Malignant Nonhematological Effusion Characterization by Flow Cytometry

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Abstract
With the exception of hematological malignancies, flow cytometry (FC) is infrequently applied as an ancillary tool in the diagnosis of malignant effusions in most institutions. However, FC may be effectively used to differentiate between epithelial cells, mesothelial cells and leukocytes using antibodies against both cell surface and intracellular proteins, offering the advantage of quantitative analysis. Additionally, FC may be applied to the quantitative detection of cancer-associated molecules, including stem cell markers, as well as assessment of critical cellular processes, such as proliferation and apoptosis. Some of the latter tests may have relevance for monitoring treatment response in the presence of metastatic disease, although this does not constitute routine practice to date. This review summarizes current knowledge regarding the application of FC to serous effusions in the diagnostic setting, as well as in research into cancer biology focusing on clinical specimens. The studies published to date suggest a role for this method in the clinical setting in the context of diagnosis, prediction and prognosis.

Introduction

The serosal cavities are frequently affected by malignancy, most commonly in the form of metastatic disease originating from the lung, breast, female genital tract or gastrointestinal tract. Malignant mesothelioma (MM), the native tumor of this anatomic site, is an important differential diagnosis. Differentiating carcinoma cells from inflammatory cells (particularly macrophages) and reactive or malignant mesothelial cells based on morphology alone can be difficult. Immunohistochemistry (IHC) has in recent years become the most widely used ancillary method in effusion diagnosis and allows for the correct classification of the majority of tumors [1]. The use of cell blocks enables the application of protocols identical to the ones used in surgical pathology.

Flow cytometry (FC) is widely applied in the diagnosis of hematological disorders, but has been infrequently applied in the characterization of epithelial and mesothelial cells in the diagnostic setting. FC has the advantage of being a quantitative method that may be rapidly applied to viable cells in fresh specimens, thereby avoiding fixation artifacts. FC reliably detects cytoplasmic, nuclear and surface antigens, and multicolor FC allows for the assessment of multiple antigens simultaneously. Recent years have seen an expansion in the use of FC for research pur-
poses, as it provides an accurate assessment of multiple biological processes. As effusions constitute metastatic disease that requires treatment by chemotherapy and/or targeted therapy, FC is of evident potential as an objective tool to assess predictive and prognostic endpoints.

The present review presents data from studies performed in the last 20 years in which FC has been applied alone or with other methods in both the diagnostic and research settings, the latter with the potential to advance treatment. Studies of hematological cancers are not discussed in this overview.

FC in Effusion Diagnosis

Earlier studies investigated the role of DNA ploidy by FC in the diagnosis of malignancy on effusions. These studies generally reported good sensitivity and specificity of this assay in differentiating benign from malignant effusions [2–7]. Some authors reported that image cytometry performed better than FC in this context [2], while others had the opposite experience [4]. Irrespective of this, the role of this method in effusion cytology has been limited in recent years.

The author’s group applied FC as a diagnostic tool in several studies. In two of these, a panel consisting of Ber-EP4, N-Cadherin, CD45 and CD14 identified carcinoma cells, mesothelial cells, leukocytes and macrophages, respectively [8, 9]. A panel including Ber-EP4, CD45 and CD14 was used by Pillai et al. [10] in a study of 83 effusions, with sensitivity and specificity at 88.15 and 97.64% for detecting malignancy, respectively, compared to 73.68 and 100% for morphology.

A third study by the author included reactive and malignant effusions, the latter including both carcinomas and MM specimens, using the 4 abovementioned markers and an antiepithelial membrane antigen antibody, detecting the same protein as MUC1 antibodies, as well as 3 nonconjugated antibodies against carbohydrate/glyco-protein moieties on the cell surface of tumor cells B72.3, the AH6 antibody against the Lewis y antigen, and the HB-Tn antibody against the Tn carbohydrate antigen. Carcinoma cells expressed 3 of the 4 epithelial markers (Ber-EP4, B72.3, AH6 and HB-Tn) in 77% of cases compared to 7% of reactive specimens, and cells positive for all 4 markers were found in 39% of carcinoma effusions versus none in reactive effusions [11].

