"MEASURING THE METASTATIC POTENTIAL OF CANCER CELLS"

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ABSTRACT

Cancer cells must secrete proteolytic enzymes to invade adjacent tissues and migrate to a new metastatic site. Urokinase (uPA) is a key enzyme related to metastasis in cancers of the lung, colon, gastric, uterine, breast, brain and malignant melanoma. A NASA technology utilization project has combined fluorescence microscopy, image analysis and flow cytometry, using fluorescent dyes, and urokinase-specific antibodies to measure uPA and abnormal DNA levels (related to cancer cell proliferation) inside the cancer cells. The project is focused on developing quantitative measurements to determine if a patient's tumor cells are actively metastasizing. If a significant number of tumor cells contain large amounts of uPA (esp. membrane-bound) then the post-surgical chemotherapy or radiotherapy can be targeted for metastatic cells that have already left the primary tumor. These analytical methods have been applied to a retrospective study of biopsy tissues from 150 node negative, stage I breast cancer patients. Cytopathology and image analysis has shown that uPA is present in high levels in many breast cancer cells, but not found in normal breast. Significant amounts of uPA also have been measured in glioma cell lines cultured from brain tumors. Commercial applications include new diagnostic tests for cancer cells, in different cancers, which are being developed with a company that provides a medical testing service using flow cytometry for DNA analysis and hormone receptors on tumor cells from patient biopsies. This research also may provide the basis for developing a new "magic bullet" treatment against metastasis using chemotherapeutic drugs or radioisotopes attached to urokinase-specific monoclonal antibodies that will only bind to metastatic cells.

INTRODUCTION

Malignant cells are characterized by abnormal levels of DNA, rapid proliferation, uncontrolled growth and the ability to invade surrounding normal tissues. The measurement of biochemical markers on cancer cells can provide valuable information as to disease-free survival, time to relapse and thus provides the physician valuable data for planning adjuvant therapy. Indirect immunoassays of markers extracted from biopsy tissues are important, but more precise measurements can be made by analytical cytometry. The current trend is towards microscopic analysis of the immunochemically stained tumor sections or dissociated cells, coupled with quantitation by image analysis. Specific markers can be directly associated with the cancer tissue, as opposed to biochemical extraction procedures. Tumor markers currently assessed include those which measure cellular proliferation, the presence of specific oncogenes, tumor-suppressor molecules, and cancer related proteins (see Table 1). Tumor related proteins include proteolytic enzymes which are correlated with recurrent disease and metastasis. These enzymes are involved in a cascade of proteolytic interactions with other enzymes and inhibitors which often culminate in the dispersal of invasive cancer cells through surrounding basement membranes and vascular systems and thereby allow them to relocate at metastatic sites distant from the primary tumor. Among these proteases are the plasminogen activators, their receptors and inhibitors, which together mediate key steps in the metastatic process.
Table 1. Examples of breast cancer prognostic markers currently used for patient assessment.

<table>
<thead>
<tr>
<th>PROGNOSTIC</th>
<th>MARKER</th>
<th>DESCRIPTION</th>
</tr>
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<tbody>
<tr>
<td>DNA CONTENT</td>
<td>Propidium iodide</td>
<td>DNA content variation from normal diploid (Aneuploidy)</td>
</tr>
<tr>
<td>Proliferation</td>
<td>% S PHASE</td>
<td>% of cells undergoing DNA replication</td>
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<tr>
<td></td>
<td>BrdU, IdU</td>
<td>DNA synthesis rate in S phase cells</td>
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<tr>
<td></td>
<td>Ki 67</td>
<td>Cycling (dividing) cells</td>
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<tr>
<td></td>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen expressed in G₁, S and G₂ phases of cell cycle</td>
</tr>
<tr>
<td>Receptors</td>
<td>Estrogen (ER)</td>
<td>ER negative tumors do not respond to ER hormone therapy</td>
</tr>
<tr>
<td></td>
<td>Progesterone (PgR)</td>
<td>PgR negative tumors indicate better disease-free survival (Stage II and beyond)</td>
</tr>
<tr>
<td></td>
<td>HER 2/neu, c-myc</td>
<td>Oncogenes amplified or overexpressed in breast cancer</td>
</tr>
<tr>
<td></td>
<td>EGFR</td>
<td>Epidermal growth factor Receptor, overexpressed in breast cancer</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Urokinase (uPA)</td>
<td>Plasminogen activator --&gt; plasmin --&gt;activates proteases</td>
</tr>
<tr>
<td></td>
<td>Collagenase IV</td>
<td>Metalloproteinase that dissolves collagen &amp; laminin</td>
</tr>
<tr>
<td></td>
<td>Cathepsin B &amp; D</td>
<td>Estrogen-related lysosomal enzymes</td>
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Abnormal DNA

