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# Procoagulant and Fibrinolytic Activity of Human Ovarian Carcinoma Cells in Culture

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**Abstract**—*We have investigated here the coordinate expression of both procoagulant (PCA) and fibrinolytic (FA) activity of cells from* 16 *human ovarian carcinoma cases. To avoid interference of contaminating host cells, we used cells isolated in primary culture from ascitic fluid or from solid tumor. The FA was determined in cellular extracts by an amidolytic assay in the presence of fibrin monomers. FA, which was plasminogen dependent in almost all of the cases, showed a wide range of activity (from* < 0.001 *to* 2.30 UK units/mg *protein). The molecular analysis of plasminogen activator (by SDS–PAGE and fibrin autography) showed a single molecular form of* 52,000 daltons, *inhibited by an antibody against human urokinase. PCA, studied with a one stage clotting assay in disrupted cells, was of tissue thromboplastin type in all instances and varied from* 12.0 *to* 1300 *thromboplastin units/*$10^4$ *cells. No simple correlation was found between FA and PCA in the cellular samples studied; moreover, for neither parameter was it possible to find any changes with the staging of the disease.*

## INTRODUCTION

FIBRIN has been repeatedly observed within the tumor cell microenvironment [1, 2]; its involvement in local invasion and metastasis formation is still a matter of controversy. Whatever its role, the formation and dissolution of fibrin around tumor cells is closely related to cellular activities such as procoagulant (PCA) [3, 4] and fibrinolytic activity (FA) [5].

Knowledge about these activities has been mainly derived so far from animal models [6], whereas information regarding humans is still very scanty [7, 8]. Most studies have been made on cell lines [9, 10]. Some human tumors of different origin have been studied in extract form for either PCA [11, 12] or FA activity [13–15].

Obviously the extracts reflected activities deriving not only from cancer cells but also from host cells such as vascular cells or tumor-associated macrophages. Moreover, only few data are available so far on the coordinate expression of both activities in the same tumor sample [9, 16].

We have here investigated PCA and FA in a series of human ovarian carcinoma cases. To avoid interference from contaminating host cells, we used tumor cells isolated in primary culture.

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## MATERIALS AND METHODS

### *Patients*

This study was performed on 16 patients (aged 40–69 yr) with histologically confirmed advanced ovarian carcinoma (F.I.G.O., type III–IV). Histological grading of tumors was evaluated in terms of well differentiated (grade 1) moderately differentiated (grade 2) and poorly differentiated (grade 3) [17] and according to Broders' criteria [18, 19]. Biopsy material was obtained from primary surgery, from second-look laparotomy or from ascitic fluid after paracentesis under sterile conditions.

All but three patients were undergoing polychemiotherapy treatment (Table 1).

### *Cell cultures*

Tumor biopsy specimens were collected in PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO Europe, Glasgow, U.K.). Tumour tissue fragments were disaggregated by treatment with a 0.3% collagenase solution (collagenase type 1, Sigma Chemical Company, St. Louis, MO) for 40 min at 37°C under continuous stirring. Cell preparations were then washed and resuspended in growth medium. Tumor cell suspensions contaminated by RBC and/or leucocytes were further processed as for ascitic fluid [20, 21]. Briefly, the ascitic fluid was collected in hepari-

*Table 1. Clinical data of patients with ovarian carcinoma*

| Pt | Age | Tumor type | F.I.G.O. Staging | Grading Grade | Grading Broders |
|---|---|---|---|---|---|
| 1 | 50 | SE | III | 3 | 4/4 |
| 2 | 49 | " | " | " | " |
| *3 | 39 | " | " | " | " |
| 4 | 61 | " | IV | " | " |
| *5 | 53 | " | III | 2 | 3/4 |
| 6 | 60 | " | " | 3 | 4/4 |
| *7 | 52 | " | " | " | " |
| 8 | 50 | " | " | 2 | 2/4 |
| 9 | 40 | " | " | " | 4/4 |
| 10 | 69 | UN | " | " | " |
| 11 | 60 | SE | " | " | " |
| 12 | 68 | " | " | " | " |
| 13 | 47 | " | " | N.D. | N.D. |
| 14 | 54 | UN | " | 3 | 4/4 |
| 15 | 60 | SE | " | 2 | 3/4 |
| 16 | 62 | MU | " | N.D. | N.D. |

*Patients with no previous therapy.
UN = undifferentiated.
SE = serous.
MU = mucinous.
N.D. not determined.

nized bottles and the cells were separated by centrifugation. A first gradient with 100% of Ficoll Hypaque ($d$ = 1.077; MSL, Eurobios, Paris) was performed (600 ***g*** for 20 min) to remove RBC contamination and debris. In a few cases, when gross lymphocyte and granulocyte contamination was present, a second discontinuous gradient (75% Ficoll–Hypaque, layered on 100% Ficoll–Hypaque) was performed. After these steps, in all cases, tumor cells were freed of macrophages by adhesion on plastic culture dishes [22].