Hartman et al. [12] applied the 6E6 antibody against the MUC1/Y protein to ovarian and breast carcinoma effusions and reported a high sensitivity in detecting tumor cells. Several other proteins have been assessed for their diagnostic role by FC in effusions. Kentrou et al. [13] analyzed 125 effusions suspected of being malignant using a two-phase analysis applying a large panel. Specimens containing cells negative for CD45 and positive for CD71 (transferring receptor), suspected of being malignant, were analyzed for the expression of Ber-EP4, antiepithelial membrane antigen, desmin (expressed in reactive mesothelial cells), the myeloid markers CD66 and CD56 (NCAM), as well as ploidy. FC and conventional cytology had a sensitivity of 85.1 and 93.2%, and specificity of 97.8 and 95.6%, respectively.

In another study, a panel including CD45 and CD326, the latter as a tumor marker, was applied to 506 ascites and peritoneal washing specimens from 333 patients, and a high tumor-to-leukocyte ratio using these markers was significantly associated with poor survival [14]. The detection of EpCAM in microvesicles by FC was recently reported to be useful in diagnosing malignant pleural effusions in the analysis of 71 malignant and 14 benign specimens, and its addition to morphology was superior to morphology alone [15].

Folate receptor-α was shown to be a sensitive marker for ovarian carcinoma in a study combining an antibody against this protein with Ber-EP4 [16]. The FOLR1 gene, encoding for this protein, was found to be overexpressed in ovarian serous carcinoma compared to peritoneal MM in a gene expression array analysis [17], and folate receptor-α expression by FC was subsequently found to be useful in differentiating ovarian and breast carcinoma from MM in an analysis of 91 effusions [18]. PINCH-2, a protein with a cellular role in adhesion and signaling, was not differentially expressed in these 3 tumor types [19], despite overexpression of the gene in peritoneal MM in the abovementioned gene expression array analysis.

FC in Effusion Research

In recent years, FC has been applied to studies related to different aspects of tumor biology, including to analyses of the host response to cancer. These studies are discussed below.

The Immune Response

The host immune response to cancer is often ineffective or even tumor promoting and the presence of leukocytes does not necessarily imply an improved outcome. Characterization of leukocyte subclasses that modulate...
the immune response to cancer has been extensively studied in recent years in practically every cancer type. FC is highly informative in this respect as it provides a quantitative analysis of these cell populations.

The diagnostic value of specific leukocyte populations in effusions was assessed in 2 studies. Cornfield and Gheith [20] compared natural killer (NK) and T-cell parameters in 30 benign and 30 malignant effusions. CD16+/CD56+ NK cell counts were higher in malignant effusions, but the difference was only weakly significant (p = 0.04). T cells carrying α/β T-cell receptor rearrangement were far more numerous than those expressing T-cell receptor γ/δ in both specimens. In contrast, Wang et al. [21] found significantly higher numbers of CD14+/CD163+ tumor-infiltrating macrophages, considered to be tumor promoting, in malignant compared to benign effusions, with a cut-off at 3.65% of the macrophage population associated with sensitivity and specificity at 81.2 and 100%, respectively.

In another comparative study, effector memory CD8+ T cells were found to be present in significantly higher levels in blood and pleural fluid from healthy controls compared to patients with malignant pleural effusion, pleural metastases or benign asbestos-related lesions [22]. Breast carcinoma specimens, including malignant pleural effusions, were shown to contain nonconventional double-positive T cells expressing both CD4 and CD8 that produced IL-5 and IL-13 [23].

