TECHNISCHE UNIVERSITÄT MÜNCHEN
Frauenklinik und Poliklinik des Klinikums rechts der Isar

Evaluation of metabolic sensor-chip measurements with MCF-7 breast cancer cells

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Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines Doktors der Medizin genehmigten Dissertation.

Vorsitzender: Univ.- Prof. Dr. E. J. Rummeny
Prüfer der Dissertation:

1. Univ.-Prof. Dr. M. B. Kiechle
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Die Dissertation wurde am 08.02.2013 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 13.11.2013 angenommen.

Abbreviations

2DG	2- Deoxy- D- glucose
2DG-6-P	2-deoxyglucose-6-phosphate
ALD	Aldolase
BSA	Bovine Serum Albumin
DMEM	Dulbecco`s Modified Eagle`s Medium- high glucose
DMEM-B	Dulbecco`s Modified Eagle`s Medium- Base
E_2	Estradiol
ER	Estrogen Receptor
ER +	Estrogen Receptor positive
$FADH_2$	Flavin adenine dinucleotide
FCS	Fetal Calf Serum
FDG	Fluorine-18-fluorodeoxyglucose
G-6-P	Glucose- 6- phosphate
G-6-PDH	Glucose- 6- phosphate- dehydrogenase
HK	Hexokinase
LDH	Lactatedehydrogenase
MHC	Major histocompatibility complex
MRS	Magnetic resonance spectroscopy
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NaOH/EDTA	Sodium hydroxide/ ethylenediaminetetraacetic acid
OxPhos	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PET	Positron emission tomography
PFK-1	Phosphofructokinase-1
pH_e	extracellular pH
pH_i	intracellular pH
PHI	Phosphohexoisomerase
PK	Pyruvatekinase
PPP	Pentose phosphate pathway
ROS	Reactive oxygen species
UPR	Unfold protein reaction

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1. Introduction

1.1. The tumor cell

An aberrant energy metabolism as a hallmark for tumor cells was postulated by Otto Warburg many decades ago (140). Since then it remains controversial: Is the energy metabolism a result of the altered extracellular milieu or is the extracellular milieu the result of the aberrant energy metabolism? Or is it all just a necessary and logical consequence of the genetic mutations in the DNA of cancer cells? Even if the origin of cancer is still not completely understood, the fact is that microenvironment, energy metabolism and the activity of certain metabolic pathways of tumor cells differ fundamentally from those of normal tissue.

1.1.1. Microenvironment of solid tumors

Tumor cells need to be supplied with nutrients and growth factors to survive and proliferate. The blood system offers the exchange area for nutrients, growth factors, hormones and cellular waste products. (Fig. 1.1.)

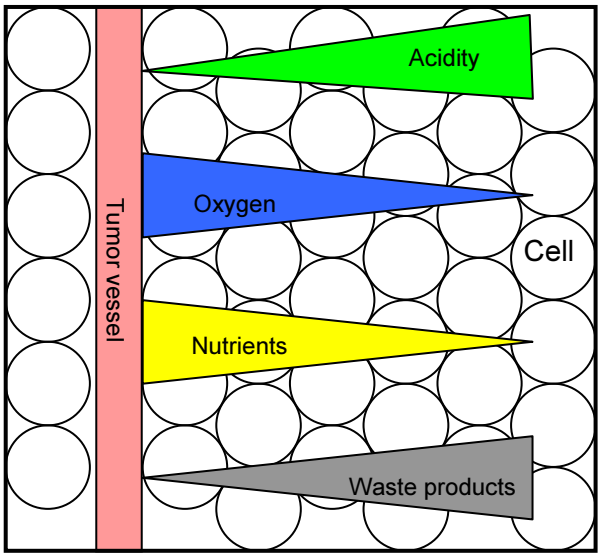

Fig. 1.1. Schematic diagram of changes in nutrient and oxygen supply, pH and concentration of waste products in a tumor tissue with distance of cells to the blood vessel.

1.1.1.1. Vascularization and blood flow

Tumor vascularization consists of two distinct vessel sytems (137). First, there are the preexisting host vessels which become to be part of the tumor. These vessels are subject to structural and functional changes such as elongation, dilatation, obstruction and reduction of the available exchange area for nutrients. The second system consists of microvessels whose growth is stimulated by tumorangiogenetic factors. Such factors are released by cancer cells, that are poorly supplied with nutrients, not sufficiently decontaminated of metabolic waste products or exposed to hypoxia (Fig. 1.1.). Neovascularization originates from host venules and is characterized by alterations in organization, structure and function. Activated by growth factors (e.g. VEGF), a vascular network is formed which is supplied and drained by venules. The newly formed

vessels can show severe architectural abnormalties, expecially in rapidly growing tumors: Elongation, dilatation, abnormal vessel walls, and arterio-venous shunts can lead to poorly supplied or even necrotic tumor areas, as well as to alterations in blood flow (137). Investigations on the blood flow in human breast cancers in situ using positron emission tomography (PET) show a higher blood flow in viable breast cancer tissue compared to the surrounding normal breast tissue and the contralateral normal breast (13, 144). However, the blood flow per weight unit decreases with increasing tumor mass; therefore, oxygen consumption and nutrient supply of the cancerous tissue is determined by the tumor perfusion (73). The critical radius for supply lies in the range of 80 µm for oxygen, 200 µm for glucose and 240 µm for glutamine (72). Taken together, tumor perfusion, vascularization and blood flow differs from normal tissue and thereby has a strong influence on the micromilieu of solid tumors.

1.1.1.2. Interstitial compartment

The interstitial compartment of solid tumors is defined as interstitial fluid and extracellular matrix which is composed of collagen and elastic fiber network (69). Bakay (8) and O`Connor (110) showed that the interstitial space is considerably enlarged compared to its host tissue. The interstitial fluid pressure is elevated and has been shown to increase with increasing tumor size (63, 117). Reasons for this could be increased permeability for macromolecules, rapid increase in cell number in areas that do not allow any further tumor expansion, ischemic cell swelling and absence of a functioning lymphatic

network due to its anatomical abnormality. Consequences of elevated interstitial pressure can be vascular occlusion, necrosis and potentially faciliated intravasation and thus vascular dissemination (69).

The composition of the interstitial fluid also deviates from normal tissue: Glucose and oxygen concentrations were found to be decreased, whereas H^+, CO_2 and lactate concentrations were elevated (56, 137). In the interstitial compartment of solid tumors, glucose concentrations were measured to be between 0.4- 2.5 mM, glutamine concentrations between 0.56- 0.67 mM (72, 103).

1.1.1.3. Extracellular pH levels

Early reports on intratumoral pH were controversial due to inexact and insensitive experimental measuring methods. There is now consensus that the microenvironment of tumor cells is likely to be more acidic than that of normal tissue. Depending on the cell type, the extracellular pH (pH_e) of most human tumors range between 6.15 and 7.4, whereas normal tissue usually has values between 7.0 and 7.4 (137). Metabolic changes may contribute to extracellular acidification, such as an increased proton pump activity (104) or oxidative energy metabolism, including the Krebs Cycle (108). However, experiments have shown that the main contribution to acidification in tumors is by lactate resulting from a high rate of anaerobic glycolysis (3, 60, 118). The acids are transported via monocarbonic acid transporters from the cytosol to the extracellular milieu. Disorganized vascularization and poor lymphatic drainage are suggested to be responsible for the insufficient clearance of metabolic

acids from the interstitial tissue (133). Lactate distribution as a measure of pH shows a very heterogenous pattern, with large concentration gradients in some tumors and flat profiles in others (74). Acidification of the extracellular environment inhibits the activity of antitumoral non-MHC-restricted cytotoxicty, promotes invasiveness, and can change the electrical charge of drugs and thus its membrane permeability and efficacy (46, 49, 134).

In the past, an acidic intracellular pH (pH_i) appeared to be a logical consequence of an increased rate of glycolysis leading to the production of lactic acid (140). However, with the improvement of magnetic resonance spectroscopy (MRS) imaging, making in vivo measurements of intracellular tumor pH possible, this assumption has been discarded. Due to a complex proton extrusion system, the cancer cell is able to maintain pH_i levels near neutrality or above (49, 133). This feature is necessary to maintain the metabolic activity of cells due to the pH dependence of many enzymes, processes of mitosis, and membrane functions. In the acidic micromilieu of cancer cells, the activity of Na^+ dependent Cl^-/HCO^{3-} exchanger and Na^+/H^+ exchanger are elevated compared to normal cells. Close to neutrality, the Na^+ dependent Cl^-/HCO^{3-} exchanger is the dominant regulator, whereas an increased activity of the Na^+/H^+ exchanger has been observed when pH_i decreased (20, 83). Further regulators with less impact on the pH_i are the monocarboxylate carrier (lactate, H^+), the ATP dependent H^+ pump and Na^+/K^+ antiport, and the Ca^{2+}/Na^+ antiport.

The feature of maintaining a stable pH_i even though the extracellular pH is by far below that of physiological levels, gives tumor cells a competitive advantage over normal cells (134).

1.1.1.4. Oxygen consumption and hypoxia

Cells require oxygen for the production of ATP. Under physiological conditions, most ATP is generated from the metabolism of glucose, by glycolysis, Krebs cycle and oxidative phosphorylation (35). Cellular oxygen uptake mainly represents the activity of the mitochondrial respiratory chain (9).

Due to the abnormal vessel system in solid tumors, especially in fast growing tumors, cells can not be appropriately supplied with oxygen. Thus hypoxia is a metabolic challenge to be faced by cancer cells. Like many other cancer cell lines studied, the human breast cancer cells increase the activity of almost all glycolytic enzymes when exposed to hypoxia, including aldolase and phosphofructokinase, while fructose-1,6-bisphosphatase, a gluconeogenetic enzyme, is down- regulated (41). Therefore it appears that in hypoxia MCF-7 cells reorganize their energetic balance by enhancing the anaerobic glycolysis and inhibiting gluconeogenesis.

