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MICRODIALYSIS - AN *IN VIVO* TECHNIQUE FOR STUDIES OF GROWTH FACTORS IN BREAST CANCER

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1. ABSTRACT

Changes in the microenvironment are important in the development of cancer and further tumor growth. Although landmark discoveries have been made regarding genetic alterations in cancer at a cellular level very little is known about protein regulation in the extracellular space. In the microenvironment many growth factors are activated at a post-translational level by interactions of different cell types such as epithelial cells, fibroblasts, adipose cells, and immune cells. The extracellular space is the bioactive site for the majority of growth factors and increased knowledge of protein activation in this compartment is of utmost importance for our comprehension of tumor biology. Microdialysis is a minimally invasive technique, which enables sampling of molecules in the extracellular space. It is applicable in human cancer as well as in experimental tumors. This review describes microdialysis, its application and the up to date literature of microdialysis for detection of growth factors in cancer with special emphasis on breast cancer.

2. INTRODUCTION

Cancer development and tumor growth are results of interactions between cancer cells and the microenvironment. Without changes in the microenvironment dysplastic cells or *in situ* cancers are not likely to develop into cancerous disease. For invasive tumor growth and metastasis angiogenesis is required (1). Proteolytic enzymes are highly involved in the angiogenesis process both by degrading basement membranes and clearing the way for new blood vessels as well as in providing escape routes for seeding tumor cells into the tissue and subsequent solid tumor growth (2). The activities of growth factors, anti- and pro-angiogenesis molecules, and proteolytic enzymes are tightly regulated at a post-translational level in the extracellular space. The pivotal role of these events in cancer development urge for further research, which considers the tumor cells as parts of a larger entity rather than single units.

Tumor tissue consists of epithelial cell, connective tissue, blood vessels, fat, and immune cells together creating a unique microenvironment *in vivo* which is virtually impossible to reproduce *in vitro*. It is therefore imperative to develop minimally invasive techniques that allows for *in vivo* investigations. Microdialysis is such a technique and an overview of the possibilities of using this technique in cancer research is provided in this mini-review.

3. MICRODIALYSIS FOR *IN VIVO* INVESTIGATIONS

The microdialysis technique has been known and used for at least four decades. It was initially used in animal studies but has developed over the years for use also in humans. The measurement of substances derived from the extracellular water space was introduced in the 1960s when a ¨push-pull¨ technique was developed (3). The liquid was pumped into the tissue through one cannula and withdrawn through another and to avoid a net liquid injection, the push and pull flows had to be balanced exactly. A ¨dialysis sac¨ has also been used for long-term sampling of substances in the extracellular space (4). The sacs were implanted in the subcutaneous tissue and brain in dogs and then surgically removed after several weeks. However, to be able to measure metabolic events during a period of time, a continuous flow of the perfusion liquid was developed (5). Ungerstedt *et al.* implanted ¨hollow fibers¨ in brain tissue to mimic the function of a blood vessel and created the microdialysis technique as it is used today (6). The technique has been further improved over the years and is now available for studies of various tissues in humans (7).

The principle of microdialysis is to mimic a blood vessel in an individual organ or tissue (Fig 1A). A double lumen catheter is implanted in the tissue and then perfused with a physiological solution. The catheter consists of a double lumen cannula with a semipermeable membrane glued to the end of the cannula. The perfusion liquid enters the catheter through the one lumen and leaves it from the other. A diffusion of substances across the membrane takes place and after an equilibration period the dialysate leaving the catheter reflects the chemical

composition of the interstitial space of the organ or tissue (Fig 1B). The chemical composition of the interstitial space is the net sum of cellular uptake/release and transport of the compounds by the regular microcirculation. The pore size of the dialysis membrane determines the size of the molecules that are detectable.

Microdialysis is not a technique for direct sampling of the extracellular water, but instead the dialysate reflects the chemistry of the extracellular space. The concentrations of the compounds in the dialysate depend on the degree of equilibration between the perfusion fluid and the extracellular fluid. This is termed recovery. The recovery of substances across the microdialysis membrane depends on several factors; the surface area of the dialysis membrane, the flow rate of the perfusion liquid, ability of the molecules to diffuse across the membrane, certain properties of the membrane such as the pore size, the blood flow and the temperature in the tissue.

