Ki-67 STAINING IN BENIGN, BORDERLINE, MALIGNANT PRIMARY AND METASTATIC OVARIAN TUMORS: CORRELATION WITH STEROID RECEPTORS, EPIDERMAL-GROWTH-FACTOR RECEPTOR AND CATHEPSIN D

Sonja C. HENZEN-LOGMANS1,3, Elly J.H. FIERET1, Els M.J.J. BERNS2, Maria E.L. VAN DER BURG2, Jan G.M. KLUIN2 and John A. FOKENS3

1Department of Pathology; and 2Division of Endocrine Oncology (Department of Medical Oncology), Dr. Daniel den Hoed Cancer Center, P.O. Box 5201, 3008 AE, Rotterdam, The Netherlands.

Ki-67 immunoreactivity was studied in relation to immunohistochemically assessed expression of epidermal-growth-factor receptor (EGFR), estrogen receptor (ER) progesterone receptor (PgR) and cytosolic levels of cathepsin D in advanced human ovarian adenocarcinomas, borderline and benign cystadenomas and normal ovaries. A significantly higher number of Ki-67-positive cells were found in metastatic tumors vs. primary adenocarcinomas and in the total group of adenocarcinomas vs. benign/borderline cystadenomas. Cathepsin-D levels were also significantly higher in metastatic tumors than in primary adenocarcinomas, which in turn presented higher levels than were found in normal ovaries. However, no significant difference was observed between cathepsin-D levels in malignant adenocarcinomas and borderline/benign cystadenomas. Immunohistochemically assessed expression of ER and PgR was detected in variable percentages of epithelial tumor cells, and stromal cells were occasionally positive as well. In the group of primary adenocarcinomas, 46% were ER-positive and 34% were PgR-positive, although there was no significant difference between primary and metastatic lesions with respect to ER or PgR expression. Concordance between immunohistochemically assessed ER or PgR data and cytosolic ER and PgR levels measured with enzyme immunoassay was relatively low. EGFR, immunohistochemically assessed with MAb-EGFR1, was positive in 76% of the primary and in 78% of the metastatic adenocarcinomas. A strong positive association was detected between ER and PgR, and EGFR was observed to present a weak positive correlation with Ki-67 and ER. Cathepsin-D levels were not found to be significantly correlated with the expression of ER, PgR, EGFR or Ki-67.

Although the precise pathogenesis of ovarian cancer remains unknown, it appears that hormones and growth factors involved in menstrual activity contribute to it. Attention has been drawn to estradiol, progesterone, epidermal growth factor (EGF) and, more recently, cathepsin D. With regard to their function in breast cancer, it has been shown that binding of estradiol to its receptor activates both PgR synthesis and cell proliferation. Binding of EGF to its receptor activates a variety of biochemical and physiological changes in the target cell, normally leading to enhanced DNA synthesis and cell division. Cathepsin D, which is over-expressed and induced by estrogen and growth factors in hormone-dependent breast-cancer cells (Westley and Rochefort, 1980), is an aspartic lysosomal proteinase and is synthesized as a 52-kDa precursor (reviewed by Rochefort et al., 1987). This cathepsin-D (52-kDa) protein shows an autocrine mitogenic effect in breast-cancer cells through binding to the insulin-like growth-factor-II receptor (Mathieu et al., 1990) and a proteolytic effect on extracellular matrix after its autolysis at acidic pH (Briozzo et al., 1988). It may therefore play a role in controlling the growth and spread of human breast cancer (Rochefort et al., 1987).

The potential function of steroid receptors, growth-factor receptors and cathepsin D is complicated by the fact that estradiol can stimulate the production of both EGF and cathepsin D, meaning that the observed mitogenic effect of estradiol can be mediated by local action of EGF or cathepsin D.