Leukocyte counts in effusions were shown to be associated with patient survival. Cytokine array analysis showed that ascites specimens in ovarian carcinoma have a suppressive immune environment. A low CD4/CD8 ratio, observed in specimens from 13 patients, was associated with longer survival [24]. In an analysis of 73 ovarian carcinoma effusions for the expression of leukocyte markers (CD3, CD4, CD8, CD4/CD8 ratio, CD16, CD19, and CD14), the presence of CD16-positive NK cells and more advanced (FIGO stage IV) disease was associated with a shorter overall survival (OS). Additionally, a higher percentage of CD19-positive B cells and stage IV disease were markers of poor survival for patients with post-chemotherapy effusions [25].

The expression and clinical role of regulatory T cells (Tregs) have been investigated in several studies. The fraction of CD4+/CD25high/Foxp3+ regulatory T lymphocytes, which suppress the immune response, among CD4+ cells was increased in cultured cells obtained from ovarian carcinoma cells at diagnosis and at disease recurrence following expansion by interleukin-2 (IL-2), and was more pronounced in the latter group [26].

Tregs (CD4+/CD25+/CD127low/) were found in ascites from gastric cancer patients, as well as at other tumor sites, and higher Foxp3 expression in these cells was associated with a more advanced TNM stage [27]. In another study [28], lymphocytes from lung cancer patients with malignant pleural effusions contained a higher number of CD4+/CD25+ cells strongly expressing Foxp3 and CTLA4 compared to pleural lavage specimens from patients with lung cancer but no effusion.

The chemokine CCL22 was overexpressed in malignant pleural effusion specimens from lung cancer patients compared to corresponding serum, and was shown to promote the influx of CD4+/CD25+ Tregs into the pleural cavity [29]. A higher ratio of Tregs/T helper IL-17-producing cells (Th17) by FC was found in malignant (n = 26) compared to parapneumonic (n = 12) pleural effusions and a higher ratio was associated with significantly shorter survival [30]. Conversely, another group reported that Th17 cells are increased whereas Tregs are decreased in effusions from patients with cancer and tuberculosis compared to benign specimens from patients with other conditions [31].

Chemokine receptor expression was analyzed in several studies using FC. Milliken et al. [32] found the frequent expression of CCR1, CCR2 and CCR5 on macrophages in ascites specimens from ovarian carcinoma patients. CCR1 was expressed by >60% of all T cells, whereas CCR2 and CCR5 expression was more frequent on CD4+ than CD8+ T cells.

Analysis of the expression of 5 chemokine receptors (CXCR1, CXCR4, CCR2, CCR5 and CCR7) by FC in ovarian carcinoma, breast carcinoma and MM effusions using FC showed the frequent expression of these proteins on leukocytes, while all receptors were rarely expressed on cancer cells. Chemokine receptor expression on lymphocytes and monocytes from ovarian carcinoma effusions had no prognostic role [25, 33, 34].

Analysis of 44 effusions constituting 27 malignant specimens and 17 specimens from patients with cirrhosis showed that malignant specimens were enriched for T cells with a ‘naïve phenotype’ (CD62L+ and CD45RA+CCR7+), ‘central memory’ (CD45RA-CCR7+) and type 2-polarized (CCR4+) cells, while being deficient in ‘effector’-type (CD45RA-CCR7+ or CD45RA+CCR7+) and presumably type 1-polarized T cells (CCR5+), as well as potentially cytotoxic CD56+ NK cells [35]. Macrophages isolated from malignant and reactive effusions were shown to have a different antigenic profile, with CCR7 expression being significantly higher in nonmalignant effusions [36].
**Adhesion Molecules and Other Cell Membrane Proteins**

FC has been applied to several studies of surface molecules involved in cell-cell adhesion or interaction between cells and the extracellular matrix. Integrins, a large family of proteins containing α- and β-subunits, have been the focus of several studies by the author’s group. In the first two studies, frequent expression of the αv, α6 and β1 integrin subunits was observed at the protein and mRNA level in ovarian carcinoma effusions [37, 38]. The absence of α6 integrin subunit protein expression using FC was associated with shorter OS, though not significantly [38]. The third study investigated whether these receptors are differentially expressed in carcinomas of various origins, MM and reactive mesothelial cells [39]. Analysis of 67 effusions (48 carcinomas, 7 MM and 12 RM specimens) for αv, α6, β1 and β3 integrin subunit expression using FC showed frequent expression of the αv, α6, and β1 subunits, with a significantly higher expression of the α6 subunit in MM. Expression of the β3 integrin subunit was not found in any of the specimens in our assay.