The recovery of a substance can be expressed as the relative recovery or the absolute recovery. The relative recovery is the percentage of the concentration of the substance in the perfusion fluid compared to the surrounding tissue, while the absolute recovery is the total amount of substances collected in the dialysate during a period of time. Several methods have been used to estimate the true tissue concentration of different compounds. One is the *in vitro* recovery value, which is obtained from experiments performed with standard solutions. However, the diffusion of substances across the microdialysis membrane is different in a solution and tissue, and the *in vitro* recovery value cannot be extrapolated to the *in vivo* recovery (7). During an *in vivo* calibration, a substance is perfused in various concentrations until an equilibrium concentration is reached. At that time the concentration of the incoming and outgoing perfusate is the same. When equilibrium is reached, the concentration of the substances is the same in the dialysate and in the tissue (7, 8). For low-molecular-mass substances such as glucose, glycerol, lactate, and urea there is a nearly complete recovery when using a long microdialysis membrane (30 mm, 20,000 mol wt cut-off) with a low perfusion rate of 0.3 μl/min (9, 10).

If the perfusion conditions remain constant, i.e. the function of the dialysis membrane and the flow of the perfusion liquid, the relative recovery is constant *in vivo* (7). The absolute recovery will, however, vary according to the metabolic events in the tissue.

Before experiments, an equilibration period is needed to establish steady-state. This depends on the initial lesion of the tissue, which causes a release of cellular molecules and the initial drainage of extracellular substances through the catheter (11). Tissue damage, measured by the amount of adenosine release and histological examination, has been shown to be minimal (12, 13). Moreover, the equilibrium point will differ for each molecule due to individual physico-chemical properties of each growth factor and the equilibration period will therefore vary. The equilibrium point for the analyte of interest can be determined by a long-term period of repeated sampling at different time points before start of the experiment.

The development of larger molecular-weight cut-off membranes (100,000 Da) has made it possible to use microdialysis in the research of growth factors. When using these membranes a colloid has to be added to the perfusion fluid to avoid ultrafiltration and loss of the perfusion liquid into the tissue (14). Larger pore size membranes (>100,000 Da) have been used in studies but at this point these are not commercially available. The recent advancement of the technique has opened for new and exiting possibilities to explore and learn more about the crucial intercellular crosstalk and signaling in the tumor tissue environment.

4. MICRODIALYSIS FOR DETECTION OF GROWTH FACTORS

The extracellular matrix (ECM) is not only an inert compartment for cell attachment but plays an important role in controlling diffusible signaling molecules. The resident components of the ECM include structural proteins necessary for the architecture of the tissue. The active components of the ECM bind and activate membrane-bound receptors that are responsible for the intercellular crosstalk. Many growth factors are secreted as precursor molecules, which are activated in the extracellular space. Some growth factors are sequestered to various molecules such as binding proteins or heparin-like compounds in the ECM. This functions as a reservoir for the growth factors in their inactive form and the balance between inducers and inhibitors in the ECM will determine the release of biologically active proteins and thus tissue growth. Our knowledge of this regulation is still limited and by studying these factors outside the cells in the interstitial space we will significantly increase our understanding of tumor biology.

One of the most important growth factors in cancer and normal tissue is insulin-like growth factor 1 (IGF-1). The role and regulation of this growth factor has been thoroughly investigated in breast cancer. IGF-1 has been shown to increase the proliferation of breast cancer cells and inhibit apoptosis (15, 16). Epidemiological studies have shown that high plasma levels of IGF-1 in combination with low levels of IGFBP-3 or high levels of free IGF-1 increase the risk of breast cancer (17-19).