The quantity of DNA in normal cells is a precise amount depending on the phases of the cell cycle. DNA can be measured by labeling with DNA specific fluorescent dyes (propidium iodide). The amount of dye (fluorescence) measured is directly proportional to the amount of DNA present. Also the cells can be exposed to DNA precursors (BrdU, IdU), then fluorescent labeled antibodies, specific for the DNA precursor, can be used to localize which cells are synthesizing DNA and how much [1]. Antibodies against proliferating cell nuclear antigen (PCNA) can also be used (see below). Fluorescent labeled cells then can be analysed in a laser flow cytometer or fluorescent microscope. A histogram of DNA content in normal cells shows a single diploid peak (at G₁ phase) and a tetraploid peak (at G₂+M phase). However, in most biopsies the abnormal DNA content of tumor cells is detected as a second G₁ peak or multiple peaks. Abnormal DNA (DNA aneuploidy) is considered as an independent indicator of tumor aggressiveness and poor prognosis that is used to supplement cytopathology grading of the tumor.

Proliferation

Flow cytometric measurement of the percentage of proliferating tumor cells that are involved in synthesizing DNA (S-phase cells) also is an independent indicator of malignancy. High percentages (15 -20%) of S-phase tumor cells usually indicates an aggressive malignancy and usually correlates well with abnormally high DNA content. The labeling index (LI) obtained by pulse-labeling cells with DNA precursors represents the rate that DNA is being synthesized in tumor cells. Usually, a LI > 4% is associated with a higher probability of recurrent malignancy [2]. Antibodies against Ki 67 and PCNA have been used as a measure of tumor cell proliferation. PCNA (also called cyclin) is an auxiliary protein of DNA polymerase-alpha [3]. PCNA normally appears in only trace amounts in G₁ and increases to maximum in S-phase then declines in G₂+M phase. In tumors, high levels of PCNA are expressed in the proliferating cells in all cell phases, whereas BrdU only labels cells in S phase [4].
Receptors

Hormone receptor density on cancer cells is often important as a marker for aggressive tumors and provides strategic information for post-surgical adjuvant therapy. In the case of breast cancer, the most common prognostic indicator for the past decade has been the number of lymph nodes that the primary cancer has spread into. More recently, hormone receptor density on tumor cells has gained importance. The lack of estrogen receptor in Stage I breast cancer has become an important predictor of earlier recurrence and poor survival. In stage II, the measurement of progesterone receptors is more important than estrogen receptors for predicting disease-free survival. There is a strong correlation between tumor receptor content, % S-phase cells and DNA aneuploidy. High proliferative activity is usually inversely related to estrogen receptor levels [6].

The protooncogenes HER-2/neu (also called erbB-2) and c-myc have normal roles in the control of cell growth and differentiation, but these are amplified and overexpressed in adenocarcinomas, lung, ovarian and breast cancer. The HER-2/neu protein appears to function as a receptor for mediators of growth and differentiation. The HER-2/neu protein has structural similarity to epidermal growth factor (EGF) which is a potent cellular mitogen. The measurement of cell surface receptors for HER-2/neu and EGF also has become important as a marker for invasive cancers and poor survival. Antibodies against HER-2/neu have been shown to arrest growth of tumor cells at late S or early G2 phase [7].

