Transforming Growth Factor-β and Nitrates in Epithelial Ovarian Cancer

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ABSTRACT: The role of transforming growth factor-β (TGF-β) and nitric oxide (NO) in ovarian neoplasia is still not clear. We studied the expression of TGF-β by enzyme immunoassay, and nitrates (as a stable end product of NO) in 127 ovarian tissues (36 normal, 37 benign, and 54 malignant). Ploidy status and synthetic phase fraction (SPF) were also assessed by flow cytometry. Mean ranks of SPF were significant among different groups (X^2 = 12.01, P = 0.0025, X^2 = 67.42, P = 0.000, X^2 = 9.06, P = 0.011 respectively). Nitrate mean ranks were significant among different FIGO stages of the disease (X^2 = 17.6, P = 0.000). A significant correlation was shown between (r = 0.24, P = 0.01), as well as in malignant tissues (r = 0.3, P = 0.026). Cutoff values were determined for both TGF-β (290 pg/mg protein), and nitrates (310 nmole/mg non protein nitrogenous substances). At these cut-offs, nitrates showed a sensitivity of 93% and 84% specificity for malignant versus normal cases, while TGF-β had 76% sensitivity, and 82.4% specificity for poor versus good outcome. Patients with epithelial ovarian cancer were followed up for a total of 40 months. Survival analysis showed that patients with TGF-β above the cut-off had worse prognosis (X^2 = 12.69, P = 0.004). The present results suggest that malignant transformation of ovarian tissues is associated with increased TGF-β and NO production. NO level is related to the development and progression of epithelial ovarian cancer, while high levels of TGF-β could be of prognostic significance.

KEYWORDS: TGF-β, NO, nitrates, synthetic phase fraction, ovarian cancer

INTRODUCTION

The majority of ovarian cancers arise from malignant transformation of the ovarian surface epithelium [2,36]. Unlike most epithelial cells, ovarian surface epithelial cells are not replaced. For this reason, they behave in the same way as generative stem cells [15], which have continued growth potential. Every increase in DNA replication, adds to the probability of a mutation. During reproductive life, ovulation could be a factor in disease initiation because of the surface epithelial mitotic activity required to repair wound created by follicular rupture [15].

The family of transforming growth factor beta (TGF-β1, β2, and β3) inhibit proliferation in a wide range of cell types including epithelial cells, hepatocytes, and hematopoietic cells [12]. Although the signal transduction pathways involved are not yet well understood, it has been shown that the biological activity of TGF-β is dependent on the presence of type I, and II serine/threonine kinase membrane receptors [29,40]. Binding of TGF-β to these receptors initiates a cascade of molecular events that are thought to culminate in decreased activity of cyclin dependent kinase 4, which prevents progression from G1 into S phase of DNA synthesis [30]. Mutations of the transforming growth factor-beta type II receptor (TGF-beta RII) gene have been detected in several human cancers [13,14,20] with loss of...
responsiveness to TGF-β. Loss of responsiveness to TGF-β is suggested to be involved in the process of immortalization of ovarian cancer cells in vitro but not in the development of human ovarian cancer [3,18]. However, in vivo studies showed that TGF-β is associated with promotion of angiogenesis in epithelial ovarian cancer [31].

Nitric oxide (NO), a potentially toxic gas with free radical properties, is generated from L-arginine by constitutive or inducible nitric oxide synthases (NOSs) [32]. NOSs are expressed in human precancerous and cancerous gynecologic lesions [39]. Tumor growth may be sustained by increased blood flow mediated by prolonged and excessive production of NO in solid tumors [27]. It has been also suggested that excess NO produced in inflamed tissues could play a role in carcinogenesis by impairing the function of many proteins, including the tumor suppressor function of p53 [6], which is impaired in about 50% of epithelial ovarian cancers [24]. Loss of p53 function due to mutations and/or deletions of its gene during the process of malignant transformation has been associated with the development of resistance to the growth inhibitory effect of TGF-β in some cell lines [37].

Synergistic expression of TGF-β, and NO was associated with malignant transformation in cell lines [26]. However, no previous studies investigated the relation between them in human gynecologic cancers. In this study, the quantitative Enzyme immunoassay (EIA) for TGF-β1, as well as enzyme-end point one step method for nitrate — the stable end product of NO — estimation were utilized to evaluate their levels in epithelial ovarian cancer. Correlation with DNA ploidy status, and synthetic phase fraction as established genetic markers was performed. The significance of TGF-β, and nitrates as predictors of poor survival was also investigated.