A cytospin preparation was made to check for the presence of different types of normal and neoplastic cells.

Final cell suspensions, containing more than 70% viable cells (erythrosine-dye test) were seeded at 70,000 cells/cm$^2$ in 25 cm$^2$ tissue culture dishes (Corning, NY, U.S.A.).

Culture Medium was 199 supplemented with 15% fetal calf serum, 2 mM glutamine, 6% of 50 × MEM essential amino acid, 3% of 100 × MEM vitamins (Flow Lab., Irvine, U.K.) and 20 mM Hepes (Merck, Darmstadt, F.R.G.).

The pH was 7.2 in air, with osmotic pressure maintained at 285 ± 10 milliosmoles.

Cells were cultured for 5–6 days when the nests of cancer cells became well spread out. Only cultures that contained typical nests of cancer cells without fibroblast-like cells and with less than 5% macrophages, (as determined by non specific esterase staining) were used (Fig 1).

Before testing, the growth medium was discarded, the cells were washed three times with PBS and maintained overnight in serum-free culture medium. The serum-free medium was removed from the cultures and stored at −20°C until used. After addition of 2 ml of fresh serum-free medium, the cells were frozen and thawed three times and tested for PCA.

To measure FA the cultures were washed three times with cold PBS and the cells, extracted with 0.5 ml of Triton X–100 (0.25% v/v in water; Bio-Rad, Richmond, CA, U.S.A.), were removed with a rubber policeman and stored at −20°C until used.

*Assay of procoagulant activity*

PCA was measured by a one-stage plasma recalcification assay. Clotting time was determined in prewarmed plastic tubes using the following test system: 0.1 ml test material, 0.1 ml normal human plasma and 0.1 ml 0.025 M $CaCl_2$. In order to characterize the type of PCA, human plasma selectively deficient in factor VII, IX or X was used as substrate. Duplicate times differed by less than 5%. Blank clotting times were always >300 sec.

PCA was expressed in arbitrary units by comparison of clotting times of cell samples with a standard curve obtained with dilutions of a human brain thromboplastin suspension.

One thousand units of thromboplastin caused normal plasma to clot in 39 sec.

*Assay of fibrinolytic activity*

FA was determined in cell extracts by an amidolytic assay in the presence of fibrin monomers