Proteolytic Enzymes and Metastasis

Cancer cells must secrete proteolytic enzymes to dissolve the basement membranes and intracellular matrix between the densely packed normal cells in order to leave the primary tumor and migrate to a new metastatic site via the blood or lymphatic circulatory systems. Serine proteases such as plasminogen activator enzymes have been linked with the invasion of tumor cells into adjacent normal tissues and with metastasis. Urokinase is not produced in most normal cells, except for low levels in certain types of normal kidney cells, colon, gastric mucosa, and endothelial cells lining small arteries. However, urokinase is produced in many tumors such as breast [8, 9], lung [10], colon [11], gastric mucosa [12], uterine [13], bladder [14], prostate [15], and malignant melanoma [16]. Both urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) enzymes have been studied using assays of the enzymes after extraction from tumor cells or assays of supernatant medium from tissue culture of the tumor cells [12, 13, 14, 15]. In most tumors, high levels of uPA, not tPA, has been correlated with metastasis or recurrent disease [13, 14].

High levels of urokinase (>3.49 ng/mg of total protein) extracted from breast tumor tissues have recently been shown to be a good prognostic indicator for high risk of recurrence and shorter patient survival times [17]. Primary lung and colon tumor cells also produce more uPA than metastatic cells, but different methods of extraction and assays often give widely variable results [13]. Total uPA measured from tumor tissue or secreted by cultured explants is difficult to quantitate, especially if the measurements are made on a large group of cells. The data obtained is an average value of all normal and cancer cells, rather than a measurement of each individual cell. Few direct measurements of intracellular and extracellular urokinase have been made [10, 18]. Urokinase (uPA) can be present in the tissues in several molecular forms. The inactive proenzyme is a single chain protein (scuPA) that is cleaved at Lys.158 to form the double chain, high molecular weight active form (HMW-uPA) that is 54 kDa. A low molecular weight form (LMW-uPA) can also be formed by cleavage of the HMW-uPA at Lys.135 - Lys.136 giving a 35 kDa active enzyme. The active urokinase enzyme converts plasminogen into plasmin, which in turn, dissolves intracellular fibrin matrix components as well as activating collagenases, laminases, and other related protease enzymes which are important to the anchorage and growth regulation of cells (see Figure 1). Recently, it has been shown that the HMW active form of urokinase, bound to the tumor cell membrane, is responsible for the local lysis of the extracellular matrix, hence the tissue invasion mechanism for metastasis [10]. Receptor (membrane) bound uPA is twice as efficient (catalytically) as free fluid-phase uPA [19]. The unbound uPA and the LMW form is not responsible for most of the local dissolution of extracellular matrix in the immediate vicinity of the metastatic tumor cell. The presence of plasminogen activator inhibitors (PAI-1, PAI-2) also are correlated with a better prognosis and are inversely related to high levels of uPA (poor prognosis). PAI-1 binds to the active HMW-uPA, but not to the inactive scuPA [20]. Also after PAI-1 binds to the membrane-bound active uPA the complex is internalized into the cell and degraded [21].
Figure 1. Schematic of interrelationships among urokinase forms, inhibitor, uPA receptor, activation of plasmin and subsequent protease steps that enable tumor cell invasion and lysis of the extracellular matrix and metastasis.

It is clear that the complexity of the many interrelationships within the cascade of proteolytic activations makes it difficult to use an average value for the level of uPA produced by all of the cells in the tumor. Especially, when most of the normal tissue do not produce uPA and many of the tumor cells do not produce uPA unless they are actively metastasizing. The challenge is to quantitatively measure uPA inside and on the surface of the cancer cells and then correlate those uPA levels with other specific markers to characterize the metastatic scenario for each tissue type and stage of cancer. No previous method has been developed to accurately measure the intracellular urokinase content, membrane-bound urokinase and cellular secretion levels and then correlate those urokinase levels with DNA content, DNA synthesis, hormone receptors and other markers of aggressive tumor growth to determine the metastatic potential. This project is developing a quantitative diagnostic test to be used first with existing panels of cytological evaluations of breast cancer and later for other types of cancer.