MATERIALS AND METHODS

This study was conducted in the period from March 1995 through September 1998. The clinical specimens were obtained from the Obstetrics and Gynecology Department, Ain Shams University Hospitals. The laboratory work was carried out at the Oncology Diagnostic Unit (ODU), Biochemistry Department, Ain Shams Faculty of Medicine.

PATIENTS

The study included a total number of 127 patients. Tissue samples were obtained from patients who suffered from malignant (n = 54), and benign ovarian tumors (n = 37). Normal ovaries (n = 36) were obtained from patients undergoing hysterectomies for non-malignant gynecological conditions. Patients with ovarian cancer were surgically staged and classified according to FIGO staging [11]. All patients with histologically proved ovarian cancer have received platinum based combination chemotherapy for three to six courses. Follow up of all patients by clinical assessment, tumor markers and medical imaging has been carried out till the end of the study or disease related death of the patient. The surgically excised tissue specimens were divided into three portions: one was processed for histopathological evaluation, the second for flow cytometric DNA analysis, and the third was washed with ice cold saline and transported in citrate sucrose dimethylsulfoxide (DMSO), pH 7.4, and stored at −80 °C until subsequent processing and measurements.

PREPARATION OF THE CYTOSOLIC FRACTIONS

Processing of the frozen tissue to get the cytosolic fractions was done according to Mabrouk et al. [27]. Protein concentrations were determined by Bradford method [5] using bovine serum albumin as the calibrator. Nitrate and TGF-β antigen were measured in ovarian cancer cytosolic fractions with adjusted protein concentrations (3 g/L).
NITRATE ASSAY

The nitrate concentration was determined by an end-point enzymatic one-step assay with β-NADPH dependent nitrate reductase [3] modified to suit nitrate determination in cytosolic fractions [22]. All chemicals were purchased from Sigma Co. (St. Louis, MO, USA). The decrease in NADPH absorbance was monitored at 340 nm for 15 minutes at 25 °C using a Beckman DU-70 spectrophotometer (Brae, USA).

Nitrate levels were expressed as nmol/mg NPN (Non-protein nitrogenous substances). NPN were measured using the automated clinical chemistry analyzer Beckman Synchron CX4,5 (Brae, USA). NPN was adjusted to 40–60 mg/L in cytosolic fractions by homogenization buffer.

TGF-β1 ASSAY (MODIFIED TO SUIT MEASUREMENT IN CYTOSOLIC FRACTIONS)

We used Quantikine™ Human TGF-β1 immunoassay (R&D Systems, Abingdon, Oxon, UK) which was designed to measure TGF-β1 in sera, plasma, and cell culture supernates. Latent TGF-β1 had been first activated to immunoreactive TGF-β1 by adding 0.1 ml of 1 N HCl to 0.1 ml of cytosolic fractions. Samples were incubated for 10 minutes at room temperature, then, neutralized by addition of 0.1 ml of 1.2 N NaOH/0.5 M HEPES to reach a pH range: 7.2–7.6 that is suitable for the assay (final dilution 1:3). The manufacturer recommended 1:30 dilution of serum samples, 1:12 dilution for platelet-poor plasma, and 1:1.4 dilution for cell culture supernates by the calibrator diluent. Based on the adjusted protein concentration of the cytosolic fractions, no further dilution of the samples was needed. The assay was then carried out according to manufacturer’s instructions.

PRECISION STUDIES

Intra-assay evaluation for TGF-β was performed using 10 replicates of two cytosolic samples. Interassay was done by 7 measurements of the same samples over 15 days. Between experiments, the samples were stored at −20 °C.

ACCURACY STUDIES

To test the accuracy of Quantikine™ Human TGF-β1 immunoassay in cytosolic fractions, serial dilutions (1/2, 1/4, 1/8, 1/16, and 1/32) of two different cytosolic samples were measured. Dilution was done using calibrator diluent.

RECOVERY STUDIES

To test the recovery of TGF-β1 from cytosolic fractions, different TGF-β1 standard solutions (1000, 500, 250, 125 pg/ml) were added to a sample of benign ovarian cytosol, and another sample of ovarian cancer cytosol.