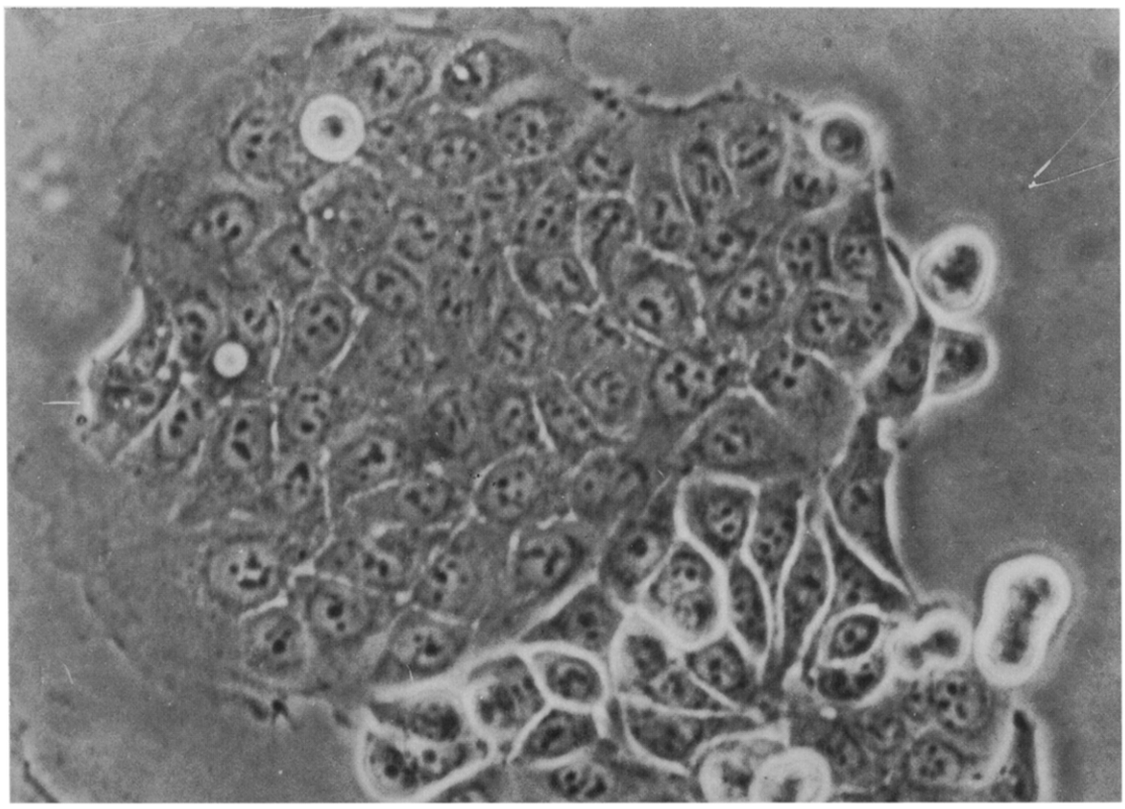

*Fig.*1. *Representative nests of cancer cells in primary culture from an ascitic fluid sample of a patient with ovarian carcinoma* (160 ×).

[23]. The standard assay mixture (1 ml) included 0.33 μM human plasminogen (AB Kabi, Stockholm, Sweden), 0.33 mM of a plasmin-specific chromogenic substrate (S-2251, Kabi Diagnostica, Stockholm, Sweden), 7 mg/l of fibrin monomers in PBS 0.15 M, pH 7.4 containing 0.25% Triton X-100 (Bio-Rad) and the cell extract or the serum-free medium. The solution was incubated at 25°C for 4 hr, the reaction stopped with 4 M sodium acetate buffer, pH 3.8, and absorbance read at 405 nm.

A curve of standard urokinase (1st International Reference Preparation of human UK, coded 66/64, a gift from Dr. Gaffney, National Institute of Biological Standards and Controls, London, U.K.) was plotted with each set of experiments and tumor-cell FA activity was expressed as urokinase equivalents units/mg of protein by linear regression analysis.

Protein content was determined by the method of Bradford [24].

Plasminogen-independent FA was determined in parallel samples, omitting the plasminogen in the standard mixture.

The molecular type of plasminogen-dependent fibrinolytic activity (PA) generated by the various cell extracts was evaluated by fibrin agarose-plate method [25] after sodium dodecyl-sulfate polyacrylamide slab gel electrophoresis (SDS–PAGE). Briefly, samples were applied to the gels and subjected to electrophoresis at 50 V at room temperature with resolving gels of 10% and stacking gels of 4% acrylamide. The PA activity was located by placing the SDS gel on a fibrin indicator gel. The apparent molecular weight (M.W.) of PA activity was estimated by comparing the lysis area with marker proteins of known M.W. [26].

For inhibitory studies anti-urokinase goat antiserum (kindly provided by Prof. B. Astedt, Lund, Sweden) was incorporated into the fibrin indicator gel.

## RESULTS

Table 2 shows the results of PCA and FA assays on cell preparations from 16 patients. From some patients tumor specimens could be obtained more than once.

PCA was detectable in all samples and ranged from 12.0 to 1300 thromboplastin equivalent units. In samples obtained from the same patients on different occasions the range of PCA was narrower than on the whole population. No detectable activity was present in the culture medium even when the sample was concentrated up to 10 times.

FA was below the detection limit (0.001 u UK/mg) in 8 out of 18 samples tested. In the remaining samples, including those from the same patients, a wide range of activity was observed. No activity was detectable in the overnight culture medium. There was no simple correlation between PCA and FA values. PCA was identified in all instances as tissue factor since it required coagulation F VII, not factor IX, for its expression (Table 3); moreover, no activity was present in factor X deficient plasma (<1%).