Furthermore, studies of ovarian tumor cell lines have shown that PgR synthesis and the proliferation of cells regulated through the binding of estradiol to its receptor can become uncoupled (reviewed by Rao and Slotman, 1991). Translating the findings in human breast cancer and ovarian tumor cell lines to the situation in vivo, several studies have reported the incidence of both ER and PgR in ovarian cancer, with percentages ranging from 38% ER and 14% PgR positivity to 100% ER and 100% PgR positivity (Rao and Slotman, 1991). With regard to EGFR, the reported incidence varies from 40% (Owens et al., 1991) to 77% (Bergluch et al., 1991). We expanded these data by studying ER, PgR and EGFR status in advanced human ovarian cancer using ELISA or ligand-binding assay techniques and found a positive incidence of 60% for ER, 35% for PgR and 65% for EGFR (Berns et al., 1992). While both ER and PgR have been described as favorable prognostic factors in ovarian cancer, PgR appears to be the most important (Rao and Slotman, 1991) and our own experience concurs (van der Burg et al., 1993). Data on the prognostic significance of EGFR in ovarian cancer are contradictory and concern relatively small series of patients (Bauknecht et al., 1988, 1990; Berchuck et al., 1991; Owens et al., 1991; van der Burg et al., 1993). With respect to cathepsin-D levels, published data are scarce. Scambia et al. (1991) found detectable levels of cathepsin D in all 88 normal and neoplastic ovarian tissues examined. Interestingly, cathepsin-D levels were significantly higher in malignant tissues than in normal ones.

Although the presence of ER, PgR, EGFR and cathepsin D suggests that ovarian cancers are endocrinologically regulated tumors, their function and biological and clinical significance for patients with ovarian cancer remain to be clarified in large series of patients.

Rutgers et al. (1987) have shown the importance of classical prognosticators such as ovarian cancer growth kinetics, including S-phase values assessed by flow cytometry. Immunostaining with MAb Ki-67 gives reliable data on the growth fraction (McGurrin et al., 1987; Kuenen-Boumeester et al., 1991). In ovarian cancer samples (Wong and Tattersall, 1989; Isola et al., 1990) high expression is associated with poor prognosis (Isola et al., 1990). We expanded these data by studying ER, PgR and EGFR expression in relation to Ki-67 expression in human ovarian cancer (Berns et al., 1992; Henzen-Logmans et al., 1992; van der Burg et al., 1993). Results obtained for 103 carcinoma specimens from 99 patients were compared with those obtained for benign and borderline malignant epithelial tumors, their function and biological and clinical significance for patients with ovarian cancer remain to be clarified in large series of patients.

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Ki-67 and cathepsin D in ovarian tissues

Material and Methods

Patients

A total of 121 epithelial tumor specimens were classified according to the WHO system, and analyzed. Tumors were classified as primary when they were taken from the ovarian tumor mass and as metastatic when the biopsy was taken from metastases in the omentum, peritoneum or diaphragm. Forty-seven patients (mean age, 58 years) had serous adenocarcinoma (38 primary, 9 metastatic); 20 patients (mean age, 59 years) had mucinous adenocarcinoma (19 primary, 4 metastatic, including 3 patients with both); 7 (mean age, 57) had endometrioid adenocarcinoma (6 primary, 1 metastatic); 10 (mean age, 56) had clear-cell carcinoma (8 primary, 2 metastatic); 8 (mean age, 54) had mixed-type adenocarcinoma (7 primary, 2 metastatic, 1 patient with both); and 7 (mean age, 57 years) had poorly differentiated carcinoma (5 primary, 2 metastatic). Overall, 103 specimens (83 primaries and 20 metastases) from 99 patients were analyzed. Apart from these carcinoma patients, 8 patients (mean age, 59 years) had benign cystadenomas (5 serous, 3 mucinous) and 10 (mean age, 56 years) had borderline malignant cystadenomas (5 serous, 5 mucinous). Furthermore, 24 non-tumorous ovaries (9 from patients between 43 and 55 years of age, and 15 from patients over 55) were analyzed.

Immunohistochemistry

Representative tissue samples were snap-frozen in liquid nitrogen and stored at −70°C until use. Serial sections were cut at 5 μm. These were air-dried and fixed in acetone for 10 min, after which the indirect immunoperoxidase technique was used for visualizing Ki-67 MAb (DAKO, Glostrup, Denmark) and EGFR MAb (Amersham, Aylesbury, UK) as described by us in detail (Kuenen-Boumeester et al., 1991; Henzen-Logmans et al., 1992). For immunostaining of ER and PgR commercially available kits were used (ER-ICA and PgR-ICA kits, Abbott, Chicago, IL). After the final washing procedure, nuclear counterstaining was achieved for all antibodies by incubation in Mayer’s hematoxylin for 1 min. Control samples consisted of known positive and negative specimens identified by the ligand-binding assay (for ER, PgR, EGFR). In addition, for negative control sections, the first layer antibody was changed to PBS or non-immune ascites fluid.