To exert its mitogenic signal IGF-1 binds to the IGF-1 receptor and the interaction of IGF-1 and the receptor is regulated by IGFBPs (20, 21). High levels of IGFBPs block the receptor binding and suppress the action of IGF-1 (20). When IGF-1 is bound to its binding protein, a stable complex forms which neutralizes the bioactivity of IGF-1 (22). Hence, IGF-1 has to be in its free form to be biologically active and it has been shown that free IGF-1 has a stronger association with breast cancer than total IGF-1 (18). Sex steroids influence IGF-1 biosynthesis as well as breast cancer development. IGF-1 is produced in large amounts in the liver under the control of growth hormone, which in turn is under the influence of sex steroids (23, 24).

However, IGF-1 is also produced locally by the stroma cells at least in the breast (25). Since IGF-1 functions in an autocrine/paracrine pathway and the sources of this growth factor are both systemic and local, a direct measurement of the biologically free fraction IGF-1 in the target tissue is needed. Early studies of rat brain have shown that IGF-1 is possible to measure using microdialysis (26, 27). It has also been shown that IGF-1 is possible to detect in human tissue (28). It has been shown that free IGF-1 in the extracellular space locally in the breast were doubled in the luteal phase, when estradiol and progesterone levels were elevated, compared with the follicular phase (28). The increased local levels of the free form of IGF-1 may promote proliferation in the breast epithelium and could be important in development of sex steroid dependent breast cancer.

Transforming growth factor (TGF)- beta is synthesized initially as a long precursor molecule which by proteolytic cleavage yields a complex in which the mature TGF-beta protein remains associated with the rest of the precursor molecule (29, 30). TGF-beta is in this complex in a latent form and acid activation releases the mature biologically active TGF-beta from this complex. Almost all forms of TGF-beta are released as biologically inactive forms (29, 30). TGF- beta 1 has been detected in microdialysis samples from humans and it is now possible to further elucidate the regulation of this peptide in the extracellular space in tumors (31).

Fibroblast growth factors (FGF) have been suggested to play a role *in vivo* in the modulation of such normal processes as angiogenesis, wound healing and tissue repair, and embryonic development. However, inappropriate expression of FGF basic and other members of the FGF family can result in tumor production (32). Many cells express FGF basic only transiently and store it in a biologically inactive form sequestered by heparin-like glycosaminoglycans (33). The mechanism by which the growth factor is released by the cells is not known. To my knowledge no microdialysis studies measuring FGFs have been published but sampling the 18-24 kDa molecule FGF basic may be possible and would gain insight in the complex regulation of this growth factor in situ.

Several cytokines and proteases are involved in the generation and regulation of bioactive growth factors. Some of these compounds have been detected in microdialysis samples from various tissues. In humans IL-6 has been detected in brain, connective tissue, and dermis (34-37) whereas TNF-alpha has been detected in microdialysis fluid of canine muscle tissue (38). Cathepsin D is a protease that also functions as a growth factor in breast cancer and by using microdialysis we have shown that the release of this protein is regulated by estrogen and tamoxifen in breast cancer in tumor explants in nude mice (39). Microdialysis had also been used in assessing antioxidant levels and free radical formation, events also involved in the regulatory process of growth factors (40-42).

5. MICRODIALYSIS AND ANGIOGENIC GROWTH FACTORS

Angiogenesis, the formation of new blood vessels from an existing capillary network, is essential for continued tumor growth and spread to distant sites. Vascular endothelial growth factor (VEGF) is a key factor in promotion of tumor angiogenesis, an important event in tumor progression and metastatic dissemination (1, 43). Several isoforms of human VEGF, containing 121, 165, 189, and 206 amino acids are produced from a single gene as a result of alternative splicing (44). VEGF 121 is a freely soluble secreted protein, VEGF 165 is also secreted, although a significant portion remains bound to the cell surface whereas the larger isoforms bind tightly to heparin and are sequestered in the extracellular matrix (45, 46). VEGFs are bioactive as freely diffusible proteins in the extracellular space where they become available to the endothelial cells, and stimulate cell proliferation, migration, and tubular organization and increase vascular permeability (46). In one report it has been suggested that the soluble 121 isoform has greater angiogenic and tumorigenic properties than the heparin bound isoforms (47).