QUANTITATIVE ANALYSIS OF INDIVIDUAL CELLS

We have used flow cytometry and image analysis of fluorescent microscopic images to measure urokinase and DNA in histopathology tissue sections of breast tumors, dissociated cells (prepared in single cell suspensions) taken from tumor biopsies and in several cell lines of malignant brain tumors (gliomas). Fresh cells are isolated from tumor tissue or cytological samples and prepared for antibody incubation in the same manner. Histology sections are prepared from frozen tissues or deparaffinized sections cut from previously embedded biopsies. The antibodies specific for urokinase are incubated with the cells or tissues first, then the cells are incubated with a second antibody having a fluorescent marker detectable by analytical cytometry techniques. DNA content and synthesis rate (based on DNA stain or uptake of DNA precursors) is measured by flow cytometry or image analysis. The same cell sample can be measured for DNA content and urokinase by staining of the DNA and labeling the urokinase with a fluorescent marker that emits at a different wavelength than the DNA dye or marker. Thus both the DNA and urokinase can be measured simultaneously using two-color image analysis or flow cytometry. The image analysis can localize and quantitate uPA in the cytoplasm and cell membrane. An advantage of the use of cell lines is the ability to study uPA expression in relation to cell proliferation and DNA replication. We also are conducting a retrospective study on biopsies from 500 Stage I, node negative, breast cancer patients in collaboration with the Ontario Oncology Working Group made up of researchers from three Canadian and three U.S. cancer centers.
Methods

Attempts have been made to study the expression of uPA during exponential growth as well as in cultures that have been placed on serum-free medium. Quantitation of uPA levels involves immunofluorescent staining with anti-uPA monoclonal antibody (#394, obtained from American Diagnostica, Greenwich, CN), as primary antibody by the indirect technique. The second antibody consisted of fluorescein-conjugated goat anti-mouse IgG, for FCM and image cytometry studies, or in some cases, rhodamine-labeled goat anti-mouse IgG for image cytometry.

Cells were scraped from flasks, in lieu of trypsinization, in order to preserve membrane-bound antigen. Cells were washed in PBS and triturated to disperse the cell pellets. Single-cell suspensions were usually achieved, which were then fixed for in 0.5% paraformaldehyde 15 min. at room temperature, followed by one hour permeabilization in 70% methanol at 4°C. Cells were then washed with PBS and stained for one hour with increasing dilutions of the primary antibody or the equivalent concentrations of naive mouse IgG, both diluted in 1% BSA in PBS. Cells were then fixed for in 0.5% paraformaldehyde 15 min. at room temperature, followed by one hour incubation in secondary antibody dilute in 4% goat serum for one hour. In later experiments, cells were pre-incubated for one hour in 4% goat serum in PBS prior to addition of the secondary antibody.

Analysis of fluorescence by FCM was conducted with the 488 nm line of an argon laser of a EPICS Profile flow cytometer (Coulter Corporation, Hialeah FL). Green light (from fluorescein emission) was directed by means of a dichroic mirror to pass through a narrow bandpass interference filter (520 +/- 10 nm) to impinge on the green-sensitive PMT. Red light (from the DNA stain PI) was deflected through a 630 long pass filter to the corresponding PMT. Bivariate, 64 x 64 channel histograms were obtained for analysis of mean fluorescence intensity.

Digital image analysis was conducted using both Nikon and Zeiss fluorescence microscopes, equipped with a high resolution video camera connected to a QuickCapture board (Data Translation, Inc.) for the Macintosh II CI and Fx. The fluorescent filters in the Zeiss microscope were matched closely with the bandpass filters of the EPICS so that image analysis and FCM data on cells from the same sample could be compared. Images were stored as TIFF files and later analysed using NIH Image Version 1.4 (public domain software from NIH). Individual cells were scanned for mean optical densities and normalized for area. Areas of concentrated uPA (including membrane-bound) were further analysed by density slicing and thresholding followed by particulate analysis of those specific areas. Data were also normalized for area and staining intensity after the background was subtracted. This allows comparisons among cells from the same samples and comparisons between cell lines and different samples. Statistical analysis of the data was performed by multivariate analysis using Statview 512 (Abacus Concepts, Inc.)

Flow Cytometric Studies of Urokinase in Cultured Glioma Cells:

In order to establish the parameters for immunofluorescent analysis of urokinase (uPA) in tumor cells, studies were initiated with U937 lymphoma and human glioma cell lines, which were found to produce high levels of the plasminogen activator.

The two glioma cell lines employed in the studies were obtained from Dr. Marylou Ingram, Huntington Research Foundation, Pasadena, CA. The two cell lines, which were cultured from patient surgical biopsy material, have different morphological characteristics and growth rates. While alterations in the cells obviously occur in culture, the consistent morphology of these cell lines during passage in culture encouraged us to pursue differences in the cells' characteristics, which can provide correlations between uPA and metastatic relationship of uPA to the biological behavior of the original tumors. The first of these lines, CS, grows very rapidly as polygonal cells in monolayers and, in the absence of serum, tends to form spheroid structures. The second cell line, HBR09, has a fibroblastoid morphology although it has the characteristic immunological marker associated with gliomas, glial fibrillary acidic protein (GFAP) [22]. This cell line grows at about one forth the rate of CS.