*Table 2. Procoagulant (PCA) and fibrinolytic (FA) activity of human ovarian carcinoma cells in primary culture*

| Pt | Cell source | FA (u UK/mg) | PCA ($AU/10^4$ cells) |
|---|---|---|---|
| 1 | Ascitic fluid | N.D. | 34.4 |
| 2a | Ascitic fluid | 0.21 | 134.6 |
| 2b | " | 0.23 | 267.6 |
| 3 | Ovary | 0.16 | 67.0 |
| 4 | Ovary | 1.90 | 206.6 |
| 5 | Ascitic fluid | N.D. | 12.8 |
| 6 | Ovary | <0.001 | 358.5 |
| 7a | Ascitic fluid | 1.02 | 643.4 |
| 7b | " | 1.33 | 351.5 |
| 7c | " | 2.05 | 1317.4 |
| 8a | Ascitic fluid | 2.30 | 608.0 |
| 8b | " | 0.50 | 564.4 |
| 8c | " | 1.57 | 204.1 |
| 9 | Ascitic fluid | N.D. | 659.6 |
| 10 | Ascitic fluid | <0.001 | 134.7 |
| 11 | Ascitic fluid | <0.001 | 1168.0 |
| 12a | Omentum | <0.001 | 308.5 |
| 12b | Ascitic fluid | <0.001 | 138.5 |
| 13 | Ascitic fluid | N.D. | 230.0 |
| 14 | Ascitic fluid | N.D. | 81.1 |
| 15a | Ascitic fluid | <0.001 | 41.5 |
| 15b | Ascitic fluid | 1.33 | 58.9 |
| 16 | Ascitic fluid | <0.001 | 70.6 |

a, b, c represent different occasions on which samples from the same patient could be studied.
N.D. not determined.

As shown in Table 4, in all but one of the samples FA was mainly accounted for by a plasminogen-dependent activity, identified as urokinase-like activity (u-PA) on the basis of the M.W. in the SDS-fibrin overlay method and of their inhibition by anti-human UK antiserum.

Plasminogen-independent activity, detectable in all samples in variable amounts, was generally low.

## DISCUSSION

Several studies in experimental tumor models have shown that, depending on the type of tumor, cancer cells may express two different types of activity capable of triggering blood clotting, namely tissue factor activity (the initiating cofactor of the extrinsic pathway) or PCA, called cancer procoagulant, that directly activates coagulation factor X [3, 4, 27]. It has been suggested that quantitative variations of these activities may influence the metastatic behaviour of some experimental tumors [28].

*Table 3. Characterization of PCA of human ovarian carcinoma in culture*

| Pt. | PCA (U/10 cells) Normal plasma | F IX def. | F VII def. |
|---|---|---|---|
| 1 | 34.4 | 32.3 | N.D. |
| 2a | 134.6 | 129.4 | 0.50 |
| 2b | 267.6 | 248.0 | 0.50 |
| 3 | 67.0 | 68.0 | 0.50 |
| 4 | 206.6 | 288.0 | 0.50 |
| 5 | 12.8 | 11.5 | 0.50 |
| 6 | 358.8 | 315.4 | 2.15 |
| 7a | 643.4 | 715.3 | 2.99 |
| 7b | 351.5 | 310.3 | 1.52 |
| 7c | 1317.4 | 1200.1 | 6.97 |
| 8a | 608.0 | 700.0 | 2.15 |
| 8b | 564.4 | 503.7 | 4.79 |
| 8c | 204.1 | 172.7 | 1.38 |
| 9 | 659.6 | 520.3 | 2.83 |
| 10 | 134.7 | 140.1 | 4.01 |
| 11 | 1168.0 | 1428.0 | 31.53 |
| 12a | 308.5 | 320.1 | 7.74 |
| 12b | 138.5 | 150.1 | 4.70 |
| 13 | 230.0 | 170.3 | 6.28 |
| 14 | 81.1 | 77.5 | 0.50 |
| 15a | 41.5 | 37.8 | 0.50 |
| 15b | 58.9 | 51.0 | 1.36 |
| 16 | 70.6 | 65.3 | 0.50 |

Much less information is available on the content and nature of PCA in human malignant tumors. Kadish *et al.* [10] reported significant amounts of tissue factor in homogenates and intact whole cells from a variety of human neoplastic cell lines but no differences were found between neoplastic and normal cells. Studies of clinical material have been conducted mainly on tissue extracts. Sakuragawa *et al.* [29] found that PCA of gastric cancers was greater than in normal samples. Gordon *et al.* [12] reported PCA similar to that described in animal models (i.e. factor X activating activity) in a number of tumor extracts. Extracts, however, may also contain activities derived from host cells, such as vascular cells, fibroblasts and macrophages.

