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Quantification of the concentration gradient of biomarkers between ovarian carcinoma interstitial fluid and blood

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ABSTRACT

Background: Tumor interstitial fluid (TIF) rather than plasma should be used in cancer biomarker discovery because of the anticipated higher concentration of locally produced proteins in the tumor microenvironment. Nevertheless, the actual TIF-to-plasma gradient of tumor specific proteins has not been quantified. We present the proof-of-concept for the quantification of the postulated gradient between TIF and plasma.
Methods: TIF was collected by centrifugation from serous (n = 19), endometrioid (n = 9) and clear cell (n = 3) ovarian carcinomas with early (n = 15) and late stage (n = 16) disease in grades 1 (n = 2), 2 (n = 8) and 3 (n = 17), and ELISA was used for the determination of CA-125, osteopontin and VEGF-A.
Results: All three markers were significantly up-regulated in TIF compared with plasma ($p < 0.0001$). The TIF-to-plasma ratio of the ovarian cancer biomarker CA-125 ranged from 1.4 to 24,300 (median = 194) and was inversely correlated to stage ($p = 0.0006$). The cancer related osteopontin and VEGF-A had TIF-to-plasma ratios ranging from 1 to 62 (median = 15) and 2 to 1040 (median = 59), respectively. The ratios were not affected by tumor stage, indicative of more widespread protein expression.
Conclusion: We present absolute quantitative data on the TIF-to-plasma gradient of selected proteins in the tumor microenvironment, and demonstrate a substantial and stage dependent gradient for CA-125 between TIF and plasma, suggesting a relation between total tumor burden and tissue-to-plasma gradient.
General significance: We present novel quantitative data on biomarker concentration in the tumor microenvironment, and a new strategy for biomarker selection, applicable in future biomarker studies.

1. Introduction

As pathological tissues are perfused by blood and drained by lymph vessels, locally secreted proteins are assumed to enter the circulation, generating disease-specific signatures in the blood [1–5]. It is likely that disease related biomarkers could be found in substantially higher concentrations closer to the source, i.e. in the tumor microenvironment [4,5]. Although the concentration gradient is hypothesized to be 1000- to 1500-fold [4], the actual gradient between tumor interstitial fluid (TIF) and plasma has to our knowledge not been quantified.

The exact quantification of potential gradients has been hampered by the lack of suitable methods for isolation of undiluted TIF. As recently reviewed in Haslene-Hox et al. [6] and Wiig & Swartz [7], most methods used for TIF isolation, such as capillary ultrafiltration, microdialysis and tissue elution add physiological buffer to extract proteins from the interstitium. Accordingly, TIF will be diluted to an unknown extent, preventing direct comparison of plasma and TIF concentrations.

The cancer antigen 125 (CA-125) is a high molecular weight cell membrane glycoprotein, with a molecular weight > 500 kDa [8,9] expressed by a variety of epithelial cells. It was introduced as the first serum tumor marker test for ovarian cancer patients in 1983 [10–13]. CA-125 is to this date one of the few proteins that is routinely used as a biomarker of cancer in the clinic and the reference standard for validation of new biomarker candidates [14]. This protein is thought to originate from the tumor tissue and a serum concentration > 35 U/ml

Abbreviations: CA-125, cancer antigen 125; EDTA, ethylenediaminetetraacetic acid; FIGO, International Federation of Gynecology and Obstetrics; PBS, phosphate buffered saline; TIF, tumor interstitial fluid

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is considered pathological. The presence of CA-125 in ovarian tumor tissue has earlier been determined mainly in a semi-quantitative manner by immunohistochemistry [15]. CA-125 concentration in blood can also be used to monitor recurrent disease in ovarian cancer patients [16].

We also wanted to extend the quantification in TIF to include additional proteins, and chose two proteins highly related to cancer in general, that were likely to be produced in the tumor. VEGF-A, which is central for angiogenesis and lymphangiogenesis in the body, and especially in cancer [17], and osteopontin, which [18] has been shown to have a role in many steps of cancer development and is up-regulated in many cancers [19]. Osteopontin has also been suggested as a biomarker for ovarian cancer [18,20] as well as renal cell [21], gastric and liver carcinomas [22].

Utilizing the centrifugation technique developed by Wiig et al. [23,24] to isolate native, undiluted TIF, we could determine the gradient of tissue specific proteins from TIF to plasma. In this manner we could demonstrate that the local production of the established ovarian cancer biomarker CA-125 resulted in several orders of magnitude higher concentration in the tumor interstitium than in the plasma at early stage disease. This is in contrast to the two smaller proteins osteopontin and VEGF-A, known to be cancer specific and to be induced in several pathological conditions [17,19], that do not show any stage-related correlation in the present cohort.

2. Materials and methods

2.1. Ethics statement

The research protocol has been approved by the Norwegian Data Inspectorate (Protocol # 961478-2), Norwegian Social Sciences Data Services (Protocol # 15501) and the Regional Committee for Medical and Health Research Ethics (REK-Vest, Protocol ID REKIII nr. 052.01). All samples were collected after obtaining the patients' written informed consent. The work conformed to the standards set by the latest revision of the Declaration of Helsinki.