RESULTS

Initially, there were some problems with non-specific fluorescence background which interfered with uPA quantitation. The high background was determined to be due to autofluorescence, since the levels of non-specific fluorescence remained the same even when PBS was substituted for the second antibody. Nevertheless, signal-to-noise was sufficient to measure significant differences in the immunostaining with the anti-uPA Mab. Dual staining with propidium iodide (PI) following ribonuclease treatment and fluorescein-labeled anti-uPA antibodies enabled bivariate analysis of DNA and uPA content as shown in Figure 2.
Figure 2. Flow cytometry immunofluorescence of glioma cells (HBR09 line) labeled with propidium iodide (PI) for DNA and fluorescein-conjugated antibodies for urokinase (uPA). Panel A shows the DNA histogram of these cells with G1, S and G2 + M subpopulations. Panel B compares the uPA and DNA fluorescence for cells in G1 (82% of total), in S phase (15% of total) and in G2 + M (22% of total).

Figure 3. Flow cytometry analysis of CS cell line for comparison with Figure 1 above. Panel A shows the CS cell cycle distribution of DNA fluorescence (PI). Panel B shows the fluorescence distribution of uPA vs. DNA. Most of the cells in G1 phase (22% of total) and in S phase (7% of total) contain significant levels of uPA.
We are comparing flow cytometric analysis of uPA levels in both CS and HBRO9 glioma lines. The relative levels of uPA as measured by flow cytometry immunofluorescence are tabulated in Table 2. Further studies are concentrating on measurements of uPA by image cytometry, in an effort to distinguish the membrane-bound (receptor) vs cytoplasmic uPA, since flow cytometry only measures fluorescence at "zero resolution". FCM studies have demonstrated that two color fluorescence can be used to measure uPA and DNA in the same cell population. There are often some non-standard cells in some individual cultures that require careful placements of the gates to get representative cells in all three phases of the cell cycle. It is important to know that the measurement methods are sensitive enough to determine the variability among replicate cultures from the same tumor source, since there is always some degree variability from patient to patient in any marker expression.

Both glioma cell lines produce uPA during growth and also during stationary (G1) phase, HBRO9 producing significantly higher levels of intracellular uPA. They also appear to produce more membrane-bound uPA (based on qualitative examinations of some 150 cells), however, quantitative measurements are still underway. It is noted that the HBRO9 cells had considerably more variability in the fluorescence measurements than did the CS cells.

Table 2. Urokinase levels in glioma cell lines measured by immunofluorescence flow cytometry.

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>MEAN FLUORESCENCE</th>
<th>+/- s.d.</th>
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</thead>
<tbody>
<tr>
<td>CS</td>
<td>6.13</td>
<td>0.10</td>
</tr>
<tr>
<td>HBRO9</td>
<td>32.25</td>
<td>11.3</td>
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</table>

Image Analysis of Fluorescent-labeled uPA in Breast and Brain Tumors

Evaluations of anti-uPA labeled breast cancer sections reveal that normal breast tissue does not contain uPA except for some endothelial cells lining the arterioles. Intraductal carcinomas, however, do express measurable quantities of uPA [23]. Quantitative measurements of uPA by absorption of immunologic stains as light passes through tumor cells is difficult since histopathology counterstains add to the absorption of the uPA antibody labels. Fluorescence is a better quantitative tool since the light is emitted only from the uPA molecules and it is emitted at a wavelength different from the incident light. Fluorescence emitted from whole cells (cytoprep) can clearly show the concentrations of uPA on the cell membrane as well as "hot spots" within the cytoplasm. Figure 4a shows an example of a breast tumor section illustrating the areas of uPA found in foci of tumor cells. Distinct areas of concentrated uPA are shown (white lines). Clearly, many tumor cells are not producing significant quantities of uPA and neither are most of the normal cells. Thresholding and image enhancements can often give size distribution and more information on the cells producing the uPA. Figure 4b shows the same tissue section as Figure 4a, however, this image has been analysed and pseudocolor added to the display to illustrate that considerable cellular detail remains obscured in the photo 4a.