Previous assessments of VEGF protein have been performed by immunohistochemistry or immunoassay of tissue extracts and these measurements appear to correlate with microvessel density in invasive ductal carcinoma of the breast (48, 49). These methods are, however, semiquantitative, tedious and have to be performed postoperatively. Therefore, VEGF measured in blood has been considered as an alternative to these methods but the interpretation of such studies has been complicated by the fact that serum VEGF to a large part is released from platelets, which are activated upon coagulation (50). Unlike VEGF measured in serum, VEGF measured in plasma has been shown to be significantly higher in breast cancer patients compared with control patients, although the plasma levels have not been shown to correlate with intratumoral VEGF assessed by immunohistochemistry (51). VEGF 121, considered to be the most potent stimulator of angiogenesis *in vivo* and the suggested predominant isoform in primary human breast cancer, diffuses freely into the extracellular space from the cells producing it and cannot be readily detected by immunostaining of tumor sections (47, 52). Moreover, longer isoforms of VEGF may also be released in soluble bioactive form by proteolytic cleavage (46). This cleavage may be especially important in tumors where the local microenvironment generally express a high proteolytic activity (53). Clearly, a direct measurement of VEGF locally in the tumor as provided by microdialysis would be more accurate for measurement of the total amount of bioactive protein released by the tumor. We have validated microdialysis as a technique for VEGF measurements *in vivo* and found that VEGF detected in locally the extracellular space correlates significantly with tumor VEGF measured in tissue homogenate contrary to VEGF measured in plasma (54-57).

In breast cancer tissue, VEGF mRNA expression is increased compared to adjacent normal breast tissue (58) and high tissue VEGF levels appear to correlate with poor

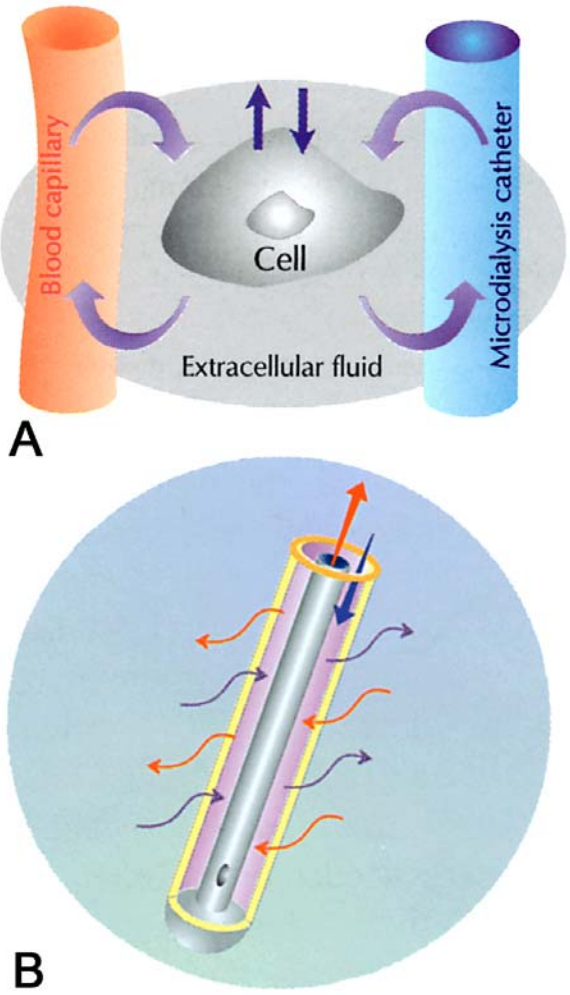

Figure 1. A. The chemical composition of the extra cellular space is the net sum of the cellular events from various cell types and the transport by the microcirculation in an organ or tissue. B. Illustration of the tip of the microdialysis catheter. The perfusion fluid enters the catheter and a diffusion of substances across the membrane takes place. The liquid leaving the catheter will therefore reflect the chemical composition of the tissue.

prognosis and decreased overall survival for both node-positive and node-negative breast cancer patients (59, 60). Plasma levels of VEGF, which have been suggested to reflect tumor-derived VEGF more than serum levels, have not been shown to associate with intratumor VEGF, indicating that circulating VEGF only to a minor degree reflects tumor derived VEGF (51). In mice, we have shown that 93% of the VEGF measured by intratumoral microdialysis was tumor derived, while only 45% of the circulating plasma VEGF originated from the tumor (56). In humans, where tumors are a much smaller part of the total body volume compared to tumor-bearing mice, the VEGF released from a tumor would probably be an even smaller part of the total circulating VEGF in plasma.