1.1.2. Energy metabolism

For mammalian cells there are multiple ways of gaining energy from various substrates. While erythrocytes rely on glycolysis and the pentose phosphate pathway (90), liver cells draw most of their energy from the respiratory chain and glycolysis (129). Tumor cells use two energy generating pathways:

Oxidative phosphorylation (OxPhos), which is dependent on oxygen supply, and glycolysis, which can provide the cell with ATP even under conditions of hypoxia, as long as glucose is available (122, 140). A high aerobic glycolysis rate due to elevated glucose transporters (24, 52, 55, 92, 98) and enzyme expression, such as hexokinase, pyruvate kinase and lactate dehydrogenase, is well documented (10–12). Another interesting feature is the ability of some cancer cells to change their energy metabolism depending on the availablility of glucose or glutamine and correspondingly, the energy gained via aerobic glycolysis or oxidative phosphorylation (40, 124). Some characteristics of key metabolic pathways will be described in the following paragraphs.

1.1.2.1. Glycolysis

Glycolysis describes the enzymatic breakdown of glucose under both aerobic and anaerobic conditions. Glucose uptake into the cell is mediatetd by faciliative glucose transporters (GLUT) in the cell membrane and the expression and function of the GLUT isoforms which are under tissue-specific hormonal and environmental regulation (90, 113, 152). Elevated GLUT transporter levels, in particular GLUT-1 and GLUT-5 transporters in MCF-7 cells (24, 52, 150, 152), the expression of atypic GLUT isoforms (98) and increased glucose uptake (25, 111) contribute to the up-regulated glucose metabolism of cancer cells. Furthermore, elevated glycolytic enzyme activities up to 3.7- to 7-fold compared to normal breast tissue are observed in breast cancer tissue (10), among these hexokinase (HK), aldolase (ALD), lactatedehydrogenase (LDH) and pyruvatekinase (PK), which was mainly present as K_4 isozyme (11).

MCF-7 cells typically exhibit a constantly high glycolytic flux (96, 111). The end-product of glycolysis is pyruvate which, depending on the availability of oxygen, may enter the citric acid cycle in the mitochondria for oxidative phosphorylation or be reduced to lactate in the cytosol (Fig. 1.2.).
The characteristic aberration in enzymatic regulation and the glucose transport system allow cancer cells to assume a survival advantage in a low oxygenated environment. Several studies show a correlation between high GLUT expression and increased malignancy, invasiveness and poor prognosis (55, 92, 98, 112, 116).

1.1.2.2. Pentose phosphate pathway

Glucose metabolism can also be diverted to serve other pathways. In the pentose phosphate pathway, glucose-6-phosophate (G-6-P) is converted to pentosephosphate (62), which is either used in nucleotid biosynthesis or completely metabolized to carbon dioxide (Fig 1.2.): The NADP produced in this process is required for biosynthesis of fatty acids and steroid metabolism (19). Here, the rate limiting enzyme is glucose-6-phosphate-dehydrogenase (G-6-PDH) (62), which catalyzes the oxidation of G-6-P to 6-phosphogluconol-actone. Elevated activity of glucose-6-phosphate dehydrogenase and 6-phos-pho-gluconate dehydrogenase in cancer cells suggests that the tumor cell uses the pentose phosphate pathway to adjust its increased demand of nucleic acids and NADPH for its increased metabolic and proliferative activity (18, 38, 122).

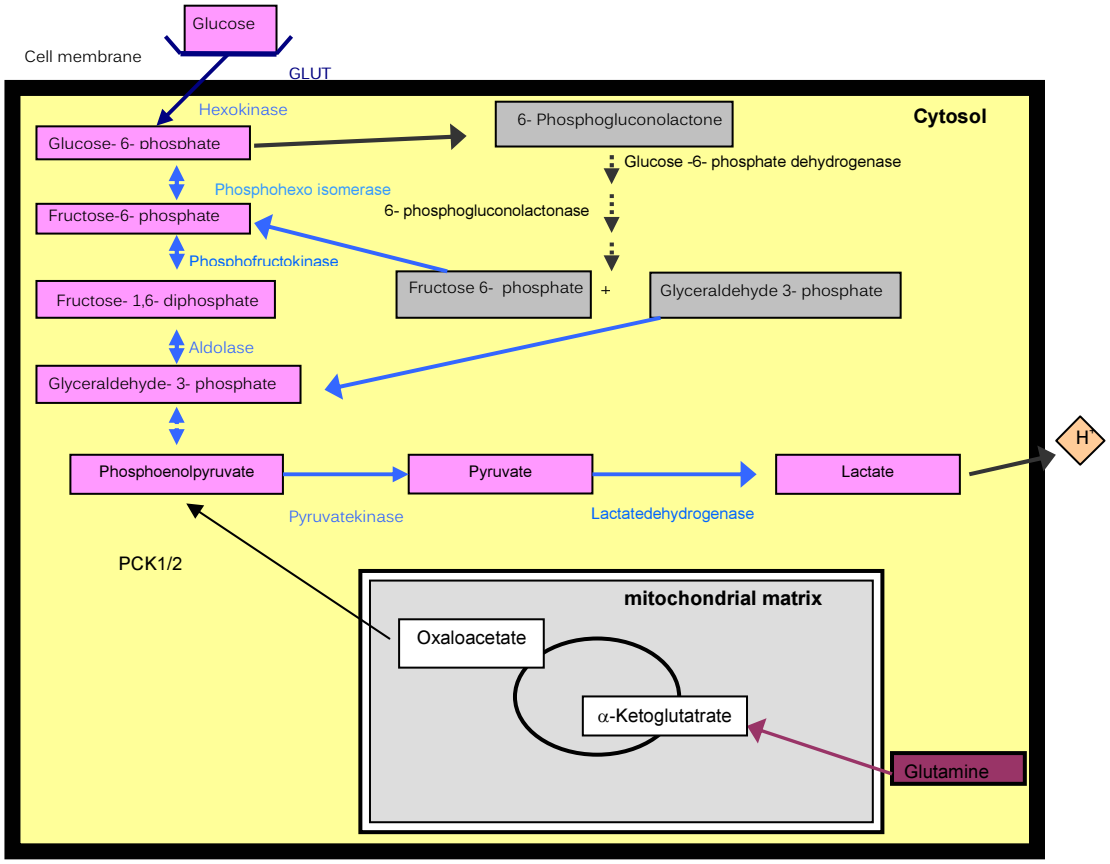

Fig. 1.2.: Interrelation of metabolic pathways. Pink: glycolysis. Grey pentose phosphate pathway. White: Krebs cycle

1.1.2.3. Glutamine in energy metabolism

Gluatmine is both a source of aminonitrate for biosynthetic purposes and a substrate for further oxidation in energy metabolism (48, 123). In the latter case, glutamine can be either used for anaplerosis or converted to lactate (by glutaminolysis in the mitochondria). The resulting NADPH can fuel the fatty acid synthesis (38). In anaplerosis, glutamine can be desaminated to α-ketoglutarate (64), as which it enters the citric acid cycle and is further

metabolized to oxaloacetate while releasing NADH, which contibutes to ATP regeneration by oxidative phosphorylation (Fig. 1.3.)

The discussion on glutamine being the major substrate for energy metabolism in cancer cells has been controversial. Reitzer et al. (122) has claimed that in HeLa cells growing in the presence of glucose glutamine provides 50% of the cells energy; and in hypoglycaemic conditions it was held responsible for even 98% of the ATP generation. This observation was contradicted by the group of Vaupel, claiming that even though the radius of glutamine supply in tissues exceeded by far that of glucose, glutaminolysis occurs only under aerobic conditions in the immediate neighborhood of tumor blood vessels (72). However, a high glutamine turnover and high steady ATP levels, as observed in cultured HeLa cells at a diminished glucose concentration (122) can be interpreted in a different way: In transformed cells the increased need of NADPH and oxaloacetate for biosythesis is met by a highly active glutamine metabolism, which is also required for the synthesis of fatty acids (38), proteins and precursors of nucleotid acids (38, 78).

Also other investigations have shown that the fraction of glutaminolysis in energy provision is very much dependent on how the cell is being supplied with glucose. The presence of glucose can influence oxidation and consumption rate of glutamine, and vice versa (6, 7, 154). A stimulation of glycolysis in the presence of glutamine was observed. On the other hand starvation lead to an increased rate of glutamine utilization.

Taken together, it appears that glutamine is an essential substrate for anabolic processes, but can also be used for ATP production if the environmental conditions require energy sources alternative to aerobic glycolysis.

1.1.2.4. Citric acid cycle

The citric acid cycle, also termed Krebs cycle or tricarboxylic acid cycle (TCA), takes place in the mitochondria. In the first steps metabolites of the catabolic metabolism of glucose, fatty acids and certain amino acids are transformed to acetyl-CoA. By further oxidation and decarboxylation reducing equivalents are provided, such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), as well as with precursors for the amino acid, haem and fatty acid biosynthesis (90) (Fig. 1.3.).

Hypoxia and nutrient deprivation can cause oxidative stress that leads to an elevation of mitochondrial reactive oxygen species (ROS) production (109). High concentrations of ROS inhibit aconitase (45, 75) catalyzing the conversion of citrate to isocitrate (Fig. 1.3.). Consequently the energy generation by aerobic glycolysis is attenuated. The coenzyme for further ATP production, NADH, can only be recovered by oxidation of pyruvate to lactate. This might be another explanation for the Warburg effect (140), which states that cancer cells gain their energy by anaerobic glycolysis even if oxygen is available.

Another metabolic source for the TCA is glutamine. Entering the Krebs cycle as α- ketoglutarat, it turns out to be an option for cancer cells to generate ATP under normoxic conditions (72, 122) (Fig. 1.3.).