Quantitative analysis of two CD44 splice variants in gastric carcinoma showed low expression of the v6 variant, whereas expression of the v9 variant was high in carcinoma cells in ascites specimens [40]. Analysis of CD44 and its variants v4/5 and v6 in ascites specimens from patients with gynecological carcinomas showed variant expression in vesicles constituting membrane fragments [41].

Expression of matrix metalloproteinases (MMP-2, MMP-7, MMP-9, MMP-14/MT1-MMP) and their inhibitors TIMP-2 and TIMP-4, the latter family required for MMP activation, was observed on the cell surface of smooth muscle actin-positive cells, identified as tumor-associated myofibroblasts, in effusions from gastric cancer patients [42].

Analysis of Her2 expression in ovarian carcinoma specimens from multiple anatomic sites, including effusions, as well as short-term cultures, showed that this method was superior to IHC in terms of sensitivity in detecting this protein [43].

MM cells from 9 effusion specimens were shown to be consistently positive for mesothelin. However, upon short-term culturing the expression of this protein was variably lost, as assessed by IHC and FC. Tumors with high expression of this protein were susceptible to the antimesothelin immunotoxin SS1P [44].

**Cancer Stem Cells**

Cancer stem cells (CSC) are increasingly regarded as a chemoresistant cell population that is the cause of failure of antitumor therapy and cancer-related death. FC is ideal for identifying cell populations expressing postulated CSC markers, and has been used in this context in several studies.

Meng et al. [45] identified CSC by the high aldehyde dehydrogenase (ALDH<sub>high</sub>) phenotype by FC and the presence of these cells was associated with shorter progression-free survival (PFS) compared to patients with specimens that had an ALDH<sub>low</sub> profile. A higher expression of ALDH1A1 was observed in ovarian carcinoma effusions compared to solid tumors, which decreased after treatment with niclosamide [46].

The detection of a side population by FC is another widely used method for isolating CSC, as in the study of ovarian carcinoma effusions by Hu et al. [47]. Yao et al. [48] analyzed the expression of 35 cancer-associated markers, including CSC markers, by FC in 12 malignant and 8 benign pleural effusions. EpCAM<sup>+</sup> tumor cells were tumorigenic in mice and had a high CD24 expression.

The cell surface proteoglycan chondroitin sulfate proteoglycan 4 (CSPG4) was detected on breast carcinoma cells from primary carcinomas and malignant pleural effusions, and in the latter specimens was observed on CSC with a CD44<sup>+</sup>/CD24<sup>low/-</sup> profile by FC [49].

**Apoptosis**

FC is a highly valuable method for analyzing apoptosis, since it allows for the simultaneous detection and quantification of several apoptosis parameters, thus ensuring the reliability of the obtained results. The author’s group published a protocol for the measurement of apoptosis on carcinoma cells in effusions. Cells were identified by Ber-EP4 and EpCAM expression and the absence of CD45, and apoptosis was quantified by FC using the intracellular/nuclear markers cleaved caspase-3, cleaved caspase-8 and incorporated dUTP [50].

A subsequent study of 76 ovarian carcinoma effusions analyzed the expression and clinical role of cleaved caspases and dUTP incorporation by FC. Caspase cleavage and dUTP incorporation were found in <10% of cells in the majority of effusions, with no significant difference between pre- and postchemotherapy effusions, suggesting little apoptosis occurs in tumor cells. Cleaved caspase-3 and cleaved caspase-8 levels, as well as the levels of both cleaved caspases and dUTP incorporation, were directly related. Higher than median cleaved caspase-3 levels correlated with a longer OS and PFS [51].