Quantification

The percentage of immunohistochemically assessed ER-, PgR-, EGFR- and Ki-67-positive tumor cells was calculated by counting the number of positive cells in a total of 3 × 100 cells in 3 different areas of the tumor section. For cytosolic ER, PgR and cathepsin-D assays, tumor tissue (0.4–0.8 g) was pulverized and homogenized as recommended by the EORTC for processing breast tumor tissue for cytosolic ER and PgR determinations. The homogenate was centrifuged for 30 min at 100,000 g at 4°C and the supernatant fraction (cytosolic extract) was used for ER and PgR determination by enzyme immunoassays (ER-EIA and PgR-EIA kits, Abbott) as described before (Berns et al., 1992). Cathepsin-D levels were measured in the same cytosols with a commercially available radiometric immunoassay kit (ELSA-CATH-D, CIS bio international, Gif-sur-Yvette, France) as described before for human breast cancer samples (Poekens et al., 1993).

Statistics

Associations between ER, PgR, EGFR, cathepsin D and Ki-67 were analyzed by Spearman rank correlation. Differences between groups were tested non-parametrically by means of the Wilcoxon 2-sample test (Mann-Whitney U-test).

Results

Ki-67 and cathepsin D in histological subgroups

Ki-67. In tumor cells, various levels of immunoreactivity for Ki-67 were observed exclusively in the nuclei. Cells expressing Ki-67 were randomly distributed over the tissue sections. Stromal elements such as fibroblasts occasionally stained with the antibody. Figure 1 (top) shows the percentage of cells stained with the Ki-67 MAb according to the histological classification of the tumors. For the total group of 83 primary adenocarcinomas, the median value of Ki-67-positive cells was 18%. Metastatic tumors contained a significantly higher (2p < 0.03) number of Ki-67-positive cells (median, 28%; n = 20) than did primary adenocarcinomas, and in the total group of adenocarcinomas the number of Ki-67-positive cells was higher than in 18 benign/borderline cystadenomas (2p < 0.01). The median percentage of the number of Ki-67-positive cells in benign tumors was 4.5% and in borderline serous and mucinous tumors 4% and 23% respectively. Normal ovarian tissues (n = 24) did not express Ki-67 (Fig. 1, top).

Histological subtype

Figure 1 – Ki-67 and cathepsin D in various histological subtypes of ovarian tissues. The percentage of Ki-67-positive epithelial cells (top panel) was determined by immunohistochemistry; cytosolic cathepsin-D content (bottom panel) was determined with an enzyme immunoassay. Abbreviations: se = serous, mu = mucinous, cc = clear-cut, pd = poorly differentiated, ot = other (metastatic) adenocarcinomas and n = normal ovarian tissues.
Cathepsin D. Cathepsin-D levels were measured in cytosols by quantitative radiometric immunoassay. The distribution of cytosolic cathepsin-D levels according to histological classification of the tumors is shown in Figure 1 (bottom). For the total group of 74 primary adenocarcinomas analyzed for cathepsin-D content, the median value was 22 pmol cathepsin D/mg cytosolic protein (range, 1–255). The 18 metastatic tumors analyzed contained significantly higher (2p < 0.01) levels of cathepsin D (median, 37 pmol/mg cytosolic protein) than did primary tumors. As in the case of Ki-67 expression, there was an overlap in the ranges of cathepsin-D values in all subgroups of primary and metastatic adenocarcinomas. Both metastatic and primary adenocarcinomas contained significantly higher (2p < 0.03) levels of cathepsin D than were present in normal ovarian tissues (median, 15 pmol/mg cytosolic protein; n = 24). The ranges of cathepsin-D values observed in borderline and benign tumors did not differ from those in either adenocarcinomas or normal tissues (Fig. 1, bottom).

ER, PgR, and EGFR

Adenocarcinomas. Immunohistochemically detectable nuclear ER and PgR were present in a variable percentage of tumor cells. Tumor stromal cells were occasionally positive, also in tumors lacking ER and PgR expression in epithelial tumor cells (ER: 6 cases, 2 with negative tumor cells; PgR: 6 cases, 5 with negative tumor cells). The occasional presence of ER and/or PgR in stromal tissue may partly explain the relatively weak correlation between data obtained by immunohistochemistry (IHC; scoring epithelial cells only) and data obtained by enzyme immunoassay (EIA) on cytosolic extracts (epithelial cells + stromal components). By defining >10 fmol/mg cytosolic protein as positive for the EIAs and by arbitrarily describing a tumor as being positive by IHC in those cases when any epithelial tumor cell reacted with the antibody, the observed concordances between IHC and EIA were 82% for ER and 70% for PgR (Table I). The Spearman correlation coefficients between the number of epithelial tumor cells stained in the IHC assay and the amount of cytosolic ER and PgR expressed by EIAs were Rs = 0.65 for both ER and PgR (n = 50, 2p < 0.0001). Immunohistochemically assessed ER and PgR data were included in further analyses.

