaved caspase-8, and deoxyuridine triphosphate (dUTP) incorporation was performed using the paired t test.

Univariate survival analyses of overall survival (OS) and progression-free survival (PFS) for 61 patients with FIGO stage III or IV disease were executed using the Kaplan-Meier method and log-rank test. For these analyses, annexin-V expression was grouped as low vs high based on median values. In the survival analysis, only expression levels for the first obtained effusion were included.

Results

OC Cells in Effusions Commonly Express Annexin-V

The presence of carcinoma cells was confirmed in all effusions by using the Ber-EP4 antibody. Annexin-V expression was observed in tumor cells in all 76 specimens, with an expression range of 1% to 65% (median, 18.5%) Image 11.

OC Cells in Effusion More Frequently Express Annexin-V Compared With Cleaved Caspase-3, Cleaved Caspase-8, and dUTP Incorporation

We recently reported on low levels of apoptosis in OC cells in effusions based on the percentage of cells showing caspase cleavage and dUTP incorporation, representing caspase activation and DNA fragmentation, in the same specimens analyzed in the present study. To evaluate whether annexin-V expression is in concordance with this observation, we compared its expression extent, ie, the percentage of annexin-V+ cells, with that of the previously studied parameters. Annexin-V expression was significantly higher compared with cleaved caspase-3, cleaved caspase-8, and dUTP incorporation (P < .001; paired sample t test), with median expression levels at 18.5%, 6.5%, 3.5%, and 8.5%, respectively.

Annexin-V Expression Is Associated With Poor Differentiation

Analysis of the association between annexin-V expression and clinicopathologic parameters showed higher annexin-V expression in histologic grade 3 compared with grade
were specifically analyzed with respect to previous exposure (a trend for higher expression in postchemotherapy effusions and disease recurrence postchemotherapy effusions showed (and FIGO stage, patient age, or the extent of residual disease owing to the small number of grade 1 tumors. Analysis of annexin-V expression in 2 different effusion specimens. Annexin-V expression in 2 different effusion specimens from patients diagnosed with ovarian carcinoma. A and C, A region was drawn around clear-cut populations showing CD45/7-AAD negativity. Cells in these regions were again viewed by generating cytograms displaying annexin-V vs Ber-EP4. Ber-EP4 expression was observed in all viable carcinoma cells, whereas the degree of annexin-V expression in carcinoma cells varied among different specimens (B and D). APC, allophycocyanin; FITC, fluorescein isothiocyanate; H, height; PerCP, peridinin chlorophyll protein; SSC, side angle light scatter channel.

1 and 2 tumors (P = .014). A 3-tier Kruskal-Wallis H test in which specimens from each grade group were separately analyzed still showed significantly higher annexin-V expression, though with a weaker association (P = .044), presumably owing to the small number of grade 1 tumors. No association was found between annexin-V expression and FIGO stage, patient age, or the extent of residual disease (P > .05; data not shown).

Comparative analysis of annexin-V expression in effusions obtained at diagnosis before administration of chemotherapy and disease recurrence postchemotherapy effusions showed a trend for higher expression in postchemotherapy effusions (P = .063). The same trend was observed when specimens were specifically analyzed with respect to previous exposure to platinum compounds or paclitaxel (P = .063 for both). No association was observed between annexin-V expression and response to chemotherapy at diagnosis or first recurrence (P > .05; data not shown).

Annexin-V Expression in OC Cells in Postchemotherapy Effusions Is Associated With Poor Survival

In univariate survival analysis of the entire cohort, patients with effusions showing a higher than median annexin-V expression level (n = 30) had a mean OS of 22 months compared with 30 months for patients with low annexin-V expression (n = 31; P = .109). Analysis of PFS for 60 patients (1 patient with no data regarding PFS) similarly showed a trend for poor PFS for patients with effusions with high annexin-V expression compared with patients with low-expressing specimens (mean PFS, 4 vs 8 months, respectively; P = .064).

In separate analyses of the data for patients with primary diagnosis (prechemotherapy) and disease recurrence (postchemotherapy) effusion specimens, no association between annexin-V expression and OS and PFS was found for the former category. However, in postchemotherapy specimens, higher annexin-V expression significantly correlated with poor OS and PFS (P = .005 and P = .013, respectively).

Discussion

Apoptosis is accompanied by a variety of characteristic cellular changes\textsuperscript{1-4} that can be identified and quantified by different FCM assays.\textsuperscript{5-14} A widely used assay detects PS exposure on the cell surface, which is a generally accepted feature of early apoptosis,\textsuperscript{5,8,19,20} by its binding to fluorochrome-conjugated annexin-V.

Application of this assay to our material documented the expression of annexin-V in all effusion specimens, with the percentage of OC cells expressing this marker varying among specimens. Carcinoma cells were detected and differentiated from other cell types present in effusions by using a combination of markers, including Ber-EP4 and CD45, as previously reported.\textsuperscript{31} The addition of the DNA intercalator dye 7-AAD,\textsuperscript{11} which penetrates and stains cells that lost the integrity of the plasma membrane, allowed us to exclude dead cells from the analysis.