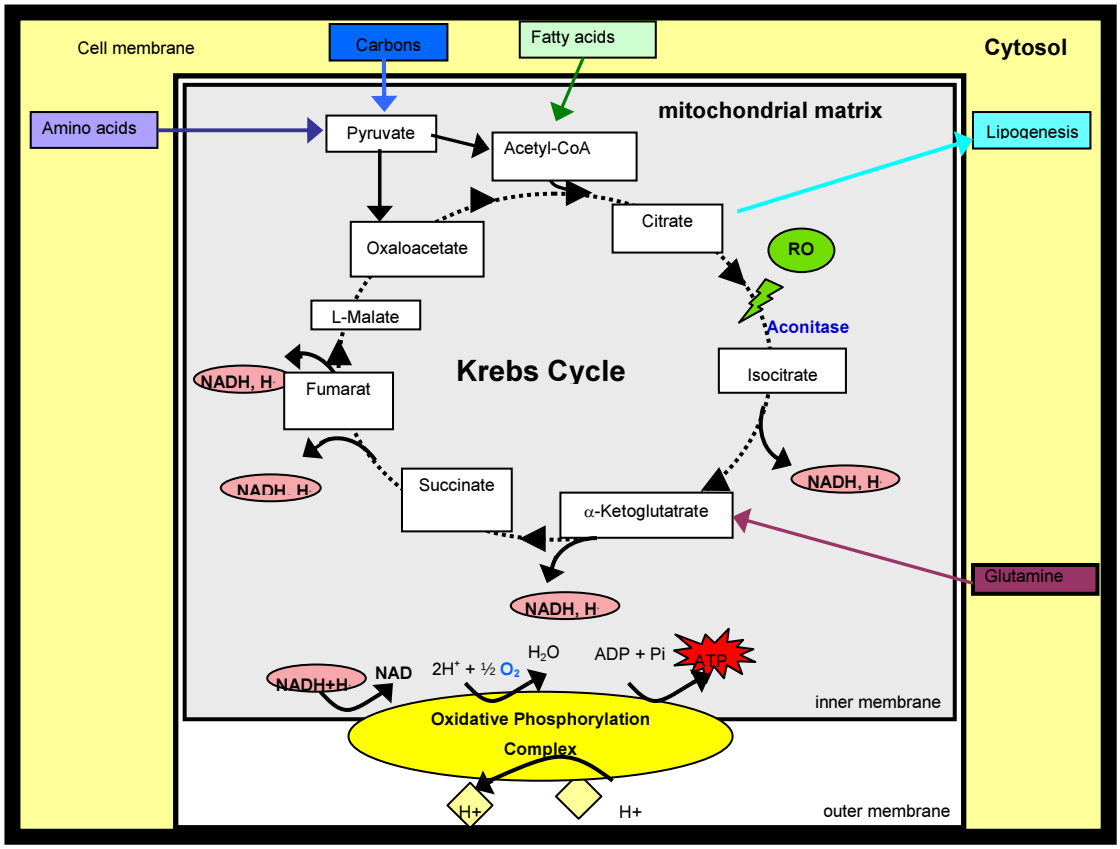

Fig 1.3.: Diagram of the main metabolic steps of the Krebs Cycle (white). Reducing equivalents of the Krebs Cycle undergo further oxidation in the respiratory chain reaction. In cancer cells reactive oxygen species (ROS) inhibit the enzyme aconitase. The citric acid cycle is fueled by multiple metabolic pathways (anaplerosis).

1.1.2.5. Oxidative phosphorylation

Reducing equivalents (NADH, FAD H_2) generated in TCA- cycle can undergo further oxidation in the respiratory chain reaction (Fig. 1.3.). The resulting electrochemical gradient across the inner and outer mitochondrial membrane activates mitochondrial carrier proteins to pass hydrogen ions into the

mitochondrial matrix. The controlled reaction of hydrogen ions with oxygen leads to ATP generation from ADP and inorganic phosphate. The primary regulating factor of the oxidative phosphorylation are the cellular ADP and the intramitochondrial NADH concentrations (68, 77, 105).

High resolution microscopy of mitochondria in cancer tissue reveales a high variability in number, form, density and lesions of mitochondria, even among cells of the same tumor tissue (16, 36, 124, 126). In the normal state, high expression levels of glycolytic enzymes are observed in the cytosol, such as glyceraldehyde phosphate dehydrogenase, lactate dehydrogenase and pyruvate kinase, which is indicative for high glycolytic activity (18, 36). Expressions of LDH isoforms in cytosol and mitochondria may indicate divergent lactate dynamics compared to normal cells (65). Furthermore, expression levels and distribution patterns of enzymes for oxidative phosphorylation, particularly of the ß-F1-ATPase and the heat shock protein 60, are downregulated (36, 66, 67). As soon as the cell switches its energy metabolism to oxidative phosphorylation, e.g. as a result of glycolytic inhibition, e.g. by 2d-glucose, the mitochondrial utrastructure changes to a more condensed state. ATP production is attenuated, but not completely inhibited by blocking glycolysis with 2d-glucose (26, 57). These findings show that cancer cells can still retrieve energy from oxidative phophorylation when glycolysis is impaired.

1.1.3. Autophagy and cell death

Obviously a prerequisite for survival is an adequate supply of nutrients. Under starvation, cells change their metabolism and, with prolonged starvation undergo cell death. Also, treatment with cytotoxic agents, such as doxorubicin and 2d-glucose, can cause cell damage ultimately leading to cell death.
Three mechanisms of cell death have been distinguished:

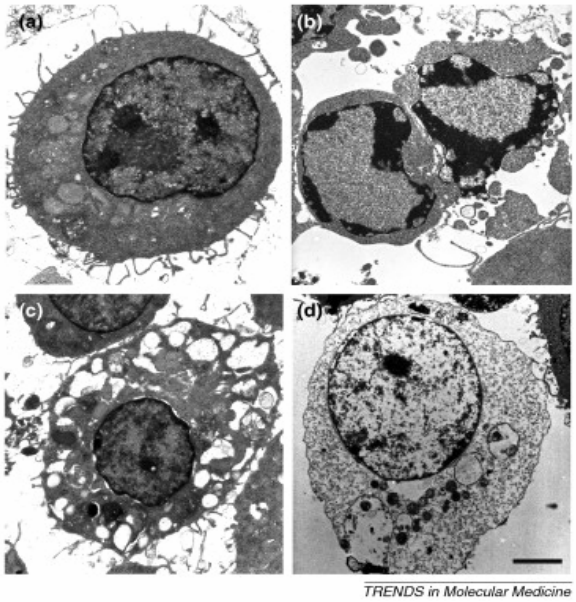

Fig 1.4.: Morphological features of cell death as observed by electron microscopy of J774A cells, deriving from mouse macrophage
a) intact cell with normal morphology
b) apoptotic cell: condensed chromatin and nucleus fragmentation
c) autophagic cell: typical vacuoles in the cytosol
d) necrotic cell: permeable cell membrane and nucleus disintegration (94)

1.1.3.1. Autophagy

Triggered by starving, cellular stress can cause damage of intracellular structures, mainly organells or proteins. To remove such structures, the cells can initiate a selfdigesting process called autophagy to remove defect proteins and lipids and thereby maintain the cellular integrity (71) (Fig. 1.4. C.). Metabolites can be recycled and fed into energy or amino acid metabolism to support cell survival (93). However, if cellular homeostasis can not be achieved by autophagy, apoptosis is induced. In autophagy low cytosolic ATP levels, hypoxia and ligand-receptor bindings at the endoplasmatic reticulum and other membranes trigger signaling cascades (51). A phagophore membrane forms, encases the target and fuses to an autophagosome. Besides the random mechanism of organelle absorption, a selective receptor mediated process is proposed (15). Eventually, the autophagosome fuses with a lysosome to form an autolysosome. Their sequestered material is digested by lysosomal proteases and recycled to amino acids or energy provider (43). The role of autophagy in cancer cells is controversial. In knockout mouse experiments a homozygous gene deletion (beclin 1 gene, chromosome 17q21) led to a complete inhibition of autophagy causing embryonic death. Heterozygous mice showed reduced autophagy and an increase in tumor development (120, 151). Evidently, autophagy, an essential feature of the prenatal development, also reduces the risk of carcinogenesis. The concept of a tumor suppressing function of autophagy is supported by the fact that certain

tumors, such as ovarian, prostate and breast cancer, show a high percentage of spontaneous monoallelic deletion of the beclin 1 gene (4, 120).
On the other hand, autophagy is shown to be a cell survival mechanism during times when growth factors and/ or nutrient supply are limited. Tumor cells with anti- apoptotic features and an efficient autophagocytosis system can use this mechanism for long term survival. The cell shrinks to a minimum size, maintains its energy production through the degradation of intracellular organells and recovers as soon as the environmental situation improves (91). High activity of autophagy can, therefore, also be a survival advantage for tumor cells.

1.1.3.2. Apoptosis

Under physiological conditions, cells usually die by apoptosis (80). It is an actively regulated, energy consuming process, which in turn means that it can only take place as long as the cell is able to provide the needed amount of energy (54, 84). In normal tissue, cellular apoptotic and anti-apoptotic factors are held in a very sensitive balance. Intra- or extracellular signals, such as hormones, proteins, cytokines, growth factors, physical and chemical stress (147) can damage DNA and can result in an imbalance of pro- and antiapoptotic regulators.
Proapoptotic signals (i.e. p53, caspases, Bax, Bad, Bid, Bim) can be inhibited, or antiapoptotic signals (Bcl- family, Akt) can be overexpressed (53, 59, 147). A genetically programmed process is started by initiating a complex cascade

during which caspases are activated (119) and cytochrome C is released from the outer mitochondrial membrane. ATP generation via oxidative phosphorylation is disrupted, the cell has to rely on energy gained from glycolysis (70). Disruption of cellular membranes, break down of cytoplasmatic and nuclear skeletons, degradation of chromosomes and fragmentation of the nucleus follow (Fig. 1.4. B.). The cellular remnants are phagocytosed by the phagocytotic cells of the immune system (128), thereby avoiding an inflammatory response.

1.1.3.3. Necrosis

Necrosis is a cell death occuring upon membrane disruption and can be caused by lytic bacteria, viral disease, tissue injuries, oxidative stress, or oxygen undersupply, as happens e.g. with heart attacks or embolic events (34, 141). Especially in large tumors, central necrotic areas due to metabolic stress can be found. In contrast to apoptosis, necrosis is less regulated and does not rely on ATP.

Upon loss of plasma membrane integrity, cells and their organells first become swollen (Fig. 1.4. D.). While mitochondrial oxidative phosphorylation becomes compromised, cellular ATP levels decline rapidly before the cellular homeostasis collapses (87, 97). This break down and the release of intracellular material into the extracellular space activate an inflammatory response.

Investigations show that there are also active, signaling pathways inducing necrosis, though their molecular flow is not yet quiet clear. A central role is attributed to TNF-α, which activates an intracellular cascade by binding to TNF-α-receptor 1. Depending on simultaneous incoming or missing signals, this ligand-receptor binding can activate metabolic pathways that lead to regulated necrosis (29, 61, 101, 139, 153).

