**Report**

**Urokinase receptor in breast cancer tissue extracts. Enzyme-linked immunosorbent assay with a combination of mono- and polyclonal antibodies**

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**Summary**

Urokinase plasminogen activator (uPA) is a proteolytic enzyme involved in degradation of the extracellular matrix during cancer invasion. The levels of uPA and its inhibitor PAI-1 in tumor extracts have previously been demonstrated to be of prognostic value in breast cancer as well as other types of cancer. We have previously characterized a specific cell surface receptor for uPA (uPAR) which strongly enhances the catalytic activity of uPA and is expressed during mammary cancer invasion. In order to quantitate uPAR in breast cancer tissue, we have now developed a sensitive enzyme-linked immunosorbent assay (ELISA), with polyclonal catching antibodies and three monoclonal detecting antibodies. The detection limit of the assay is approximately 0.16 fmol of uPAR in a volume of 100 µl (1.6 pM). There is a linear relationship between signal and uPAR concentration up to at least 6.6 fmol per 100 µl (66 pM). Both free uPAR and uPAR in complex with uPA is detected. The recovery of an internal uPAR standard in breast cancer tissue extracts is above 87%. The intra-assay and inter-assay variation coefficients are 7% and 13%. In order to find a suitable buffer for extraction of various components of the uPA-system from breast cancer tissue, we tested buffers which previously have been used for optimal extraction of estrogen receptor (A), uPA (B), and uPAR (C). Buffer A and B extracted approximately 30% and 50%, respectively, of the amount of uPAR extracted with buffer C. Extracts of samples of breast cancer tissue from 94 patients all contained uPAR in amounts above the detection limit of the present assay, which appears suitable for studies of the potential prognostic value of uPAR in this disease. Significant correlations were found between uPAR, uPA and PAI-1 tumor levels.

**Introduction**

The plasminogen activation system is a complex proteolytic enzyme cascade, which together with other enzyme systems participates in degradation of the extracellular matrix during tissue remodeling under normal and pathological conditions, including cancer invasion [1–3]. Plasminogen, an abundant proenzyme, is converted to the active serine protease plasmin by urokinase plasminogen activator (uPA). A specific cell surface uPA receptor (uPAR), which binds both uPA and its proenzyme...
pro-uPA with high affinity ($K_d = 0.1 \text{--} 1.0 \text{ nM}$), was first demonstrated on monocytes [4] and since on a variety of different cell lines of normal and neoplastic origin [5, 6]. uPAR is an extensively glycosylated protein with an $M_r$ of $\approx 55,000 \text{--} 60,000$ [6, 7]. It is anchored to the cell membrane by a glycosylphosphatidylinositol moiety [8, 9] and consists of three homologous repeats, of which the NH$_2$-terminal constitutes the uPA-binding domain I [10, 11]. Concomitant binding of pro-uPA to uPAR and of plasminogen to abundant low affinity lysine binding sites on cell surfaces leads to a strong acceleration of plasmin formation [12, 13], and the uPA/uPAR interaction appears to play a crucial role in cancer invasion [14].

The levels of uPA and its type 1 inhibitor (PAI-1) in breast cancer tissue as determined by ELISA's are both strong prognostic parameters [15-19]. We have previously developed a panel of monoclonal antibodies to uPAR [11] and used two of these to develop a quantitative ELISA suitable for measuring uPAR in extracts of cultured cells [11, 20]. With the aim of studying the possible prognostic value of uPAR levels in breast cancer tissue, we have now by immunization with a recombinant human uPAR variant raised polyclonal uPAR antibodies, established a more sensitive ELISA based on a combination of the polyclonal and three monoclonal antibodies, and found that this assay is suitable for quantitation of uPAR in breast cancer tissue extracts.

**Materials and methods**

**Materials**

Streptavidin-peroxidase complex was purchased from Zymed, CA, USA, biotinamidocaproate N-hydroxysuccinimide ester from Sigma Chemical Co, St. Louis, MO, USA, and peroxidase conjugated swine anti-rabbit IgG, P217, from Dako, Glostrup, Denmark.

**Tissue**

Invasive ductal breast carcinomas, obtained from the Department of Pathology, Bispebjerg Hospital, Copenhagen, were pre-cooled in liquid nitrogen and pulverized in a Micro-Dismembrator (Braun-Melsungen). For a part of this study tissue powders from 20 individual tumors (mean weight $330 \text{ mg} \pm 154 \text{ (SD)}$) were pooled and mixed. The pooled material was then divided into 9 equal parts (approximately $200 \text{ mg}$ each), and suspended in ice-cold extraction buffer (3 parts with buffer A, three parts with buffer B, and three parts with buffer C, see Table 1) in a ratio of 1:4 (i.e. $200 \text{ mg}$ tissue plus $600 \mu$-buffer). The particle-free supernatant resulting from centrifugation at $105,000 \text{ g}$ for 1 h at $4^\circ \text{C}$ is designated as the tumor extract. In addition, tissue powders from 94 invasive ductal breast carcinomas (obtained from the Department of Medicine, University of Texas Health Science Center at San Antonio) were individually extracted in the same way with buffers A and B.

**Protein analysis**

The Bradford method for protein analysis [21] was employed using the Bio-Rad kit with bovine serum albumin (BSA) as a standard.

**Extraction buffers**

The composition of the three different buffers is indicated in the legend to Table 1.

**Antibodies**

Mouse monoclonal antibodies against human uPAR, designated R1 to R10, were obtained from a single fusion [11]. Anti-uPAR R3, anti-uPAR R5, and anti-uPAR R9 are directed against different epitopes in domain 1 of uPAR, while anti-uPAR R2 and anti-uPAR R4 are directed against different epitopes in either domain 2 or 3 of uPAR (ref.11 and unpublished results). The monoclonal antibodies