We found a significantly higher percentage of OC cells expressing annexin-V compared with the percentage expressing the apoptotic markers cleaved caspase-3 and cleaved caspase-8 and exhibiting DNA fragmentation represented by dUTP incorporation, on which we recently reported.\textsuperscript{28} This finding is consistent with the study results by Pepper et al,\textsuperscript{22} showing a higher estimation of apoptotic cell percentage by the annexin-V assay compared with...
assays detecting DNA fragmentation or exposure of the mitochondrial membrane protein 7A6 antigen on the cell surface in cultured B-cell chronic lymphocytic leukemia cells treated with chlorambucil.

Increased PS exposure can be the result of membrane damage inflicted on cells by specimen handling, previously demonstrated to occur during various adherent cell-harvesting procedures. Such induction of membrane damage is unlikely to occur in our material because effusions are suspensions of single cells that require little handling in the annexin-V-labeling protocol, yet this issue remains to be determined and is currently under investigation in our laboratory. Alternatively, the higher expression of surface PS compared with the aforementioned apoptotic markers may be an indication that PS externalization is an early apoptotic event preceding the occurrence of other characteristic changes.

The most plausible explanation in our opinion for this difference, though, is dissociation of PS surface expression from apoptosis. Several lines of evidence from the literature support this notion. First, it has been demonstrated that PS exposure on the cell surface can occur before commitment to apoptotic death in some cell systems and may be reversible on withdrawal of the apoptotic stimulus. Second, although activation of caspases, as well as of other proteases, has been implicated in PS externalization in several studies of apoptotic cell lines, translocation of PS to the cell surface has also been shown to occur independently of the apoptotic process. Fadeel et al demonstrated that PS exposure was not an essential component of apoptosis, but a cell type–specific event. Moreover, alkylation of free thiol groups that are necessary for the activity of flippase, translocating PS from the outer to the inner leaflet of the membrane against its concentration gradient, resulted in PS cell surface exposure in the absence of other markers of apoptosis. Similarly, Balasubramanian et al reported that PS externalization following treatment of cells with sulfhydryl-modifying agents can occur through a mechanism that is distinct from the one leading to the typical events of apoptosis, including cytochrome c release, caspase activation, and DNA fragmentation, and that requires a sustained elevation of cytosolic Ca²⁺ levels.

Third, PS expression on the cell surface has been shown to have nonapoptotic functions in viable lymphocytes, facilitating B-cell selection during maturation and modulating the activities of membrane proteins in T lymphocytes. Finally, externalized PS on malignant cells may essentially be used for recruiting inflammatory phagocytes and cytokines and promoting tumor growth and progression, rather than tumor cell removal by the immune system. Although the question of whether inflammatory infiltrate promotes or hinders tumor growth is not resolved, there is increasing consensus that the former effect prevails. We have previously reported that monocytes/macrophages constitute a large cell population in effusions and that natural killer– and B-cell infiltration correlates with worse outcome in OC metastatic to effusions, suggesting contribution of

**Figure 1** High annexin-V expression in ovarian carcinoma cells in postchemotherapy effusions correlates with poor survival. **A**, Kaplan-Meier survival curve showing the association between annexin-V expression in viable carcinoma cells and overall survival (OS) for 27 patients with ovarian carcinoma effusions. Patients with effusions showing high annexin-V expression (above median; n = 16; dashed line) had a mean OS of 21 months vs 39 months for patients whose effusions showed low annexin-V expression (n = 11; solid line; P = .005). **B**, Kaplan-Meier survival curve showing the association between annexin-V expression in viable carcinoma cells and progression-free survival (PFS) for 26 patients (1 patient with no data regarding PFS). Patients with effusions with higher annexin-V expression (n = 16; dashed line) had a mean PFS of 5 months vs 10 months for patients whose effusions showed low annexin-V expression (n = 10; solid line; P = .013).
immune system components to OC progression. It is possible that surface exposure of PS on OC cells facilitates this deleterious function. Our findings regarding the clinical relevance of annexin-V expression, which has not been analyzed to date in OC effusions, are consistent with this theory. High annexin-V expression was associated in the present study with parameters of aggressive clinical behavior, including histologic grade 3 disease and poor OS and PFS in postchemotherapy effusions. This argues against a technical factor responsible for inducing PS surface exposure in our specimens. In further support of the involvement of cell surface PS in nonapoptotic functions is our recent observation that OC cells in effusions undergo little apoptosis, based on the low rates of caspase cleavage and DNA fragmentation observed in the same specimens. Of note, as opposed to the negative prognostic significance of high annexin-V expression, high expression of the apoptotic marker cleaved caspase-3 was associated with improved patient survival in this cohort, suggesting that externalized PS may promote tumor cell survival. Although preliminary, this hypothesis may be supported by the observation that enrichment of Neuro-2 mouse neuroblastoma cells with docosahexaenoic acid (22:6n-3), leading to an increase in PS content, mediates cell survival rather than cell death in vitro. Additional studies of clinical specimens are necessary to resolve this issue.

The present study is the first to document annexin-V expression in OC cells in effusions. Annexin-V expression is higher than that of apoptosis parameters, such as caspase cleavage and dUTP incorporation, suggesting that PS surface exposure may be involved in cellular processes other than apoptosis, although this hypothesis requires further research. Higher expression of annexin-V in postchemotherapy effusions is associated with more aggressive disease, reflected in shorter patient survival.

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