Glioma cells that have been labeled with rhodamine-conjugated antibodies for uPA are shown in Figure 5a. Note that these cells (CS line) contain large amounts of urokinase per cell and that the uPA concentration is quite varied throughout the cytoplasm and there is a lot of cell to cell variation. Each cell can be scanned to obtain optical density levels that can be compared among cells after normalizing with area of each cell measured. Some cells exhibit "hot spots" of concentrated uPA, especially on the membrane. It is possible to measure the membrane-bound uPA by differential analysis using the mean density level of the weaker cytoplasm subtracted from that of the whole cell containing the membrane bound enzyme. Specific areas of uPA also can be measured by selecting a density slice(s) that represent the major portion of concentrated uPA. The particle size (number of pixels) to be counted is defined, then that particular density slice of fluorescence can be measured automatically by particle analysis. This will give the number of particles, average particles per group, area, perimeter of the cells and location of particle groups larger than a defined size (see Figure 5b).
Figure 4a. Digital image of breast histology section showing tumor cells and antibody labeled urokinase areas (white lines) selected by density slicing for quantitative measurement.

Figure 4b. Pseudocolor image of same section as 4a above, showing the additional morphological information contained in the recorded grey levels in the original digitized image.
Figure 5a. Photomicrograph of human glioma cells stained for urokinase (uPA) by rhodamine labeled antibodies. Note areas of concentrated uPA in selected areas of the cytoplasm and in some areas of the cell membranes.

Figure 5b. Digital image of glioma cells labeled with rhodamine-conjugated anti-urokinase antibodies. Image has been density sliced, particle size selected from 50 to 5000 pixels, then particle analysis performed, with the major areas of urokinase counted and labeled. The area and mean optical density (related to fluorescence intensity) is also recorded for statistical comparisons between cell lines and patient biopsies.
DNA can also be quantitated on a per-cell basis using image analysis. However, when using PI for DNA and fluorescein label for uPA, the amount of DNA fluorescence was often more predominant than was the uPA-related fluorescence (green wavelength) since PI fluorescence overrides FITC emissions. This required adjustment of the incident light intensity to keep both fluorescence signals in the same range so as to avoid resetting the video camera sensitivity between measurements on the same field of view. DNA quantitation can be performed more effectively by staining a dye excited in the U.V. (Hoechst 33258) and analysed in the blue region.

**DISCUSSION**

The importance of urokinase as a key enzyme in the initial mechanisms leading to tumor cell invasion and metastasis has been underscored in the past three years. Previous methods of measuring extracted uPA /mg. of protein or measuring secretion levels in cultured explants have provided statistical correlation with disease-free and overall survival [23]. There also is a strong correlation between uPA production and lymph node status in breast cancers and multivariate analyses have shown that high levels of both uPA and PAI-1 means a maximum risk of relapse. It is now time to develop more specific tests that can accurately determine the active uPA vs. the inactive scuPA, the membrane bound uPA and the PAIs that appear to have interlinked, critical roles in the migration and metastasis of breast and other cancers.

Correlations of uPA with other markers require more precise knowledge about uPA and the multiple biochemical interactions that affect its proteolytic action. Figure 6 illustrates the current methods of measuring average levels from all tumor cells vs. our method for measuring uPA directly in the cells. These methods can be used in retrospective studies where the time to reoccurrence, degree of metastasis and morbidity are known. Cumulative data on many patients (>50) can then be used to provide a prognostic indicator for the presence and degree of active metastasis occurring in primary tumors. A study of uPA in node negative breast cancer is underway.