FLOW CYTOMETRY DNA ANALYSIS

Fresh tissue was mechanically dissociated in RPMI media. The resulting cell suspension was processed for DNA analysis as described previously [9]. Samples were incubated at room temperature for 60 min, in darkness, prior to flow cytometric analysis with a Coulter EPICS® Profile II flow cytometer, configured with a 488 nm argon ion laser as described by Eissa et al, [10]. A modified exponential debris function was used to subtract the debris in the DNA histograms. DNA aneuploidy was defined as any population with a distinct additional peak (s), or the presence of a tetraploid population greater than 15%. The synthetic phase fraction (SPF) was defined as the proportion of cells in the DNA histograms with intermediate DNA content between that of G0/G1 and G2/M.

STATISTICAL ANALYSIS

Univariate analyses were performed using a Chi square test of association of categorial variables,
whereas a t test was used for continuous variables. Mann–Whitney, and Cruskal–Wallis non-parametric tests were used for comparison of different parameter between various groups. To analyze the simultaneous effect of all variables and control for varied follow up, the assumptions of Cox’s proportional hazards model were assessed, including interactions and proportionality of hazard over time [8]. A Kaplan Meier curve was created for TGF-β [21]. These analyses were performed using a statistical package for the social sciences (SPSS) on an IBM PC computer. Cut-off values were determined using receiver operating characteristic curves, areas under the curves were calculated according to Henderson 1993 [16].

RESULTS

Patient results: The study included 36 patients with normal ovaries as confirmed by histopathology (23–50 yrs., mean 39.9), 37 patients with benign ovarian conditions (21–55 yrs., mean 40.3), and 54 patients with different ovarian epithelial neoplasms (25–64 yrs., mean 41.5). Of the malignant group, 13/54 (24.1%) patients were FIGO stage I, 8/54 (14.8%) stage II, 25/54 (46.3%) were stage III, and 8/54 (14.8) stage IV. Grades 1 comprise 25/54 (46.3%), grade 2 were 8/54 (14.8%), while grade 3 comprised 21/54 (38.9). By histopathology, 23 tumors were serous, 15 were mucinous cystadenocarcinoma, and 16 belonged to other different pathological types. The clinical follow-up period was 4–40 months (mean 23.1). Over this period, 25 patients (46.3%) of the malignant group died from the disease. By flow cytometry, all normal and benign samples were found to be diploid, while 15/54 (27.8) of the malignant samples were aneuploid.

PERFORMANCE CHARACTERISTICS OF TGF-β EIA METHOD

Precision: We tested the precision of the assay by measuring two cytosolic fractions ten times in one assay (intra-assay), and in seven consecutive assays (inter-assay). The results of precision studies are shown in Table 1. Within and between-run CVs ranged from 1.8–4.7%.

| TGF-β Conc. | Intra-assay (n = 10) | Interassay (n = 7) |
|-------------|---------------------|--------------------|
|             | mean ± SD %CV       | mean ± SD %CV      |
| Sample 1 (680 pg/ml) | 684.8 ± 12.6 1.8 | 677.9 ± 31.8 4.7 |
| Sample 2 (485 pg/ml) | 482.0 ± 18.7 3.9 | 489.7 ± 20.6 4.2 |

Table 2
Accuracy of Quantikine™ Human TGF-β1 immunoassay in cytosolic fractions

|                  | Expected | Observed | Percent accuracy |
|------------------|----------|----------|------------------|
| Undiluted        | 980      | 983      | 100.3            |
| 1/2              | 490      | 482      | 98.4             |
| 1/4              | 245      | 234      | 95.5             |
| 1/8              | 123      | 115      | 93.5             |
| 1/16             | 62       | 67       | 108.1            |
| 1/32             | 31       | 34       | 109.7            |
| mean             |          |          | 100.9            |

* Samples are expressed in pg/mg protein.
Accuracy: The estimation of TGF-β in cytosolic fractions was found to be accurate (Table 2).

Analytical recovery: TGF-β standards added to the cytosolic pools had 89.3–103.2% (mean 92.3%) recovery over the entire range tested (125–1000 pg/ml).

Lower detection limit: The minimum detectable level of TGF-β in cytosolic fractions was 9 pg/ml. It was determined by adding three standard deviations to the mean value of 10 zero calibrators.

Clinical sample analysis: The mean ranks of the different parameters studied were highly significant among different groups as shown by Kruskal Wallis one way ANOVA, Table 3. A significant correlation only between FIGO stage and nitrate concentrations, Table 4. Levels of nitrates and TGF-β were significantly correlated in all cases (r = 0.24, P = 0.01), as well as in malignant cases (r = 0.3, P = 0.026). No significant correlation was found between ploidy status, SPF and either of TGF-β, or nitrate levels in the malignant group.

