Use of DNA Image Cytometry in Addition to Flow Cytometry for the Study of Patients with Advanced Ovarian Cancer

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ABSTRACT

Forty-five patients with advanced ovarian cancer were studied with both DNA flow cytometry (FCM) and automatic DNA image cytometry carried out with the Leiden Television Analysis System (Leytas). There was a significant difference in survival between the diploid and nondiploid cases as determined by FCM. Furthermore, the presence of nuclei with a high DNA content (defined as a DNA content higher than 5C) as determined by Leytas indicated a poor prognosis. When the combined results of FCM and Leytas were taken into account, three different groups of patients could be distinguished. The group of patients with a diploid malignancy (n = 12) had a median survival of more than 60 months. The group of patients (n = 11) with a nondiploid tumor having fewer than 100 nuclei with a DNA content per 1600 microscope fields formed an intermediate group (median survival, 42 months), whereas the median survival of the remaining patients (n = 22), who had a nondiploid malignancy combined with more than 100 of these nuclei per 1600 microscope fields, was only 15 months. In addition, comparison of the clinical parameters by means of a multivariate analysis (Cox regression model) showed that the combined results of FCM and DNA image cytometry had the largest influence on survival. It is concluded that DNA image cytometry appears to be supplementary to FCM for the study of DNA ploidy abnormalities and that the combined results of these methods have a major influence on the clinical outcome.

INTRODUCTION

In advanced ovarian cancer, much effort has been given to the identification of prognostic factors (1, 2). Clinically, the main prognostic factors are the volume of the residual tumor mass after cytoreductive surgery and the presence or absence of ascites (1, 2). Recently, it has become clear that tumor ploidy, as determined by FCM,2 is of considerable importance in a variety of malignancies (3, 4) including ovarian cancer (5, 6). In our previous study, a multivariate analysis confirmed the importance of tumor ploidy as a prognostic parameter in advanced ovarian cancer by comparison with the clinical risk factors (7). In that study, tumor ploidy and the presence or absence of ascites appeared to be the only significant parameters for survival in a Cox regression analysis. Unfortunately, tumor ploidy was not of definitive prognostic value for each individual patient. Although the majority of the patients with a nondiploid tumor died during the follow-up period, some long-term survivors did occur in that group. This raised the question as to whether further refinement could be achieved in the prediction of the clinical outcome.

Since FCM performed in tumor tissue does not supply morphological information on the individually measured cells, it is not possible to discriminate between benign diploid cells and diploid tumor cells. Conventional image cytometry permits visual identification but is often based on not more than 100 randomly selected cells and is rather slow. With the Leiden Television Analysis System automatic image cytometry can be carried out more rapidly on a large number of cells and includes visual interaction. Besides tumor stem cell assessment, the presence of minor subpopulations of aneuploid tumor cells not identified by FCM can be determined with this technique (9). In this way, the number of nuclei with a DNA content exceeding 5C can be quantified.

Those cells with a high DNA content could be of clinical importance, as indicated recently in breast cancer patients (4). In the present study, we report 45 patients with advanced ovarian cancer, whom paraffin-embedded tumor blocks investigated by FCM were also examined by Leytas. The relationship between the results of FCM and Leytas is discussed and the additional value of Leytas versus FCM with respect to the clinical outcome is dealt with. Furthermore, the use of a multivariate analysis to compare the results provided by FCM and Leytas with the main clinical parameters is described.

MATERIALS AND METHODS

In the period between 1979 and December 1984, 110 patients with advanced ovarian cancer (FIGO stages IIb, III, and IV) were referred to the Leiden University Hospital. For investigation by FCM alone, patients were selected for whom paraffin-embedded blocks taken from the primary tumor at laparotomy were available, as reported previously (7). For the present study a further selection of patients was made to obtain a series of cases with a follow-up period of at least 24 months and cases of early death due to ovarian cancer (within a period of 12 months). On this basis, 46 patients were selected.

All of these patients had been treated with cytoreductive surgery and chemotherapy according to the studies of the Netherlands Joint Study Group for Ovarian Cancer (1, 10). The analysis of the 46 patients included data on survival, menopausal status, FIGO stage, the presence or absence of ascites, the volume of the residual tumor mass, as well as the histological grading on the basis of the papillary and/or glandular pattern (11). The patients ranged in age from 20 to 72 years; the median age was 55 years.