Exposure of phosphatidylserine at the cell surface, detected by annexin-V, is widely used as an assay for detecting apoptosis, but in fact occurs on stressed cells in gen-
eral. Analysis of annexin-V expression in ovarian carcinoma effusions (n = 76) using FC showed a significantly higher percentage of cells expressing this marker compared to the previously studied cleaved caspases and dUTP incorporation. Additionally, a higher percentage of annexin-V-expressing cells in postchemotherapy specimens was significantly associated with poor OS and PFS, suggesting it is a survival rather than apoptosis marker [52]. Annexin-V assay measurement similarly correlated poorly with cleaved caspases and dUTP incorporation in a subsequent study of nonovarian malignant effusions and reactive effusions [53].

Expression of the antiapoptotic protein cellular FLICE-inhibitory protein (c-FLIP) was universally seen in the analysis of 69 ovarian carcinoma effusions using FC. c-FLIP expression was unrelated to clinicopathologic parameters, including chemoresponse and survival, but was significantly inversely related to that of cleaved caspase-3 [54].

Death receptor family members are most often regarded as proapoptotic markers, but mediate both apoptosis and survival in cancer cells. Analysis of 95 ovarian carcinoma effusions and a smaller series of breast carcinoma effusions for DR4, DR5, Fas, and the tumor necrosis factor (TNF) receptors TNFR1 and TNFR2 expression using FC showed DR4, DR5 and Fas expression on tumor cells in the majority of specimens, with less frequent expression of TNFR1 and TNFR2. Unexpectedly, higher DR4, DR5 and Fas expression was significantly related to a poor chemoresponse, and DR4 expression was associated with poor OS and PFS in both univariate and multivariate survival analysis [55].

In agreement with this observation, Koyama et al. [56] found frequent expression of DR4, DR5 and the decoy receptor DcR2 on gastric carcinoma in both the primary tumor and metastatic cells in effusions, with little apoptosis detected in these cells. Another TNFR family member, CD40, was reported to be frequently expressed on ovarian carcinoma cells and its activation by an agonist antibody in vitro resulted in reduced cell growth and increased apoptosis [57].

Other Molecules

Few additional studies have analyzed the expression of various cancer-associated molecules in effusions using FC. Equilibrative and concentrative nucleoside transporters (ENTs and CNTs) mediate the cellular uptake of nucleosides used as anticancer agents. Analysis of ENT1, ENT2, ENT4 and CNT3 expression in a series of 66 ovarian carcinoma effusions showed the frequent expression of all 4 transporters in tumor cells, but no association with previous exposure to chemotherapy and response to chemotherapy at diagnosis, and no significant relationship to survival [58]. Another study by the author’s group documented frequent expression of the phosphatidylinositol-3-kinase pathway proteins AKT, mTOR and DJ-1 in ovarian carcinoma effusions using FC, Western blotting and IHC [59].

Conclusion and Future Directions

FC immunophenotyping of epithelial and mesothelial cells in effusions is a quantitative and highly informative method. This, however, requires knowledge of the cell populations found in these specimens and an understanding of their immunoprofile. Common errors, like equating cytokeratin, EpCAM or CA-125 expression with epithelial differentiation (all are present on mesothelial cells) or interpreting annexin-V staining as evidence of apoptosis, may skew results considerably. The future role of FC in the management of patients with malignant effusions rather than in a pure research setting is yet to be decided. The potential is nevertheless evident. FC may be an efficient tool in identifying isolated tumor cells in a reactive background using reliable epithelial markers. It may also aid in assessing therapy response, for example through quantitative analysis of apoptosis or proliferation. In the context of targeted therapy, FC may identify the fraction of the tumor cell population expressing the protein to be silenced or inhibited. All this, however, depends on the availability of instruments and expertise, which remains to be resolved in the future.

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