1.2. Cytotoxic agents in tumor therapy

Cytotoxic agents are drugs that can interfere with energy metabolism and the processes of the cell cycle, leading to inhibition of cell growth or even cell death. They are particularly effective in tissues with an elevated proliferation rate, e.g. in tumors. Due to their clinical relevance, two cytotoxic agents were chosen to investigate the effect of microenvironmental parameters on the chemosensitivity of MCF-7 cells: doxorubicin, which plays an important role in breast cancer treatment, and 2- deoxy-D-glucose (2d-glucose), which when labelled with ^{18}F is also used as tracer in tumor imaging by positron emission tomography (PET).

1.2.1. Doxorubicin

Doxorubicin (Fig. 1.5.) is an anthracycline antibiotic with cytostatic and cytotoxic effects and used in the therapy of mammary carcinomas. But mono- or combined therapies with anthracyclines are also indicated for numerous other cancers, such as breast, bronchial, gynaecological and bladder

carcinoma, soft tissue and osteo-sarcoma, tumor of the testis or thyroid, Ewing`s sarcoma, Wilms` tumor, Non-Hodgkin`s and Hodgkin`s lymphoma, acute lymphoblastic and myeloblastic leucaemia, neuroblastoma, and synovialoma (156). Cell death and toxic side effects are observed when treated with doxorubicin, though not all molecular effects are evident, yet. The known effects of doxorubicin are summarized in Table 1.

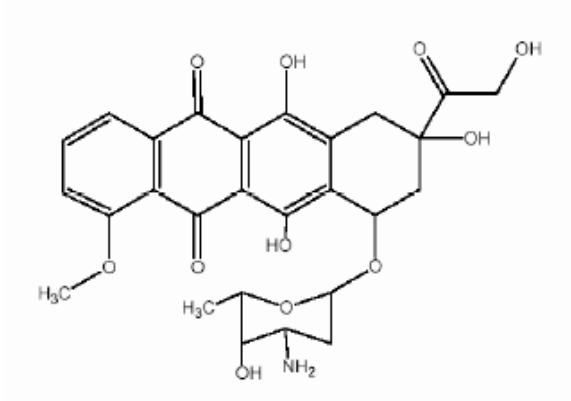

Fig. 1.5. Structure of doxorubicin

Molecular effects of doxorubicin on tumor cells (102)
DNA related • intercalation into DNA, leading to inhibited synthesis of macromolecules; • generation of free radicals, leading to DNA damage or lipid peroxidation • DNA binding and alkylation • DNA cross-linking • interference with DNA unwinding or DNA strand separation and helicase activity

• initiation of DNA damage via inhibition of topoisomerase II
apoptosis inducing • topoisomerase II inhibition • accumulation of undegraded proteins
Membrane related

Table 1.

Especially for breast cancer, doxorubicin is one of the most important antineoplastic drugs and is used in neoadjuvant, adjuvant and palliative therapy (39). The dose intensity of the most common chemotherapeutic schemes, such as the French FEC and American FAC, recommended up to 20 mg/ m^2/ week (39). Therapy with doxorubicin is however limited by its severe side effects, such as cardiotoxicity, neutropenia, myelosuppression, and emesis. For the experiments in this study a maximum of 1- 2 µM have been used in vitro to simulate in-vivo conditions (102).

The role of metabolic activity in response to doxorubicin as a consequence of variable microenvironment has not been reported.

1.2.2. 2-Deoxy-D-glucose

2-deoxy-D-glucose (2d-glucose, Fig. 1.6.) was recognized as an antagonist of glucose in the 1950´s (146). It is known to directly compete with glucose for GLUT-1 transporters on the plasma membrane (23, 24).

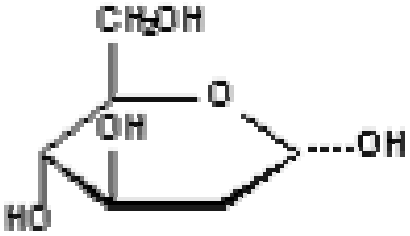

Fig 1.6. Structure of 2-deoxy-D-glucose

After transport into the cell, 2d-glucose is phosphorylated to 2d-glucose-6-P by the glycolytic enzyme hexokinase, but can not be further metabolized by phospho- hexoisomerase (Fig 1.2.). In addition to the competitive inhibition of phosphohexo isomerase, accumulation of 2d-glucose- 6- phosphate leads to a non-competitive inhibition of hexokinase (31, 81) (Fig. 1.7.).

This partial metabolization of 2d-glucose is also seen as a phosphate trap: 2d-glucose-6-phophate results from dephosphorylation of ATP, which leads to a decrease in intracellular phosphate and an increase in AMP levels. This is an explanation for the reduced rate of glycolysis and the reduced ATP levels in cancer cell lines treated with 2d-glucose (42). The reduction of ATP leads to an inhibition of anabolic processes within the tumor cell, along with cessation of cell growth, decreased clonogenity and apoptosis in breast cancer cell lines, including MCF-7 (2). Furthermore, expression of the GLUT-1 transporter and

uptake of glucose were increased in cells treated with 2d-glucose; this was explained by increased oxidative stress.

Under normoxic conditions, 2d-glucose also inhibits N-linked glycosylation by inhibiting the GDP-manose synthase and incorporation of sugar into dolichol-pyrophosphate-linked oligosaccharides (81, 82) . The lack of glycosylation disturbs the folding of glycoproteins and induces the unfolding protein reaction (UPR), resulting in endoplasmatic reticulum stress. Both, low ATP levels and endoplasmatic reticulum stress can lead to autophagy (149), which is capable of saving the cell from apoptosis.

Moreover, it is asumed that 2d-glucose also reacts with other energy providing or anti-apoptotic pathways: While several studies have reported that 2d-glucose-6-phosphate is not further metabolized (130, 142), other more recent studies show that it indeed can be metabolized in the pentose phosphate pathway, where NADPH contribute to energy generation (50, 136) and gluthatione reduction reduces oxidative stress. This concept of 2d-glucose-6-phosphate being involved in other metabolic pathways and metabolized in tumor cells is supported by results showing that the rate of decrease in the cellular 2d-glucose level is greater than the rate of increase in 2d-glucose-6-phosphate (107).

2d-glucose is commonly used to simulate glucose-deprived growth conditions in cell culture and also has been combined with chemotherapeutic drugs inhibiting oxidative phoshorylation. Such combinations showed increased efficacy in vitro (89) and in vivo (95). Ongoing phase II clinical trials are

examining the effect of 2d-glucose as a single treatment regimen in the therapy of solid tumors and hormone refractory prostate cancer (155). But although the elevated glucose uptake and glycolysis activity seem to be a typical feature for cancer cells in general, there are reports which show that 2d-glucose does not always have an anti- cancer effect, namely that the inhibitory effect of 2d-glucose is reduced in radioimmunotherapy of colorectal adenocarcinomas (37).

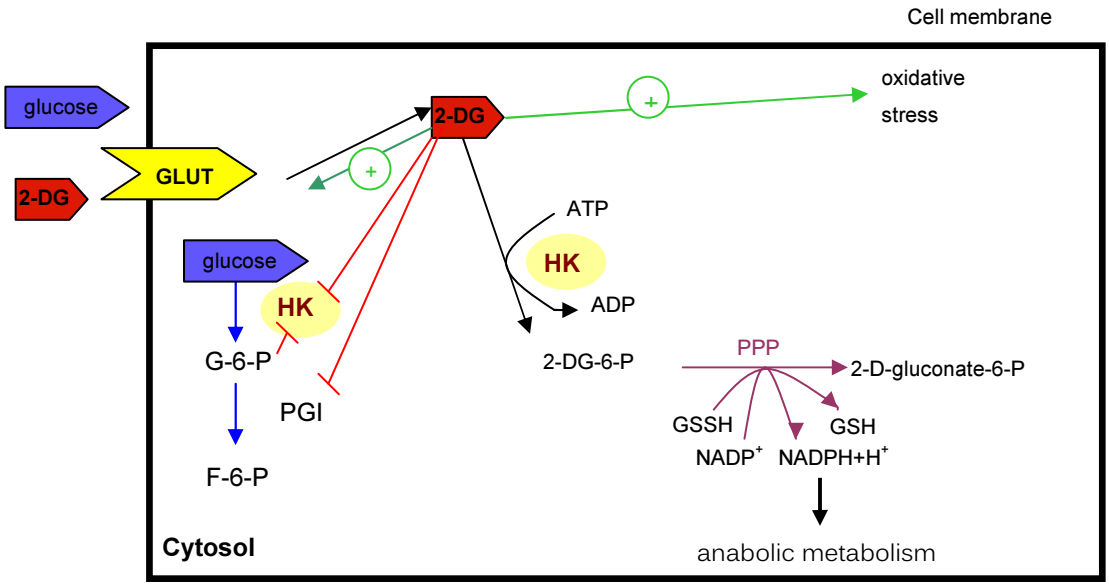

Fig 1.7. Scheme showing the potential pathways and interactions of 2-deoxy-D-glucose. 2-DG: 2-deoxy-D-glucose, 2-DG-6-P: 2-deoxy-D-glucose-6-phosphate, ADP: adenosine diphosphate, ATP: adenosine triphosphate, F-6-P: fructose-6-phosphate, G-6-P: glucose-6-phosphate, GLUT: glucose transporter, GSH: gluthatione, GSSG: oxidized gluthatione, HK: hexokinase, PGI: phosphoglucose isomerase, PPP: pentose phosphate pathway,
→ glycolysis; → pentose phosphate pathway;

Another application for 2d-glucose is in medical imaging. Due to the high glucose turnover of tumor tissue, 2d-glucose is used as radioactively labelled 2-deoxy-2-(^{18}F)fluoro-D-glucose (FDG) in PET- imaging (32, 79).

1.3. Metabolic monitoring

Various assays are used to describe the metabolic activity of tumor cells. Enzymatic activity or amount of certain metabolites, for example ATP, are used as equivalent for the metabolic activity of a cell. These assays are mainly for chemosensitivity testing of tumor cells, standardized and easy to perform. Moreover, its frequent use in literature enables comparison to other results of research. However, these assays only reflect changes in enzymatic or metabolite expression at a certain time, and therewith only give indirect hints of metabolic changes. In addition, only a one-time measurement of metabolic changes of one cell culture is possible, since cells are either destroyed, metabolically influenced by components of the assay or the assay itself is only representative in a certain time corridor.

New measuring techniques, such as sensor chips, allow a continuous monitoring of metabolic parameters of one viable cell culture over hours and days without interfering with the cell metabolism. Regarding these results in combination with the common metabolic activity assays, new insight into the cell metabolism is provided.