DNA Flow Cytometry. Fixed paraffin-embedded blocks were used for performing FCM. A 30-μm section was cut if a section routinely stained with hematoxylin and eosin showed that at least one-third of the cells were cancer cells. The sections for FCM were dewaxed and suspensions of nuclei were prepared, after which DNA staining was performed as described elsewhere (12). Samples were measured on an ICP 22 flow cytometer (Ortho, Westwood, MA). A lesion was considered to be diploid when its DNA profile showed a single G0,1 peak in combination with a CV $\leq$5.5. Suspensions with a single G0,1 peak showing a CV >5.5 were designated as peridiploid and considered as nondiploid, because of the possibility that these cases may represent low aneuploid tumors, as discussed elsewhere (7, 13). Suspensions with an additional G0,1 peak (aneuploid) or more distinct peaks (multiploid) were also designated as nondiploid.

DNA Image Cytometry (Leytas). Image analysis performed with Leytas (Leitz, Wetzlar, West Germany) was done using the next 50-μm sections cut after each section used for FCM, originating from the

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2 The abbreviations used are: FCM, DNA flow cytometry; Leytas, Leiden Television Analysis System; FIGO, Federation Internationale des Gynecologues et Obstetrites; CV, coefficient of variation.
same paraffin-embedded tumor block. The procedure for the preparation of monolayer smears from paraffin-embedded tissue has been described in detail elsewhere (14). Briefly, the sections were dewaxed by xylene, rehydrated in decreasing concentrations of ethanol, and resuspended with Pronase. Further preparation included estimation of the cell density by means of light scatter measurements. The cell suspension was diluted if the scatter reading was higher than a certain fixed arbitrary value corresponding with about 20,000 epithelial cells/ml. A monolayer smear was prepared with a special centrifugation bucket. Staining was performed with acriflavine-Feulgen. Automated image analysis was carried out with Leytas as already described in detail by 2 of the authors (8, 9, 15). In this way, cells are automatically selected on the basis of gray level and size criteria. The settings for these criteria can be adapted; i.e., all epithelial cells can be selected or only a subset of cells with a higher density and greater size. The automated selection procedure was limited to a maximum number of objects (300) or microscope fields (1600); the mean number of cell particles amounted to 58/field. A visual step was included to reject overlapping nuclei and other artifacts not eliminated by the automatic artifact rejection procedure. This visual elimination was done by inspection of the stored gray value images of the selected objects on a television monitor. The DNA measurements were performed automatically on the selected nuclei after relocation at high magnification. The measured absorbances were converted into C units on the basis of the values obtained for trout RBC, present on the same slide but in a different place. For each histogram, both the ploidy class and the number of nuclei with a DNA content of more than 5C/1600 microscope fields was calculated. For survival analysis, the number of the nuclei with a DNA content exceeding 5C/1600 microscopic fields was taken into account.

Statistics. Survival was calculated from the start of the treatment and survival time was defined as the period between the first laparotomy and death due to ovarian cancer. The cutoff date for analysis was September 1, 1985. Survival analysis was performed with the SPSS-X program (16). A Cox regression model was performed stepwise with the SAS program (17) to evaluate the effect of FCM and Leytas on survival, taking the clinical prognostic factors into account.

RESULTS

DNA Flow Cytometry. Twelve samples showed a single G0,1 peak with a CV ≤5.5 and were classified as diploid. Eleven had a single peak in combination with a CV >5.5 and were classified as peridiploid. The remaining cases were aneuploid (n = 18) or multiploid (n = 4). Of the 46 cases studied, one could not be evaluated because of the poor quality of the obtained histogram. The 12 patients with a diploid tumor had a median survival of more than 60 months, whereas the median survival for the 33 (11 + 18 + 4) nondiploid patients was only 20 months (see Table 1).