Fibrinolytic activity has been studied in a large variety of tumor cell lines of animal and human origin. Human clinical material has been mainly studied in the form of tissue extract or organ cultures [5, 15, 30]. In many instances a urokinase-like PA was found, although tissue-type PA was reported in melanoma and in prostatic carcinoma [5]. Attempts to correlate PA with the metastatic potential of cancer cells gave conflicting results [14]. It must be emphasized that in general FA and PCA have been studied independently.

It has been repeatedly suggested that PCA and FA of cancer cells play a role in fibrin deposition and dissolution at the tumor–host interface. Conceivably the net amount of fibrin in the tumor microenvironment depends, besides other less well defined factors, on the balance between clot promoting and fibrinolytic activites of cancer cells. It is therefore regrettable that so far these activities have been studied separately.

The present experiments were designed to investigate the simultaneous expression of both PCA and FA on the same clinical material. In order to minimize any interference of contaminating host cells we used here primary cultures from ovarian carcinoma.

*Table 4. Characterization of plasminogen activator activity associated with human ovarian carcinoma cells in culture*

| Pt | Fibrinolytic activity Total (uUK/mg) | Pmg dep. (%) | Pmg indep. (%) | M.W. | Inhibition by anti-UK |
|---|---|---|---|---|---|
| 2a | 0.21 | 90 | 10 | N.D. | N.D. |
| 2b | 0.23 | 90 | 10 | N.D. | N.D. |
| 3 | 0.16 | 10 | 90 | 52,000 | Yes |
| 4 | 1.9 | 91 | 9 | 52,000 | Yes |
| 6 | <0.001 | – | – | – | – |
| 7a | 1.02 | 66 | 33 | 52,000 | Yes |
| 7b | 1.33 | 93 | 7 | 52,000 | Yes |
| 7c | 2.05 | 61 | 39 | 52,000 | Yes |
| 8a | 2.3 | 67 | 33 | 52,000 | Yes |
| 8b | 0.5 | 97 | 3 | 52,000 | Yes |
| 8c | 1.57 | 99 | 1 | 52,000 | Yes |
| 10 | <0.001 | – | – | – | – |
| 11 | <0.001 | – | – | – | – |
| 12a | <0.001 | – | – | – | – |
| 12b | <0.001 | – | – | – | – |
| 15a | <0.001 | – | – | – | – |
| 15b | 1.33 | 90 | 10 | 52,000 | Yes |
| 16 | <0.001 | – | – | – | – |

PCA was present in cells from all tumor specimens tested but varied widely from one sample to another. In all cases it was of the tissue-factor type. The lack of activity in the culture supernatant suggests that the material is not secreted from the cells. In some transformed or malignant cells from experimental tumors and in extracts from human tumors a peculiar type of procoagulant (PCA) has been described [6, 12, 27], which has never been found in normal adult tissue. On the basis of these observations it has been suggested that PCA may represent a marker of malignancy.

The findings of the present study do not support this concept, at least in human cultured cells. Further challenge to the concept of PCA as marker of malignancy comes from recent evidence in our laboratory that transformed cells, like their normal counterparts, do express the same type of PCA (tissue thromboplastin) [31].

Similarly to PCA, FA too varied widely among patients and within the same patients' repeated samples. In all cases but one FA was mostly dependent on plasminogen and characterized as urokinase-type plasminogen activator as described some years ago on organ cultures [15].

The possibility cannot be excluded that the ample interindividual variability found in both PCA and FA be ascribed to random selection of clones with different biological activities.

No simple correlation was found between PCA and FA of each sample. It must be underlined that most of the patients were, at the time of study, under polychemotherapy and the possibility that such treatment influenced the parameters studied here cannot be excluded.

In conclusion, both PCA and FA can be easily demonstrated and characterized in cultured cells from a human tumor; however, their actual relevance to clinical malignancy appeared much more difficult to establish. Although on the basis of animal experiments, many correlations have been proposed so far between PCA or FA and the degree of cancer cell invasiveness; our findings clearly warn against ready extrapolation from animal data to human malignancy.

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