For chemosensitivity testing of MCF-7 cells, tetrazolium salt-based assays were here used in parallel with a sensor chip system. Both methods are specified below.

1.3.1. Tetrazolium salt-based assays

Tetratzolium salt-based assays are commonly used as cell proliferation and cytotoxicity test to indicate cell viability (17, 106, 127). The commonly used salts have been MTT, XTT and WST-1. All these assys are primarily dependent on the net rate of glycolytic NAD(P)H production by cells. The intermediate electron acceptors are used to produce formazan, which can be measured by a photometric microplate reader. The detailed biochemical interactions of these tests are not completely revealed, but the signal generated appears to correlate with the mitochondrial dehydrogenase activity of the cells (127). There is also indication for tetrazolium salt interaction on the surface of the cell membrane (17).

In various investigations, a reduced formazan absorbance was detected after adding cytotoxic agents to diverse tumor cell cultures (5, 135). Due to the known cytotoxic impact of the agents, the results were interpreted as reduced viability of the tumor cells. Other investigations indicated that diverse factors can influence the reduction of tetrazolium salts. Until now, only few factors have been tested (33, 47, 138), such as magnesium, glucose concentration and pyridine nucleotides. However, an extensive analysis of such factors is not easy

due to lack of information on the actual metabolic processes taking place in tetrazolium salt-based assays.
Due to its widespread dissemination, results of the tetrazolium salt-based assay served as a reference for the sensor chip measurements in this study.

1.3.2. Sensor chips

New possibilities of a continuous, dynamic and direct monitoring of cell metabolism are raised with the implementation of sensor chips. Recently developed silicon sensor chips of the Heinz- Nixdorf Lehrstuhl für Medizinische Elektronik (Technische Universität München) (21, 145) allow the simultaneuos measurement of

- extracellular acidification (pH-ISFET)
- cellular oxygen consumption (amperometric sensor) and
- morphological changes (impedance sensor, IDES)

(Fig. 1.8.).
The sensors are non-invasive, do not interfere with the functional activity of the cells or tissues and allow measurements in real-time. Cell metabolism and vitality is assesed via the acidification rate and oxygen consumption, which mainly reflect the rate of glycolysis and cellular respiration.
A fluid system enables a regular exchange of medium, ensures the supply with fresh (or altered) medium and the removal of extruded metabolites and acids which could attenuate cell vitality. This system allows maintaining the cells under cell growth conditions for several days.

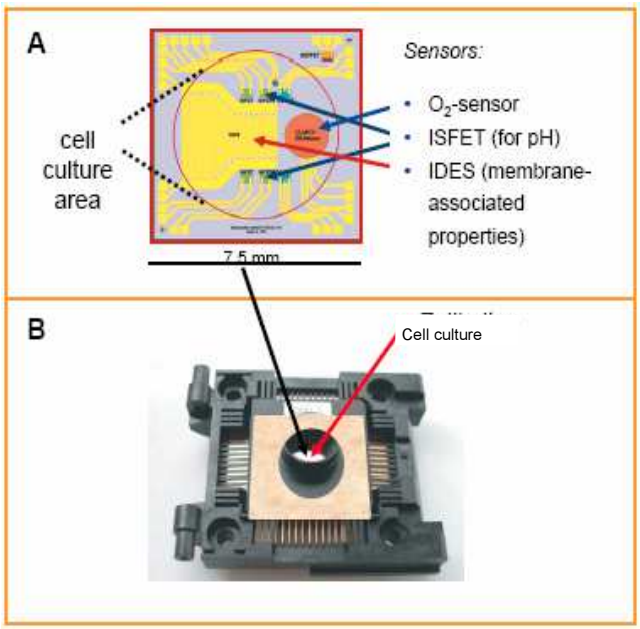

Fig. 1.8.: Silicon sensor chip
A) layout and positioning of electronis structures
B) Chip as produced by Micronas Ltd, Freiburg i.Br., Germany

1.4. MCF-7 cells - an in vitro tumor cell model

MCF-7 cells are commonly used as a breast cancer model and have been used to study different molecular, metabolic and functional aspects of tumor cells. This cell line was originally derived from a pleural effusion of a patient with metastatic breast cancer who had been treated with Tamoxifen and radiotherapy for 3 years (131), and is the first-hormone responsive breast cancer cell line on which extensive research has been done (85). MCF-7 cells are non- or moderately invasive in mice, estrogen sensitive, and express progesteron (PR) and estrogen receptors (ER) (22). MCF-7 cells exhibit the insulin dependent GLUT4 and the insulin independent GLUT1 transporter (14). Moreover, the cell line has retained various differentiated features and an

epithelial-like, cobblestone-like cell morphology (Fig. 1.9.). All these features make it a suitable cell line for investigating numerous processes of tumor cell growth.

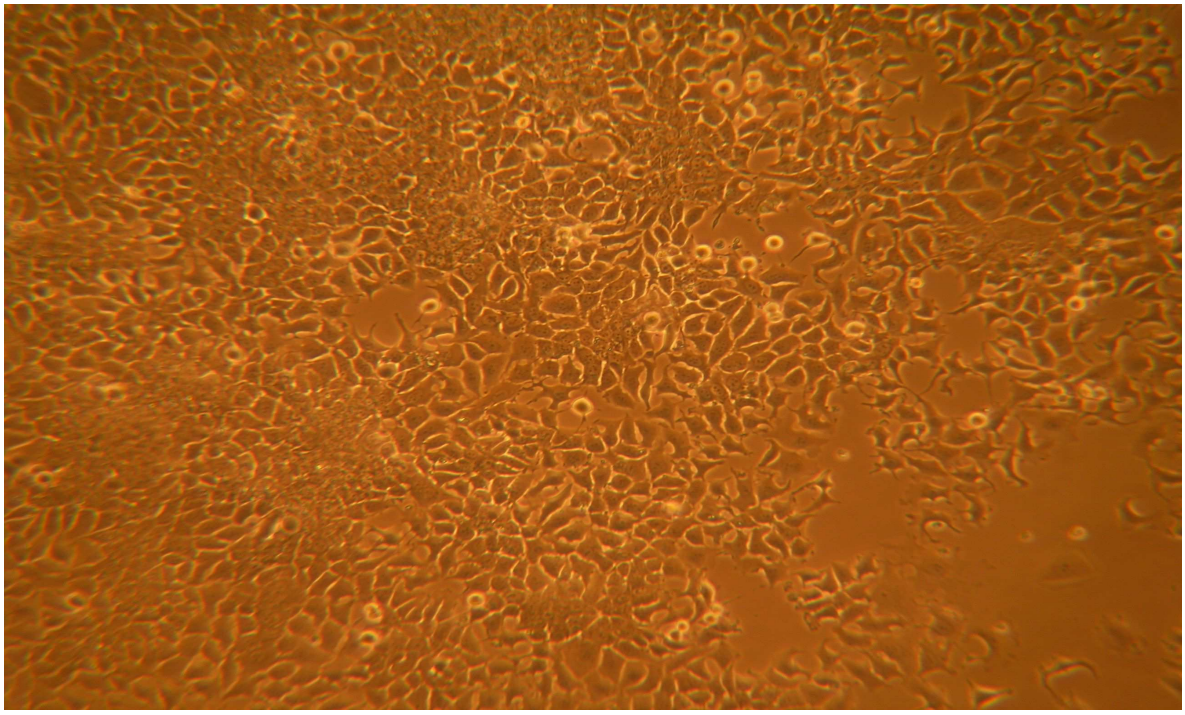

Fig. 1.9.: Microscopic image of an almost confluent monolayer of MCF-7 cells cultured in a 24-well plate. The bright round cells are in mitosis. The cell formation shows the typical epithelial-like, cobblestone- like cell morphology.

1.5. Aims

The underlying aims of this project were

- to study the metabolism of tumor cells under diverse microenvironmental conditions,
- to investigate its interrelation with chemotherapeutics and

- to gain data as reference for the potential establishment of the sensor chip system in the clinical use.

In the experimental approach, growth medium was adjusted to a more tumor-like environment to systematically analyze the tumor cell metabolism under starvation. This was mimicked by minimizing the amount of serum, lowering glucose and glutamine concentrations, and varying the pH of the growth medium to represent various possible states of a hostile microenvironment. Further experiments were performed using cytotoxic agents, as doxorubicin and 2-deoxy-D-glucose to investigate the effect of microenvironmental parameters on the chemosensitivity.

In addition to the one-time measurements of the cell culture, sensor chip measurements were performed to obtain a continuous monitoring of the cell metabolism during starvation and/ or cytotoxic treatment. Besides reference data of the sensor chip system, new evidence on the induction or inhibition of metabolic pathways under these microenvironmental conditions was obtained.

2. Materials and methods

2.1. General instruments

FLUOstar Galaxy	BMG Labtechnologies, Offenburg, Germany with absorption filters (595nm, 450 nm)
Casy® cell counter	Schaerfe GmbH, Reutlingen, Germany
Pipette Tips	Eppendorf, Hamburg, Germany
Silicon sensor chip	Micronas Ltd, Freiburg i.Br., Germany[1)]
Steamsterilizer	H+P Labortechnik GmbH, Oberschleißheim, Germany

2.2. Consumables

2- Deoxy- D- glucose	Sigma- Aldrich Co., Munich, Germany
Test Plate 24	TPP, Switzerland
Test Plate 96	TPP, Switzerland
Tissue culture Flasks	TPP, Switzerland
Eppendorf Tips	Eppendorf AG, Hamburg, Germany
Accutase	PAA laboratories GmbH,:Pasching, Austria
Acetic Acid	Fa. Janssen,
Bradford Reagent	Bio- Rad Laboratories, Hercules, California, USA
Casy® ton	Schaerfe GmbH, Reutlingen, Germany
DMEM	Sigma- Aldrich Co., Munich, Germany

DMEM- B	Sigma- Aldrich Co., Munich, Germany
	(without gluc, glut, phenolred)
Doxorubicin	Sigma- Aldrich Co., Munich, Germany
EDTA	Sigma- Aldrich Co., Munich, Germany
Estradiol	Sigma- Aldrich Co., Munich, Germany
Fetal calf serum	Sigma- Aldrich Co., Munich, Germany
Gentamicin	Sigma- Aldrich Co., Munich, Germany
Glucose	Merck, Darmstadt, Germany
Insulin	Sigma- Aldrich Co., Munich, Germany
L- Glutamine	Sigma- Aldrich Co., Munich, Germany
NaOH	Merck, Darmstadt, Germany
Dulbecco`s PBS1)	Invitrogen, Karlsruhe, Germany
Sodium Bicarbonate	Merck, Darmstadt, Germany
Sodium Pyruvate	Sigma- Aldrich Co., Munich, Germany
Triton X 100	Sigma- Aldrich Co., Munich, Germany

2.3. Cell culture and growth media

2.3.1. Cell culture and basic media

The human breast cancer cell line MCF-7 has been routinely cultured in our laboratory. The presence of mycoplasma infection were regularly excluded by cell staining using bisbenzimid. Cells were maintained in Dulbecco`s Modified

Eagle`s Medium- high glucose (DMEM; powder, 4500 mg/L glucose, equivalent to 25 mM glucose; pH 7.4) supplemented with 5% FCS and incubated in a CO_2-incubator (10% CO_2). Cells were transfered weekly to new flasks.