2.2. Collection of blood, ascites and TIF samples

Blood samples were collected from the patients 1–2 days before surgery and EDTA was added as the anticoagulation agent prior to the isolation of plasma by centrifugation. Ascites and tumor samples were collected during surgery from patients operated for epithelial ovarian cancer tumors at the Department of Obstetrics and Gynecology at Haukeland University Hospital. Ascites were collected in centrifuge tubes, centrifuged at 3220 *g* for 20 min and the supernatant was extracted and stored at −80 °C until further processing. Tumor samples were taken from the surface of the primary tumors, in an area without any apparent necrosis or inflammation. The surface region was selected for sampling, as this has been shown to result in a lower contribution from intracellular fluid to the isolated TIF [23]. Tumor samples (0.2–0.5 g) for biobank storage were snap-frozen in liquid nitrogen and transferred for storage at −80 °C. Tumor samples for TIF analysis were placed on ice and transported to the laboratory. TIF was isolated from the fresh tissue (tissue weight mean = 0.32 ± 0.02 g, range 0.13–0.53 g) approximately 60 min after extirpation by centrifugation through a mesh-filter at 106 *g* for 10 min [23,24] and stored at −80 °C until further processing. Samples larger than 0.5 g were cut in multiple samples which were centrifuged in parallel. The isolated fluid has earlier been validated as representative for interstitial fluid [24]. Selected samples were taken from the biobank and thawed and subsequently centrifuged as explained above (samples indicated by [a] in Table 1). Two samples were used as references with fluid isolated both from fresh and biobank tissue.

2.3. Tissue elution

One sample (Patient no. 14, Table 1) with ample amounts of tumor tissue was cut in two. One half was used for the isolation of TIF as described above, and the other was eluted as described by Celis et al. [24,25]. In brief, 0.31 g of tumor sample was washed in PBS, cut into small pieces of approximately 1–3 mm^3 and incubated at 37 °C in a pre-weighed 15 ml conical plastic tube containing 1 ml PBS with 0.128 TIU aprotinin to inhibit proteolysis. After 1 h, 350 μl of eluate was extracted (E1). The remaining buffer and tumor sample was further incubated overnight and the supernatant was extracted (E24). Both eluate samples were centrifuged immediately after extraction at 4622 *g* for 20 min, and supernatants were frozen at −20 °C for later analysis.

2.4. ELISA analysis

Before CA-125 analysis, samples were diluted in 50 mg/ml bovine serum albumin to maintain a similar matrix for all sample types. Plasma and ascites were diluted 1:2, TIF 1:100 and eluates 1:20 (E1) and 1:34 (E24) to match the protein mass in plasma, to a total volume of 400 μl for all samples. CA-125 concentration was measured with the Elecsys CA 125 II tumor marker assay (Catalogue number 11776223 322, Roche Diagnostics, Mannheim, Germany).

Osteopontin was quantified in plasma and TIF with an osteopontin human ELISA kit (Cat. no. ab100618, Abcam, Cambridge, UK), following the manufacturer's recommendations. Plasma was diluted 1:100 and TIF was diluted 1:1000 in buffer A and run in duplicate. Briefly, samples and standards were added to the coated 96-well plate and gently rotated overnight at 4 °C. The plate was washed four times with washing solution, and incubated with biotinylated osteopontin detection antibody for 1 h. Subsequently, the plate was washed and incubated for an additional 45 min with HRP–streptavidin solution. The washing step was repeated, and One-Step reagent solution was added and the plate was incubated in the dark for 30 min. Finally, the stopping solution was added and the optical density was read spectrophotometrically at 450 nm.

VEGF-A was quantified in plasma and TIF with a VEGF-A human ELISA kit (Cat. no. ab100662, Abcam, Cambridge, UK), following the manufacturer's recommendations, and as briefly summarized for osteopontin. Plasma was diluted 1:10 and TIF was diluted 1:100 in buffer A and run in duplicate.

2.5. Data and statistical analysis

GraphPad Prism (Software version 6.0, GraphPad Software Inc., La Jolla, CA) was used for statistical analysis. $p \leq 0.05$ was considered statistically significant when not stated otherwise. Values are given as mean ± SEM. Wilcoxon matched pairs signed rank test was used to compare paired TIF and plasma concentrations, and the non-parametric Mann–Whitney test to compare concentrations in early and advanced stage patients. To evaluate whether the values were changing with stage, linear regression for concentrations and linear regression on log-transformed values for ratios were performed.

3. Results

Selected proteins were measured in matched TIF and plasma from 31 patients. All patients had either serous (n = 19), endometrioid (n = 9) or clear cell (n = 3) tumors. International Federation of Gynecology and Obstetrics (FIGO) stage of disease ranged from IA to IV, with an even number of samples from patients with advanced (n = 16) and early stage (n = 15) disease (Table 1). The grades ranged from high to low differentiation, with the majority of samples having low differentiation (grade 3, n = 18, Table 1). Ascites was investigated when available (n = 4). Histological data are given in Table 1. Having limited access to fresh early stage tumors we isolated fluid from frozen and thawed tumor samples for 11 early stage tumors. To assess the influence of