In 47% of 102 adenocarcinomas studied (primary and metastatic tumors), ER was detectable by IHC; a histoscore (percentage of positive epithelial cells) of 15 ± 22 (mean ± SD) was observed for primary tumors (46% positive, n = 83) and of 20 ± 26 for metastatic tumors (53% positive, n = 19). PgR was detectable in 32% of the adenocarcinomas, with a mean percentage of PgR-positive cells of 14 ± 26 (± SD) for the primary tumors (34% positive), and of 6 ± 15% for the metastatic tumors (26% positive). Neither for ER nor for PgR were these differences between primary and metastatic tumors statistically significant.

With MAb EGFR1, a heterogeneous level of expression was seen and staining was predominantly cytoplasmic. In all, 76% of 80 primary tumors and 78% of 18 metastatic adenocarcinomas analyzed for EGFR by IHC were positive for EGFR1 staining. In accordance with previous reports, this difference was not statistically significant (Henzen-Logmans et al., 1992).

Benign and borderline tumors and normal ovarian tissues.

Among borderline tumors, all 5 mucinous tumors were negative for ER and only a small number of the cells of 1 mucinous tumor were positive for PgR. By contrast, in all 5 borderline serous tumors, ER (mean percentage of positive cells ± SD: 25 ± 25%) and PgR (53 ± 12%) were detectable. The reverse was observed with respect to Ki-67 expression in mucinous vs. serous borderline tumors (Fig. 1, top). All 5 benign serous tumors studied were positive for ER (63 ± 25% positive cells) and PgR (54 ± 34%), whereas ER (4 ± 3% positive cells) and PgR (2 ± 2%) were either absent from or barely detectable in the 3 mucinous benign tumors studied. In non-tumoral ovarian tissues, stromal cells were occasionally positive for ER and/or PgR (ER, 6 out of 32 cases; PgR, 23 out of 32 cases). This high incidence of stromal expression of PgR probably accounts for the high (median, 152 fmol/mg protein) levels measured by EIA in cytosols prepared from normal ovarian tissues as described before (Berns et al., 1992).

All 5 borderline serous (median, 60%; range, 30–90% positive cells) and 3 out of 5 mucinous tumors (60%, 60%, and 100% positive cells) studied were positive for EGFR1 expression. Of the benign neoplasms studied, 3 out of 5 serous tumors (40%, 60%, and 100% positive cells) and all 3 mucinous tumors (10%, 70%, and 100% positive cells) were positive for EGFR1 expression. When surface epithelial cells were present in normal ovarian tissues, EGFR1 immunoreactivity was localized on these cells. In addition, as reported before (Henzen-Logmans et al., 1992), non-tumoral spindle cells and vessel walls sometimes stained as well.

Correlations between Ki-67, ER, PgR, cathepsin D and EGFR

Correlations between the percentage of Ki-67-positive cells and immunohistochemically assessed ER, PgR, and EGFR and cytosolic levels of cathepsin D were studied in primary adenocarcinoma tissues. Tumors containing high levels (above the median) of cathepsin D were found to be equally distributed among ER-, PgR- and EGFR-positive and -negative tumors and among tumors expressing high or low numbers of Ki-67-positive cells (Table II). Similarly, primary adenocarcinomas expressing high or low numbers of Ki-67-positive cells were found to be equally distributed among ER- and PgR-positive and -negative tumors. However, a trend towards a positive correlation between EGFR and Ki-67 was observed: 54% of EGFR-positive tumors vs. 37% of EGFR-negative tumors contained a high number of Ki-67-positive cells (Table II). The respective Spearman correlation coefficients between the various cell biological parameters listed in Table III show weak positive associations of EGFR with Ki-67 and ER (2p < 0.05) and a strong positive correlation between immunohistochemically assessed ER and PgR (2p < 0.0001). None of the other associations were statistically significant.

### Table I—ER and PgR Status in Primary Ovarian Adenocarcinomas: Immunohistochemistry vs. Enzyme Immunoassay

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<th>ER-EIA 1</th>
<th>PgR-EIA 1</th>
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<tr>
<td>IHC 2</td>
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<td>11</td>
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<td>Total</td>
<td>44</td>
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1EIA: enzyme immunoassay; positive defined as ≥10 fmol/mg cytosolic protein.