For the experiments, cells were plated at a titer of 2 x 10^4 /ml in the respective medium on a 24- well plate (500 µl).

For experiments DMEM (25 mM glucose) was used. Generating conditions with different pH and/ or glucose concentrations, a supplementation of Dulbecco`s Modified Eagle`s Medium Base (DMEM-B) with the respective amount of glucose and sodium bicarbonate, L- glutamine and sodium pyruvate has been performed. DMEM- Base powder was reconstituted in accordance to the manufacturer`s protocol, then filter sterilized and stored in aliquots of 500 ml at 4°C.

Each experiment was performed in triplicats.

2.3.2. Supplements

Estradiol

Estradiol was dissolved in ethanol and stored as a stock solution of 1x10^{-5} M in aliquots of 1 ml at 4°C. Estradiol was diluted in sterile H_2O before it was pipetted into the culture medium at an end concentration of 1 nM.

Fetal calf serum

Fetal calf serum (FCS) was stored in aliquots of 20ml at -20° C.

Insulin

A stock solution of 0.1 M insulin was prepared in 0.1 M HCl, filter sterilized, and stored in aliquots of 1 ml at +4° C. Before use it was diluted in sterile PBS and added in volumes to obtain end concentrations of 1 µM, 250 nM, 50 nM and 10 nM insulin in culture.

Glucose

For experiments with reduced glucose concentration, a stock solution of 1.25 M (equivalent to 225 g/L) was prepared in 100 ml DMEM-Base, filter sterilized and stored at 4°C. For further use it was diluted to concentrations of 25 mM (as standard in DMEM- high glucose), 5.56 mM (as standard in DMEM-low glucose), 2.5 mM and 0.56 mM in culture.

L-Glutamin

L- glutamin was stored as a stock solution of 200 mM in H_2O (filter sterilized) in aliquots of 10 ml at -21° C. For DMEM-B based media a concentration of 4 mM was used as it is standard in DMEM.

Sodium bicarbonate

A stock solution of 1.19 M was prepared by diluting sodium bicarbonate powder in H_2O. After filter sterilizing, it was directly pipetted into the medium. Cell cultures were incubated in a humidified atmosphere with 10% CO_2. In standard medium the sodium bicarbonate concentration was added to obtain a pH of 7.4. Experiments were performed at pH of 7.0 and 6.6, the equivalent amount of $NaHCO_3$ was determined by a seromed® normogramm (Biochrom KG, Berlin). The stock solution was stored at -20° C.

Sodium pyruvate

To DMEM-Base media a sodium pyruvate concentration of 110 mg/L was added as used in DMEM. It was directly added during reconstiution of the medium powder in an amount to obtain a concentration of 4 mM.

2.3.3. Chemotherapeutic compounds

2.3.3.1. 2-Deoxy-D-glucose

For the stock solution a concentration of 1 M was prepared in H_2O. It was filter sterilized and stored at 4° C. 2-deoxy-D-glucose concentrations between 10 mM and 0.5 mM were used in the experiments.

2.3.3.2. Doxorubicin

The agent was delivered as a 3.45 mM solution (2mg/ml 0.9% NaCl). To obtain a 100 µM stock solution it was further diluted with sterile PBS and stored at 4° C. The concentrations in the cell cultures were between 0.2 µM and 0.05 µM, the same as reported in the publications of Ramji (121) and Chen (30).

2.4. General cell biological techniques

2.4.1. Determination of cell number

Cells were incubated in 24-well plates in the indicated medium for 2- 6 days. At the end of the experiments, medium was removed along with the detached dead cells. The plate was carefully washed with PBS and cells were incubated with a hypotonic buffer (20 mM HEPES, pH 7.4; 1 mM $MgCl_2$; 0.5 mM $CaCl_2$).

After 20 minutes a lysis solution (5% benzalkonium chloride in 10% acetic acid) was added. Preparing a suspension of cell nuclei has the advantage of avoiding incorrect cell numbers due to cell aggregation (27). The suspension was diluted in a standardized buffer (Casyton) and counted using an electronic counter (Casy). The cursor was set to cover a particle range between 4-15 µM, the average size of nuclei was 5.6 µM. The cell numbers in repeated experiments were very similar. To faciliate the comparison of results, they are put in relation to the original number seeded and presented in relative values. Standard deviation reflects the deviation of the mean cell number between the three experiments.

2.4.2. Determination of cellular protein content

Cells were cultivated in 24- well plates according to the individual protocol of the relevant experiment. The medium was carefully removed, the cultures were washed with PBS, and 250 µl of 10 mM NaOH/EDTA was added and well mixed. Of the dissolved cell solution 50 µl was pipetted into a 96- well plate containing 250 µl of Bradford Reagent. A calibration curve was prepared: defined concentrations of bovine serum albumin (BSA) (0 µg/ml - 2000 µg/ml) in PBS were added to 50 µl of NaOH/EDTA and mixed with 250 µl of Bradford Reagent. The protein solution was incubated at room temperature for 30 minutes, thereafter the absorption was measured in a FLUOstar Galaxy at

595 nm. Using the concentration curve and the cell number per well, the absorption values were converted into an average protein content per cell number.

2.4.3. Determination of dehydrogenase activity

Practical motives lead to the decision to use the XTT assay instead of the more prevalent MTT assay: the formazan of XTT is water soluble and can be quantitated in culture medium without the necessity of extraction with organic solvents. Cells are not devitalized during the test, so that the culture can be used also for other cell assays.

Dehydrogenase activity is measured in living cells as a function of tetrazolium salt transformation to formazan. XTT powder and the reconstituted solution was stored at -20°C as mandated from the producer. A XTT- solution was prepared from a XTT powder reconstituted with DME-Base and the supplements of the experiment (without phenol red). Before adding to the culture (500 µl) 100 µl medium were carefully removed, and then 100 µl XTT solution were added. The cell cultures were incubated in the CO_2- incubator for 4 hours.

Absorbance at the reference wavelength was measured at 595 nm and subtracted from the result measured at 450 nm. Values are given as mOD (10^{-3}OD).

2.5. Microsensor measurements

A detailed description of the novel sensor chip and the measuring apparatus has been published elsewhere (21). The basic set-up as used for experiments is as described below. Sensor chips are manufactured at Micronas (Zürich, Switzerland).

2.5.1. Microsensor chips

Prior to use, the chips were sterilized by autoclaving. A suspension of MCF-7 cells ($1x10^5$ in DMEM + 5% FCS) was seeded directly onto the chip surface and preincubated for 3 days in a humidified cell culture incubator with 5% CO_2. until the cells formed a semi-confluent monolayer. The chip was then inserted into a semiautomated test module. Each sensor chip was connected to a fluid system as described in Cap. 2.6.2. Cells were provided with a basic perfusion medium (DMEM + 5% FCS + Gentamycin). After a preset interval the medium was exchanged (flow modus). No bicarbonate ($NaHCO_3$) was used in the medium to avoid buffering of extracellular acidification. At the beginning of the experiments, cells were supplied with the basic perfusion medium for at least the first 6-8 hours until a stable signal was measured. Then the medium was changed according to the experimental protocol. At the end of the measurements, 0.2% Triton X 100 was added to the medium to receive a basal signal of non-viable cells.

2.5.2. Sensor chip module

The test apparatus consisted of six modules, each one providing sensor chip contacts (decribed below), and was housed in a non-gased incubator maintained at 35°C, to avoid overheating of the sensor chips in the modules. The silicon chip was connected to an electronic module, the perfusion head placed directly on top of the cell culture, regularly providing it with fresh medium and drug addition (Fig. 2.1.). Via a second port in the perfusion head, the medium was actively pumped out after a preset interval. To avoid large pressure changes with possible adverse effects to the cell culture, the perfusion head and the chip were not sealed. The software allowed to set the pump rates (200 µl/min) as well as the stop and flow intervals (3 minutes flow and 7 minutes stop). Prior to the chip insertion, the perfusion system was sterilized by pumping 70% ethanol through the tubing system. Thereafter, it was rinsed with sterile, deionized water and culture medium.

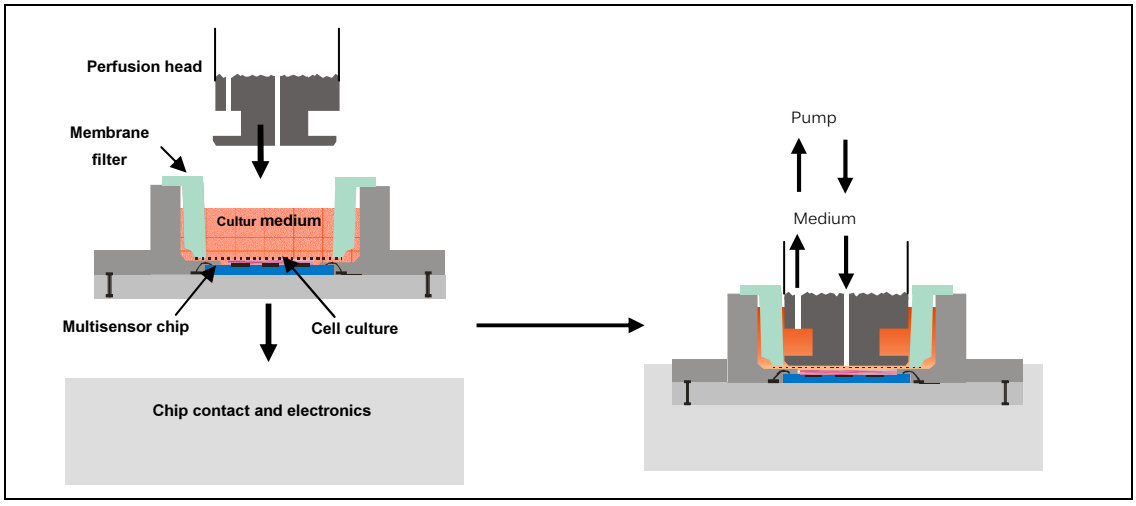

Fig. 2.1. Cross section of the chip- perfusion system

2.5.3. Recalculation and processing of data

A specially developed software for this setup recalculated the metabolic signals recorded in each stop- phase into the rate of oxygen consumption and acidification (Fig. 2.2.). During a stop- interval, the extrusion of acidic metabolic products and consumption of dissolved oxygen by the cells growing on the sensor was measured. The raw data plots showed a periodic oscillation of pH and pO_2, according to the stop and flow mode of the fluidic system. To calculate the acidification rate and oxygen consumption these raw data were calculated versus the time as dU/dt and dI/dt, correlating with the slope of the graph.