DNA Image Cytometry (Leytas). Besides the ploidy class, the number of nuclei with a DNA content exceeding 5C was calculated on the basis of the histograms obtained with Leytas. The number of nuclei with such a high DNA content ranged from 0 to 1584/1600 microscope fields among the samples, with a median value of 65 and a mean value of 259 of these nuclei. When a cutoff level of 100 nuclei with a DNA content above 5C/1600 microscope fields was applied, a significant difference proved to exist with respect to survival, median survival amounting to more than 60 months for the 24 cases with <100 (range, 0–66) of these nuclei. In contrast, the median survival for the 22 cases with >100 nuclei (range, 108–1584) with a high DNA content was only 16 months (see Table 1). A representative example of a DNA histogram with many nuclei with a high DNA content is shown in Fig. 1.

FCM Combined with Leytas. The ploidy class results obtained with FCM and Leytas show excellent correlation (Table 2).

However, a difference between FCM and Leytas was observed in 4 cases. This divergence could be explained on methodological grounds. In 3 of these 4 cases, a tumor determined as peridiploid by FCM was classified as a diploid malignancy by Leytas. In one case, a low aneuploid peak determined by FCM was not detected by DNA image cytometry.

All of the 12 diploid cases (FCM) showed a low number of nuclei with a high DNA content (range, 0–30; median, 4 nuclei) as determined by Leytas. In contrast, the 33 nondiploid cases (FCM) had a large variation (range, 2–1584; median, 136 nuclei) in the number of such nuclei per 1600 microscope fields. This led us to divide the nondiploid cases (FCM) into 2 groups: 11 with less than 100 nuclei (range, 2–66) with a high DNA content; and 22 with more than 100 of these nuclei (range, 108–1584). The median survival for these 2 subgroups of patients differed significantly, amounting to 42 months for the former and 15 months for the latter group (see Table 1). Survival curves for both of these groups as well as for the diploid cases as determined by FCM are shown in Fig. 2.

Multivariate Analysis. As already reported (1, 2), the presence or absence of ascites and a residual tumor mass larger than 15 mm are the main clinical prognostic factors for advanced ovarian cancer. In addition, histological grading is also strongly correlated with the clinical outcome (18). The simultaneous influence of these parameters as well as the results provided by FCM and Leytas (with a cutoff point of 100 nuclei with a high DNA content per 1600 microscope fields) were studied with a proportional hazard analysis (Cox regression model). For 44 of the patients the following parameters were known and could be included in the analysis: FIGO stage; histological grade; presence or absence of ascites; residual tumor mass; ploidy class as determined by FCM; number of nuclei with a high DNA content as determined by Leytas; and the last two combined.

Comparison of the clinical parameters with the results of FCM in this analysis showed that only tumor ploidy and the presence or absence of ascites had a statistically significant effect on survival. FIGO stage, histological grade, and residual tumor mass did not have additional prognostic value.

When the clinical parameters were analyzed simultaneously with the results of Leytas, i.e., the number of nuclei with a high DNA content at a cutoff level of 100 of these nuclei per 1600 microscope fields, the results of Leytas, in combination with
UNFAVORABLE SUBGROUP OF CASES WITH TUMOR CELLS WITH HIGH DNA CONTENT

Fig. 1. DNA histogram based on automated cell selection and DNA measurements performed with Leytas. For this histogram the total preparation was screened according to criteria for the selection of nuclei with an elevated DNA content.

Table 2 Relationship between ploidy class as determined by DNA flow cytometry and Leytas

<table>
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<th>FCM</th>
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<td></td>
<td>Diploid</td>
<td>Aneuploid</td>
<td>Total</td>
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<tr>
<td>Leytas</td>
<td>Diploid</td>
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<tr>
<td>Diploid</td>
<td>12</td>
<td>0</td>
<td>12</td>
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<tr>
<td>Aneuploid</td>
<td>4</td>
<td>29</td>
<td>33</td>
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<tr>
<td>Total</td>
<td>16</td>
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The residual tumor mass and the presence or absence of ascites, influenced the survival significantly. Grading and FIGO stage did not provide additional prognostic information.