![Diagram](676x1024px)

**CURRENT TECHNOLOGY**

- CANCER CELLS
  - EXTRACT UROKINASE FROM TUMOR CELLS
  - RIA or ELISA ASSAYS
  - AVERAGE VALUE
  - >3.5ng/mg = HIGH RISK (reccurrence)
  - <3.5ng/mg = LOW RISK

**NASA TECHNOLOGY UTILIZATION**

- BREAST & BRAIN CANCER CELLS
  - Fluorescent monoclonal antibodies against urokinase and DNA labels
  - DIRECT MEASUREMENTS OF UROKINASE AND DNA IN INDIVIDUAL CANCER CELLS (FLUORESCENCE)
  - LASER ACTIVATED FLOW CYTOMETRY (large numbers of cells)
  - IMAGE ANALYSIS OF MICROSCOPE IMAGES (individual cells)

**COMMERCIAL APPLICATION**

- BIOPSY TESTING SERVICE
  - TO HELP PLAN CANCER THERAPY FOR EACH PATIENT
  - MEASURE ABNORMAL DNA (AGGRESSIVENESS) AND UROKINASE / CELL (METASTATIC POTENTIAL)

Figure 6. Schematic of new methods for quantitative measure of tumor markers and metastatic potential.
Research validation: correlations of uPA and other markers

The first research step has been to compare the DNA measurements and the intracellular levels of urokinase in tumor cells and normal cells. The initial FCM analyses will determine the effect of cell cycle on those cells having elevated uPA and the general relationship between abnormal DNA and uPA in breast and brain tumors. Additional data is being collected on DNA synthesis rates and uPA levels using more specific flow cytometry and/or image analysis techniques. Urokinase levels can be determined in those subpopulations of tumor cells that have abnormal DNA (using two parameter flow cytometry). We currently are measuring the intracellular and membrane-bound levels of urokinase per cell using fluorescent anti-uPA antibodies and image analysis. Next, different forms of uPA and uPA receptor complexes will be measured using molecular-specific antibodies. Finally, PAI-1 or PAI-2 will be measured per cell and compared to the abnormal DNA and high uPA to determine the final relative metastatic potential. Correlations with hormone receptors and other proteolytic enzymes also can be made to provide additional prognostic information for custom design of adjuvant therapy following surgical removal of the primary tumor.

COMMERCIAL APPLICATIONS

Many intricate biochemical interactions are involved in dissolution of the extracellular matrix which enables metastatic cells to leave the primary tumor. Intracellular metabolism appears to have a major role in the initiation of cellular metastasis. The complexity of these interactions makes it too complicated for laboratory test kits to be effective in routinely measuring the DNA, uPA, PAIs, hormone receptors, etc. necessary to develop a comprehensive prognostic panel for a particular cancer patient. Such a task requires a specially equipped, expert medical testing service where pathologists, surgeons and oncologists can send patient biopsies for complete analysis. Several companies already offer this type of cancer testing as a commercial service, however, tests for uPA as a prognostic marker of metastatic potential are not offered yet. Once the metastatic relationships are characterized at the cellular level, clinical studies will be required to statistically correlate these biochemical tests with recurrent disease and survival. Quantitative measurements of uPAs, uPA receptors and inhibitors can be added to the existing panel of breast cancer cytological tests. The first use of these tests will be in providing additional information that indicate active metastasis at the time of initial surgery. This information can help oncologists design better, more effective follow-up therapy for those patients that have high levels of multiple markers indicating metastasis is already underway even though clinical manifestations are still undetected.

This NASA sponsored project is developing methods for a routine analytical test of intracellular and membrane-bound uPA that can be added to the existing panel of breast cancer markers. Each year more than 170,000 new breast cancers are discovered in the U.S. alone. Unfortunately, about 30% of these patients will die from their breast cancer [24]. The current tests for DNA content, DNA synthesis and hormone receptors cost about $350. More complicated tests will likely cost $450 each. A practical test for urokinase combined with other metastatic markers of breast cancer would create a significant new market for cancer testing laboratories. And of course, uPA is important in many other types of metastatic cancers. Better adjuvant therapy, used only when critical markers are known to indicate active metastasis, could make a significant impact on the survival of cancer patients and reduce medical costs required to treat recurrent disease.

Finally, the use of antibodies specific against urokinase can be used for more than diagnosis of the beginning steps of metastasis. As the entire scenario is better understood, it may be possible to develop treatments targeted against just those metastatic cells that have large amounts of membrane-bound urokinase or large concentrations of inactive scuPA. Anti-uPA antibodies could be conjugated with anti-tumor drugs or radioisotopes to treat specific metastatic cells that are actively trying to invade adjacent tissues. This could be the basis for the first therapy directed against metastatic cells that were not removed by cancer surgery or began migration prior to removal of the primary tumor.
REFERENCES