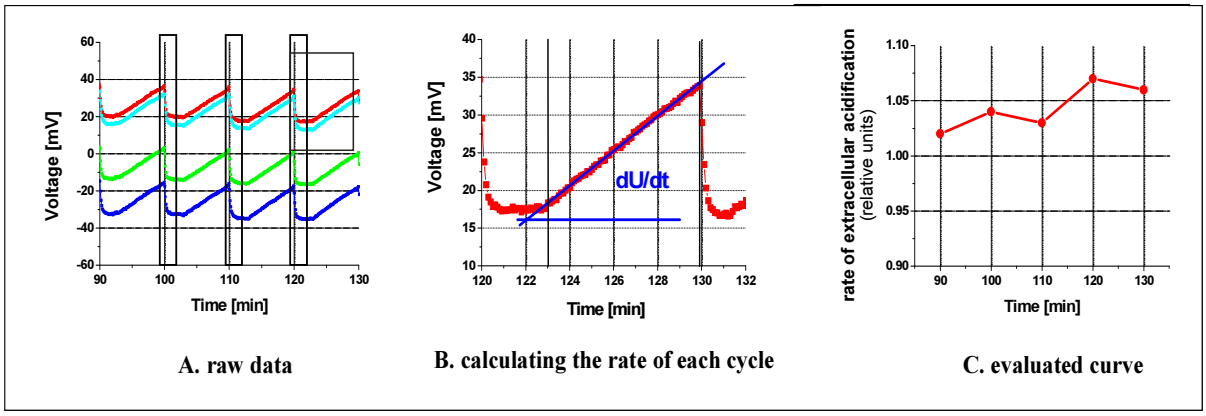

Fig. 2.2. Recalculation of the raw data. No absolut values of pH and pO_2 are measured, the calculation is based on the determination of relative changes during short time intervals.

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3. Results

3.1. Simulation of a tumor-like environment

The standard growth medium for tumor cells in culture is Dulbecco`s Modified Eagle`s Medium containing high nutrient levels for prolonged growth (25 mM glucose, 4mM glutamine). However, the supply of tumor cells with nutrients is dependent on perfusion and their distance to a blood vessel. Rapid tumor growth and comparatively slow vessel growth causes undersupply of nutrients, which is the rule rather than exception in tumor tissue. Blood concentrations of nutrients are not archieved in these regions (Fig 1.1.).
To more closely mimic limiting conditions of a tumor microenvironment, the concentrations of growth factors and nutrients as well as pH were reduced.

3.1.1. Reduction of growth factors and hormones

The serum content was reduced to 1% FCS, just enough to maintain a low level of cell growth. Experiments were performed with 10% and 1% FCS in DMEM to test the difference in proliferation at serum- reduced conditions. As shown in Fig. 3.1. cells grown in DMEM+ 10% FCS showed a sixfold increase in cell number on day 3, and a 25-fold on day 6, relative to the initial cell number. In comparison, cells growing in DMEM +1% FCS had a 40% slower growth rate. Their cell number increased four fold by day 3, by day 6 there was an increase of almost 15 fold.

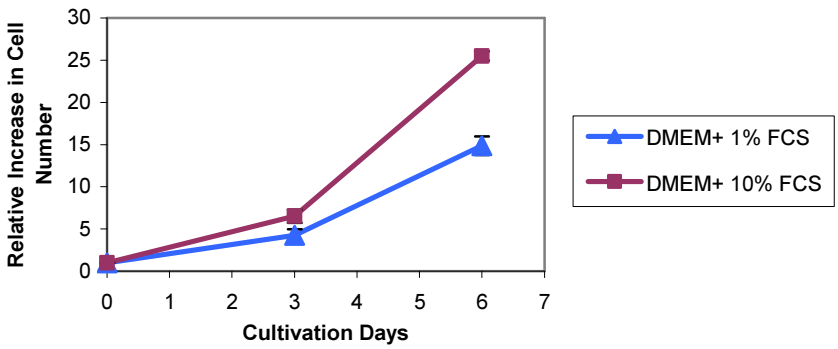


Fig. 3.1. Comparison of cell proliferation in DMEM with 10% FCS and 1% FCS. Results are averages and ranges of three independent experiments.

FCS naturally contains growth hormones, including estrogen, at concentrations sufficient to exert a growth stimulatory effect (88). To retain cell growth, well-defined amounts of estradiol and insulin were added to the medium, the latter to ensure that glucose-dependent processes were not impaired by a lack of this hormone. At all concentrations (10 - 1000 nm) tested, insulin increased cell growth (Fig. 3.2.). Saturating levels for glucose-uptake were reached with 50 nM insulin, which lead to a more than 16 -fold increase in cell number. Since cultures with 250 nM and 1000 nM insulin showed similar results, 50 nM insulin were used for the following experiments.

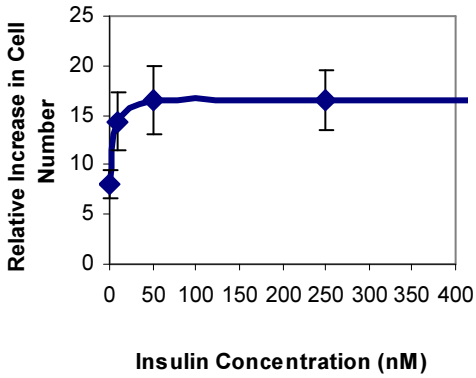


Fig. 3.2. Increase in cell number with various concentrations of insulin. MCF-7 cells were cultured in DMEM with 1%FCS and supplemented with concentrations of insulin ranging from 0 nM to 1.000 nM (1000 nM insulin not shown) for 6 days. Results are averages and ranges of two independent experiments.

Estradiol is known to stimulate proliferation of many positive estrogen- receptor cells (90), such as breast tissue and some breast cancer lines, including MCF-7 cells. In the following experiments, defined amounts of estradiol were added to analyze its effect on cell growth since the reduction of estradiol with low FCS concentrations could attenuate proliferation of MCF-7 cells (114). As shown in Fig 3.3., cell number doubled with 1nM E_2, a similar increase as in medium supplemented with 0.25 µM insulin alone (Fig. 3.3.). The combination of estradiol and insulin did not further enhance proliferation compared to the cells growing with insulin or estradiol alone. These results led to the decision to perform experiments with DME medium supplemented with 1% FCS and 50 nM insulin only. This combination will be named *low growth medium* below, unless indicated otherwise.

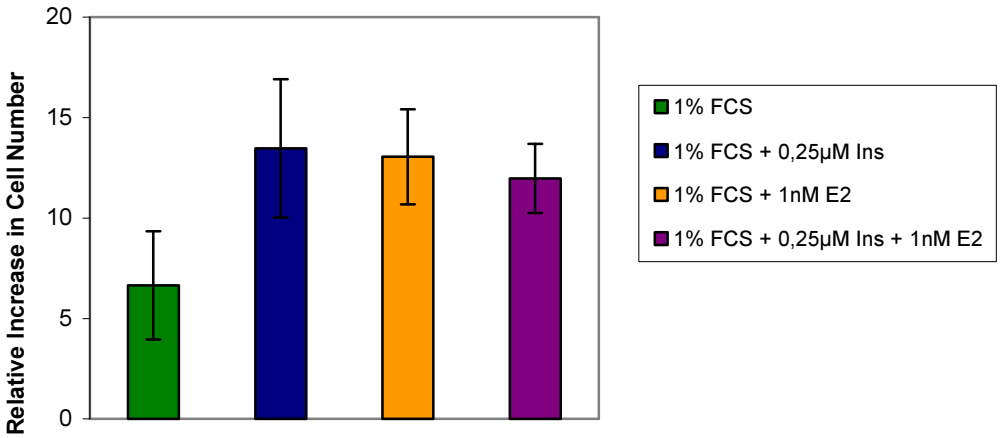


Fig. 3.3. Relative increase in cell number of cultures grown in DMEM + 1% FCS and insulin and/or estradiol as growth factors. Results are averages and ranges of three independent experiments. Cells were incubated for 6 days.

3.1.2. Effects of limiting nutrient conditions

3.1.2.1. Limiting glucose concentration

To test at which concentrations glucose is limiting for growth, cells were incubated in low growth medium with various glucose concentrations for 2 and 5 days (Fig. 3.4. A). After 2 days no significant differences between cell number of cultures growing in high or low glucose medium were evident. However, after 5 days of incubation, cultures grown with high glucose concentrations (25 mM and 5.56 mM) showed a confluent monolayer, sporadically even small conglomerates, while cultures grown in 2.5 mM glucose were subconfluent. Under glucose-free conditions, cells formed small clusters with a high number of dead, rounded cells floating in the medium.

Morphologically there were no differences detectable between the attached viable cells at the diverse glucose concentrations.

The protein content per cell was very similar at all glucose concentrations on day 2, whereas average levels on day 5 were overall lower (Fig. 3.4. B.). On day 5, the protein content decreased with increasing glucose concentration, until it reached a plateau at glucose levels higher than 5.56 mM. Highest protein levels after five days were found in MCF-7 cultures grown in glucose-free medium. These results show, on one hand, that in low-growth medium glucose levels of 2.5 mM still sustain cell proliferation. On the other hand, the increased protein levels in cultures growing at low glucose concentrations indicate changes in protein metabolism, depending on the presence of glucose.