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**Table 3**
The relation of the different parameters to the type of ovarian tissue as analyzed by Kruskal-Wallis 1-way Anova

| Mean rank | Normal (n = 36) | Benign (n = 37) | Malignant (n = 54) | X² | P    |
|-----------|----------------|-----------------|-------------------|----|------|
| TGF-β     | 52.58          | 56.14           | 77.08             | 12.01 | 0.0025 |
| Nitrate   | 32.33          | 50.91           | 94.08             | 67.42 | 0.000  |
| SPF       | 49.85          | 62.97           | 73.39             | 9.06  | 0.011  |

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**Table 4**
The relation of the different parameters to the stage as analyzed by Kruskal-Wallis 1-way Anova

| Mean rank | Stage I (n = 13) | Stage II (n = 8) | Stage III (n = 25) | Stage IV (n = 8) | X² | P    |
|-----------|-----------------|-----------------|-------------------|-----------------|----|------|
| TGF-β     | 24.15           | 21.00           | 28.62             | 35.94           | 4.38 | 0.22 |
| Nitrate   | 13.54           | 23.56           | 32.16             | 39.56           | 17.6 | 0.000 |
| SPF       | 24.73           | 30.50           | 26.96             | 27.75           | 0.66 | 0.88 |

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Fig. 1. Receiver operating characteristic (ROC) curves for TGF-β (a), and nitrates (b). The best cut-offs (circles) for TGF-β is 290 pg/mg protein, and 310 nmol/mg NPN for nitrates. Areas under the curve = 0.679, and 0.939 respectively.
Cut-off points for TGF-β, and nitrates: Cut-off values were determined for both TGF-β (290 pg/mg protein) and nitrates (310 nmol/mg NPN) using ROC curves, Fig. 1. At the determined cut-off, nitrate level was superior in discrimination of malignant from non-malignant cases with a sensitivity of 93%, and a specificity of 84%. Only 4 malignant samples were below the chosen cut-off. Above the cut-off, 16 were stages I&II, while 34 were stages III&IV ($X^2 = 7.3, P = 0.015$). On the other hand, the chosen cut-off for TGF-β revealed 44% sensitivity, and 92% specificity in discrimination between malignant and non-malignant cases. However, this cut-off maximized the sum of sensitivity (76%), and specificity (82.4%) in predicting bad prognosis, Table 5. No significant relation was noticed between TGF-β above the cut-off and any of the other parameters.

Survival analysis: Cox multivariate analysis showed that The FIGO stage, TGF-β (above 290 pg/mg protein), as well as the type of operation whether optimal or suboptimal debulking were significantly correlated to survival of the ovarian cancer patients, Table 6. Fig. 2 showed the significant difference between survival in patients with TGF-β above, and below the determined cut-off. Mean TGF-β in non-survivors (mean ± SD, 345.3 ± 160.8) was significantly higher than survivors (mean ± SD, 188.6 ± 93.8), $P < 0.001$. The mean nitrate level was also significantly higher in patients who died over the follow up period (mean ± SD, 605.0 ± 160.6) than patients who survived (mean ± SD, 453.4 ± 171.2), $P = 0.002$.

DISCUSSION

Much evidence suggests that the initiation and progression of cancer depend upon diverse processes, including multiple genetic alteration, altered response to peptide growth factors, activation of oncogenes, and inactivation of tumor suppressor genes [36,15]. Both TGF-β, and NO are related to angiogenesis and enhanced vasculature permeability, and increased blood

| Parameter       | Wald chi-square | $P$-value | Risk ratio |
|-----------------|-----------------|-----------|------------|
| FIGO stage      | 14.65           | 0.0001    | 10.2       |
| Histological grade | 1.29        | 0.25      |            |
| TGF-β           | 7.6             | 0.006     | 3.7        |
| Nitrate         | 1.38            | 0.25      |            |
| Operation       | 10.2            | 0.004     | 4.2        |

Table 5
Sensitivity and specificity at different cutoff points of TGF-β for discrimination between good outcome (survivors), and poor outcome (non survivors)

| TGF-β* | Sensitivity | Specificity |
|--------|-------------|-------------|
| ≥ 340  | 60%         | 89.7%       |
| ≥ 320  | 68%         | 86.2%       |
| **≥ 290| 76%         | 82.8%       |
| ≥ 250  | 76%         | 79.3%       |
| ≥ 220  | 76%         | 58.6%       |

* Values of TGF-β are expressed in pg/mg protein.
** The cut-off that maximized the sum of sensitivity, specificity.
flow [25,31]. NO is related to accumulation of the tumor suppressor gene p53 which occurs in about 50% of epithelial ovarian cancer. We studied TGF-β, and NO association with epithelial ovarian cancer in which p53 accumulation, as well as angiogenesis affect its biology, and peritoneal dissemination.