Breast cancer is a sex steroid dependent disease as a majority of breast cancers express estrogen receptors and respond to anti-estrogen treatment. We have shown that angiogenesis in murine breast cancer tumors is promoted by estrogen treatment (61). VEGF expression is also regulated by sex steroids at least during normal physiological angiogenesis in the uterus and ovaries, as well as during angiogenesis in endometrial cancer. In the VEGF gene an estrogen responsive element (ERE) has been identified (62). Our results have shown that extracellular VEGF in solid tumors is increased by over 200% after estrogen treatment whereas in cell culture estrogen increased VEGF secretion by 42% (56). This suggests that the accessory stroma and/or inflammatory cells may contribute to the total tumor VEGF and that a local measurement is very important. We have shown that tamoxifen increases VEGF mRNA and intracellular protein in a similar manner as estrogen. Despite this, tamoxifen decreased angiogenesis significantly in experimental solid tumors. This was explained by a significant decrease of extracellular levels of VEGF after tamoxifen treatment to tumor bearing mice (57). These results emphasize the need for studies on the regulation of proteins where they are biologically active, in the case of VEGF in the extracellular space. The advances in molecular biology research have gained a huge amount of knowledge in the regulation of proteins at the gene level. However, after gene transcription and mRNA production proteins have to be generated, activated, and transported to their bioactive site. For many proteins and growth factors this is the extracellular space.

Long-term exposure to sex steroids increases the risk of breast cancer but the pathophysiological mechanisms behind this increase are still not understood (63). Angiogenesis and VEGF may play a role in breast cancer development. Very little is known about the effects of sex steroids on the balance between VEGF and other important angiogenic factors in the normal breast and in breast cancer. It has been shown that VEGF in normal human breast tissue increases in the luteal phase of the menstrual cycle when the levels of estrogen and progesterone are elevated (54). This may contribute to the carcinogenic effects of sex steroids on the breast but this remains to be elucidated in future larger studies.

The pro-angiogenic environment necessary for cancer development is the net effect of overproduction of angiogenic stimulators and reduced levels of angiogenic inhibitors. Two important endogenous regulators of angiogenesis are the soluble VEGF receptor-1 (sVEGFR-1) and interferon-inducible protein 10 (IP-10). sVEGFR-1 is a naturally occurring VEGF antagonist while IP-10 is suggested to be a potent inhibitor of angiogenesis *in vivo*. Angiostatin and endostatin are other well-known endogenous angiogeneic inhibitors currently in clinical trials as a potential cancer treatment. The production of angiostatin and endostatin are dependent on the proteolytic cleavage of plasminogen and collagen locally in tumor tissue. A local measurement would therefore be an appropriate approach for detection and research of theses compounds. To this point IP-10 is the only anti-angiogenic compound that has been measured in microdialysis fluid but this area of research is in expansion (54).

6. SUMMARY AND PERSPECTIVES

Tumor growth is dependent on many different events in a tissue. Several genes that are critical for transformation of normal cells into cancer cells have been identified. However, there is increasing evidence that cancer cells interact with the host microenvironment and that this interaction is pivotal for cancer development. The interaction of cancer cells with the microenvironment may permit and enhance tumor formation or prohibit dysplastic cells to transform into a tumor tissue. We need to learn more about these interactions and the regulation of proteins in the compartment where the majority of proteins have their bioactive site, namely the extracelullar space. Microdialysis has been used in tumor tissue mostly for pharmacokinetic studies of chemotherapeutic drugs (64, 65) but up to this point very few studies have used microdialysis in the analysis of growth factors in cancer. Using this tool in the research of growth factors in cancer has gained novel insight in the compartment where many growth regulators are bioactive. This recent data has shown that microdialysis is a technique that has to be added to our arsenal of instruments used in mechanistic studies of cancer biology.

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