When the combined results of FCM and Leytas were taken into account and analyzed concomitantly with the remaining variables, the combination of FCM and Leytas had the strongest impact for survival. The presence or absence of ascites also had a statistically significant effect, but this was not the case for grading, FIGO stage, or the residual tumor mass. These findings are illustrated by the different relative risks as determined by FCM alone and FCM and Leytas combined, according to the Cox regression model. The relative risk of dying from ovarian cancer is 36 for a patient with a nondiploid tumor (determined by FCM) compared to that for a patient with a diploid malignancy of the ovary. Moreover, the relative risk for ovarian cancer-related death for a patient with a nondiploid malignancy as determined by FCM in combination with >100 nuclei/1600 microscope fields with a high DNA content as determined by Leytas is 62, also in comparison with the situation for a diploid case.

DISCUSSION

In this study we evaluated both FCM and a new method for automatic image cytometry (Leytas) in patients with advanced ovarian cancer. In the first place, the results indicate excellent correlation between the ploidy class determined by FCM and
Leytas, both using paraffin-embedded tissue, as had been found earlier for freshly cut tumor tissue of breast cancer patients (9). The 2 methods only gave divergent results in 4 cases. In 3 of these 4 cases, the histogram obtained with FCM revealed a single \( G_0 \) peak, which was combined with a wide coefficient of variation (a peridiploid tumor). Although these 3 peridiploid cases in FCM were identified as diploid by Leytas, it is not excluded that these cases represent low aneuploid malignancies. In fact, the fourth divergent case, a low aneuploid tumor according to FCM not identified as aneuploid by Leytas, shows that the resolution of Leytas for ploidy determinations is still lower with Leytas than with FCM, especially in near diploid cases. Furthermore, the 3 peridiploid tumors illustrate the difficulties encountered in performing FCM in paraffin-embedded material. Aware of these problems, Hedley et al. (13) considered such peridiploid samples as nenevaluable and omitted them from the survival analysis in their study on breast cancer patients. On the other hand, Leytas procedure permits elimination of doublets and other artifacts, in a histogram covering only single noncut nuclei. Thus, Leytas especially provides additional information on the tumor stemline in cases where the histogram by FCM shows a wide coefficient of variation.

Secondly, the results confirm the reported benefit of Leytas, compared with FCM, in detecting nuclei with an abnormally high DNA content (9). The preparation procedure applied guaranteed that all cellular material centrifuged on the slides had about the same cell density, which meant that an absolute number of nuclei with a high DNA content could be taken as a parameter. For about one-half of the patients, more than 100 nuclei with a high DNA content per 1600 microscope fields could be demonstrated. These nuclei occurred especially in the nondiploid cases as determined by FCM.

Third, it was shown that the presence of nuclei with a high DNA content was of clinical importance; on the basis of a cutoff level of 100 of such nuclei per 1600 microscope fields, a highly significant difference was found with respect to survival. Moreover, when FCM and Leytas combined were taken into account, 3 distinct groups could be discerned: patients with a diploid malignancy with a median survival of more than 60 months; patients with a nondiploid tumor and <100 nuclei with a high DNA content per 1600 microscope fields (median survival, 42 months); and the remaining patients with both a nondiploid malignancy and >100 of these nuclei, for whom the median survival was only 15 months. The clinical relevance of these findings was confirmed by performing a multivariate analysis, including the main clinical prognostic factors for advanced ovarian cancer, i.e., the presence or absence of ascites and the volume of the residual tumor mass.

In this study we determined the number of nuclei with a high DNA content by Leytas, after determination of the tumor stemline by FCM as reported elsewhere (7). Nevertheless, it proved to be justifiable to determine both the tumor stemline and the number of nuclei with a high DNA content by Leytas, because of the excellent correlation found between the results obtained by the 2 methods. At present, however, Leytas is more expensive and time consuming than FCM. Genetic instability resulting in polyploidization might account for the presence of the highly aneuploid cells determined by Leytas. The higher frequency of these aberrant cells in nondiploid malignancies might be related to the higher proliferative activity of the nondiploid tumors compared with that of diploid malignancies. As reported recently, these nondiploid tumors generally have a larger proportion of S-phase cells than is found in diploid malignancies (19). On the basis of the present observations, we think that Leytas supplements FCM for the study of DNA ploidy abnormalities, especially with respect to the clinical outcome. A longer follow-up period as well as further studies done in other malignancies too are needed using this promising combined modality.

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