Previous studies of cancer were performed by immunohistochemistry, which is an important, but subjective method. We found that quantitative estimation of TGF-β in ovarian cytosolic fractions was accurate and reproducible using EIA. This allows us to have precise results for TGF-β.

In ovarian cancer, TGF-β is regarded as a potent anti-mitogen during early tumor development by blocking the late G1 activation of cdkks, thereby preventing pRb phosphorylation and S phase entry [1,18]. On the other hand, Cell-autonomous TGF-β signaling is required for both induction and maintenance of in vitro invasiveness and metastasis during late-stage tumor-genesis [34]. In this study, TGF-β showed higher levels in malignant ovarian tissues. This may be due to loss of responsiveness of tumor tissues to TGF-β as a result of TGF-β II receptor inactivation by mutations, or may be a contribution of TGF-β in the process of tumor development. It is widely accepted that TGF-β is a potent angiogenic factor in vivo, although an inhibitory effect can be observed in vitro. TGF-β induces connective tissue and inflammatory cell chemotaxis, and it has been suggested that these cells are capable of releasing direct-acting angiogenic cytokines [35]. It is also likely that TGF-β stimulates several proteases, protease inhibitors, matrix proteins, and their receptors [19,30] that may increase the potential capacity of TGF-β to enhance tumor dissemination. Nakanishi et al, [31], suggested that angiogenesis is equally stimulated regardless of stage of ovarian cancer. This supports our findings that TGF-β was not correlated to FIGO staging of the tumors. Hurteau et al. [18] reported that 11 of 18 ovarian cancer cell lines expressed immunohistochemically detectable TGF-β, and that TGF-β significantly inhibits [3H]-thymidine incorporation into ovarian cancer cell lines. They also suggest that loss of TGF-β production may interrupt the TGF-β autocrine inhibitory loop and play a role in the development of some ovarian cancers. However, information obtained only from in vitro cancer cell proliferation might not properly reflect the in vivo action of TGF-β in
epithelial ovarian cancer.

NO can affect p53 gene by deaminating DNA nucleobases [31]. Deamination of 5-methylcytosine to thymine is responsible for C to T transition mutations that are frequently observed in p53 [38]. NO may also affect p53 protein by oxidizing SH groups of cysteine residues leading to the formation of disulfide bonds. Also it complexes with metal ions as zinc [17]. P53 protein is a zinc dependent transcription factor, which binds specific DNA sequences and transactivates the expression of genes under promoters containing p53 binding sites, therefore playing a critical role in mediating cell cycle arrest in G1 [23], or apoptosis [7] in response to DNA damage. All of these effects could be important for the contribution of multistage process of carcinogenesis. These reports perfectly explain our finding that nitrate— the stable end product of NO— increases significantly from normal ovarian tissues, to benign, then to malignant. Moreover, they explain the significant relation between nitrate levels and FIGO stage of epithelial ovarian cancer.

The positive correlation shown here between TGF-β and nitrate levels in all the groups as well as in malignant group can be explained by the relation of both to angiogenesis [25,31,35]. This relation could also explain the higher mean levels of both parameters in poor over good outcome.

The determined cut-off for TGF-β (290 pg/mg protein) is not useful as a marker for epithelial ovarian cancer because of its low sensitivity, (44%). On the other hand, the cut-off for nitrates (310 nmol/mg NPN) discriminates well between malignant ovarian tissues and both benign and normal which matches with the postulate that NO induces p53 accumulation [6]. This also suggests the contribution of NO in multistep carcinogenesis of epithelial ovarian cancer. The same cut-off for TGF-β (290 pg/mg protein) discriminated patients who had poor outcome with a sensitivity of 76% and a specificity of 82.8%. Above this cut-off, TGF-β was found to be an independent prognostic indicator of poor survival as demonstrated before by Nakanishi et al. [31] who reported that patients with positive TGF-β (by immunohistochemistry) had more disseminated disease and showed a lower survival rate in epithelial ovarian cancer.

In conclusion, the current study indicates the association of TGF-β, and NO with epithelial ovarian cancer. The expression of TGF-β is a prognostic indicator, while NO is more related to the process of multistep carcinogenesis which makes nitric oxide synthases potential targets for therapeutic intervention in human tumorigenesis.

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