2IHC: immunohistochemistry; positive defined as >0 stained epithelial tumor cells.
reported by Isola et al., (1990). who likewise observed a lack of association between expression of cathepsin D and ER in the latter (Rao and Slotman, 1991), although we have found a very weak, but significant trend towards a positive correlation between EGFR and ER. This finding is puzzling since both tumors are supposed to be endocrinologically regulated. One explanation may be related to the fact that the pathways of ER and/or EGF regulation are not necessarily the same in breast and ovarian cancers, with a possibly reduced functionality of ER in the latter (Rao and Slotman, 1991). As to clinical outcome, data on the prognostic value of EGFR are conflicting with respect to both ovarian cancer (Bauknecht et al., 1988, 1990; Berchuck et al., 1991; Owens et al., 1991; van der Burg et al., 1993) and breast cancer (Klijn et al., 1992).

When comparing our EGFR data with proliferative activity by counting Ki-67 reactivity in adenocarcinomas, we noted a trend towards a positive correlation between EGFR and Ki-67, i.e., 54% of EGFR-positive tumors vs. 37% of EGFR-negative tumors contained ≥ 18% (median) Ki-67-positive cells. Such a positive relationship between EGFR and Ki-67 has also been reported in a few studies on breast cancer tissues (Klijn et al., 1992).

Concerning cathepsin D, significantly higher levels were measured in adenocarcinomas vs. normal ovarian tissues and in metastatic lesions vs. primary tumors. Higher levels of cathepsin D in malignant tumors vs. normal tissues have also been reported by Scambia et al. (1991). Since cathepsin D is a proteolytic enzyme that may facilitate tumor invasion when it is secreted by cancer cells (Rochefort et al., 1987), differences in levels of expression might represent a biochemical characteristic reflecting biological tumor aggressiveness. In our series, however, no significant differences in levels of expression were noticed between benign/borderline adenomas and adenocarcinomas. Cathepsin D may have mitogenic activity through an autocrine mechanism (Mathieu et al., 1990). However, no relationship between Ki-67 activity and cathepsin-D levels was found in our study. In ER-positive breast-cancer cells, estradiol stimulates the production of cathepsin D (Westley and Rochefort, 1980). In our study however, which concords with data reported by Scambia et al. (1991), no positive correlation was found between the expression of cathepsin D and ER in ovarian carcinoma tissues. This lack of association between cathepsin D and ER is also observed for breast tumors (Rochefort, 1990), although we have found a very weak, but significant (p < 0.05), association between ER and cathepsin D in a series of 710 breast tumors (Fooke et al., 1993).

We have shown that the percentage of Ki-67-positive cells in adenocarcinomas varies without any correlation to tumor type but with a significant trend to higher expression in metastatic lesions. We also observed that Ki-67 expression in the total group of adenocarcinomas was significantly higher than in...
adenomas. Wong and Tattersall (1989) have also reported a positive correlation between high Ki-67 activity and distant metastases. Correlating Ki-67 reactivity with the level of expression of ER, PgR, EGFR and cathepsin D as markers of possible mitogenic functionality, we have shown that the levels of expression of these factors vary in adenocarcinomas without relation to tumor type. Apart from a weak positive association of EGFR with ER and Ki-67, and a strong positive correlation between ER and PgR, no statistically significant correlations were found.

Further studies are in progress to assess the prognostic significance of Ki-67 and cathepsin D in ovarian carcinomas in relation to progression-free survival and death. The lack of correlation between Ki-67 and ER, PgR, and cathepsin D suggests that hormone- and cathepsin-D-mediated pathways and cell proliferation are not directly linked in ovarian carcinomas.

ACKNOWLEDGEMENTS

This study was made possible thanks to the collaboration of St. Clara Hospital, Zuider Hospital, Bergweg Hospital, Refaja Hospital Dordrecht, Lievensberg Hospital Bergen op Zoom and Beatrix Hospital Gorinchem, The Netherlands. It was supported by a grant from the Dutch Cancer Society ("Koningin Wilhelmina Fonds", project DHDK 90-05).

We thank Mrs. M. Stuurman-Smeets, Mrs. E. Binnendijk-Noordegraaf and Mr. Henk Portengen for their excellent technical assistance and Mrs. A. Braber for preparing the manuscript.

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