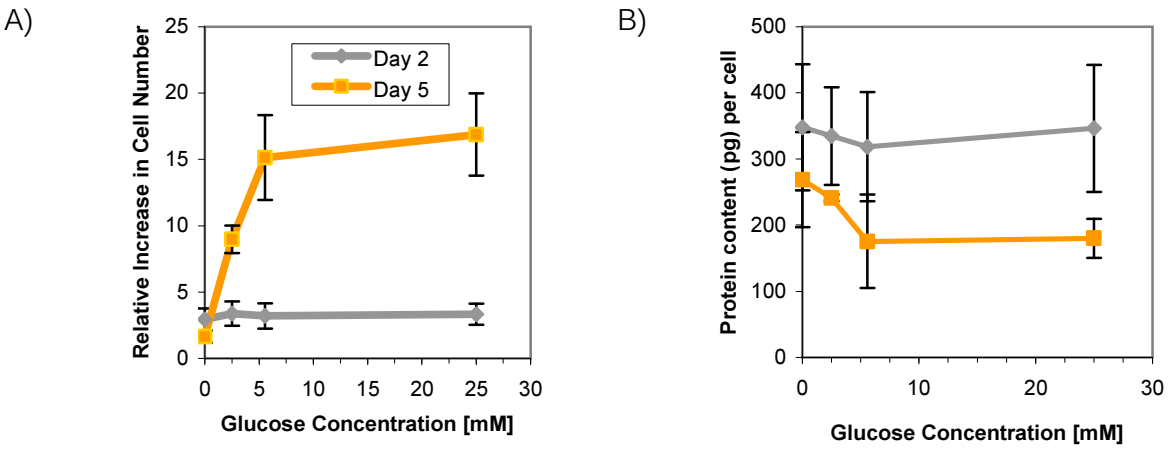


Fig. 3.4. Effect of reduced glucose concentrations on MCF-7 cell growth and protein content (glutamine concentration 4 mM). A) Relative increase in cell number B) Protein content per cell. Cultures were measured on day 2 and day 5. Results are averages and ranges of three independent experiments

3.1.2.2. Limiting glutamine concentration

Glutamine is discussed as an alternative metabolite for providing energy in tumor cells, especially in conditions with a poor glucose supply. To investigate the limiting concentration of glutamine, which can enhance cell growth in low glucose clutures, MCF-7 cells were incubated in low growth medium with 1 mM or 2.5 mM glucose and glutamine concentrations up to 0.5 mM. After 2 days there was a continuous increase in cell number with rising glutamine concentrations. There is no significant increase in cell number after two days of cells growing in 1 mM or 2.5 mM glucose (Fig. 3.5. A.). After 5 days a completely different pattern was observed: up to glutamine levels of 0.1 mM, cell number developed similar in both glucose concentrations (1 mM and 2.5 mM). With higher glutamine concentrations, a difference in cell number of 2.5 mM and 1 mM glucose cultures developed (Fig. 3.5. B.). The cell number of MCF-7 growing in 2.5 mM glucose further increased with increasing glutamine concentrations and reached a plateau at a concentration of 0.5 mM, where cell number had increased seven-fold. Cells cultured in 1 mM glucose showed a peak at 0.1 mM glutamine, where the cell number had increased almost four-fold. At higher glutamine concentrations, however, cell number decreased again.

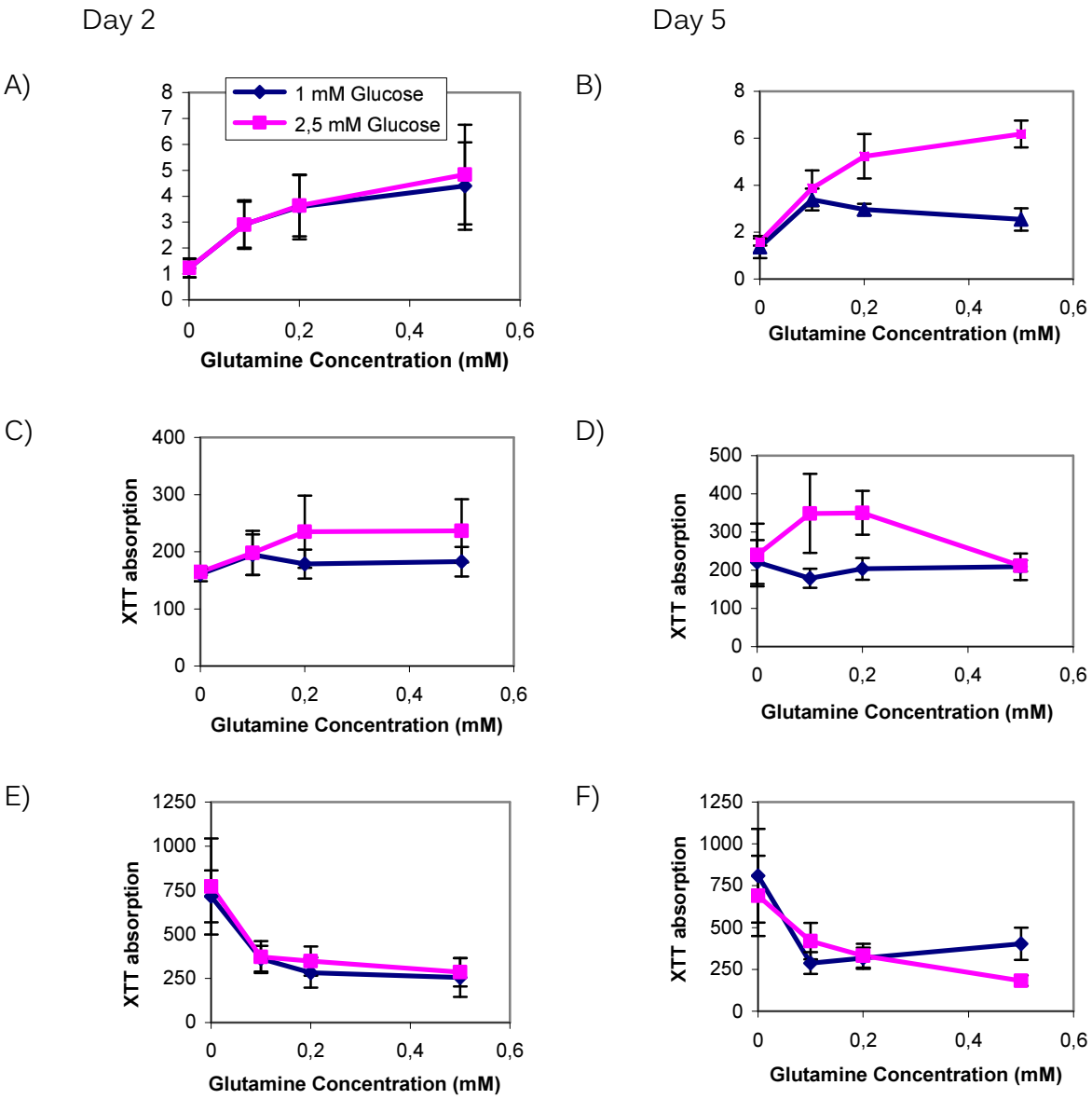


Fig. 3.5. Effect of glutamine at glucose concentrations of 1 mM and 2.5 mM incubated for 2 days (A, C, E) and 5 days (B, D, F). Increase in relative cell number on day 2 (A) and on day 5 (B). Dehydrogenase activity represented by XTT absorption at 450 nm per culture (C;D) and per cell number (E,F). Results are averages and ranges of three independent experiments

To test for the metabolic activity, dehydrogenase activity was measured with the XTT assay. When analyzing the absorption values of the cultures after two days, the curves of 1 mM and 2.5 mM glucose appear to diverge with increasing glutamine concentrations (Fig. 3.5. C.). However, both curves are similar when the dehydrogenase activity is related to the cell number (Fig. 3.5. E.). In contrast to the cell number, the specific mitochondrial dehydrogenase activity per cell decreased with rising glutamine concentrations.

On day 5, the absorption per culture with no glutamine was independent of the glucose concentration. Cultures grown in 1 mM glucose kept a steady signal independent of the glutamine concentrations, whereas dehydrogenase activity increased in cultures supplemented with 2.5 mM glucose in combination with 0.1 mM and 0.2 mM glutamine (Fig. 3.5. D.). The curve progression of the dehydrogenase activity per cell showed, similar to the results from day 2, an initial drop of activity after adding glutamine in both, cultures with 1 mM and 2.5 mM glucose (Fig 3.5. F.). Glutamine concentrations higher than 0.1 mM led to an increase in dehydrogenase activity per cell in cultures with 1 mM glucose, whereas dehydrogenase activity per cell continued to decrease in cultures with 2.5 mM glucose. Again, the curve progression of dehydrogenase activity per culture was not congruent with the cell number and the dehydrogenase activity per cell behaved opposite to the cell number.

3.1.3. Effect of extracellular pH at limiting glucose levels

As described in the Introduction, the extracellular pH in a tumor microenvironment can be very variable and is often more acidic than in normal tissue. The aim of the following experiments was to investigate cell growth (Fig. 3.6. A./C.) and dehydrogenase activity (Fig. 3.6. B./D.) of MCF-7 cells growing at various pH levels.

Maximum cell growth was observed in cell cultures cultivated at pH 7.4 and 25 mM glucose with an increase in cell number of 15-fold (Fig. 3.6. C.). When MCF-7 cells were supplied with glucose concentrations between 1 mM and 5.56 mM, the highest increase in cell number was observed in cultures growing at pH 7.0 (Fig 3.6. C.). In medium with 0.56 mM or no glucose, cells growing at pH 7.4 showed the lowest increase in cell number compared to more acidic conditions (Fig 3.6. A.). Overall, the growth rate of MCF-7 cells growing in pH 6.6 remained constant showing no dependence on glucose concentration (Fig 3.6. A.).

Dehydrogenase activity was low in cultures with glucose concentrations higher than 5.56 mM and there were no significant differences between cultures grown in 25 mM and 5.56 mM glucose at various pH levels (Fig. 3.6. B.). However, with the reduction of the glucose concentration, the growth droped (Fig. 3.6. A) and dehydrogenase activity increased (Fig. 3.6. B.). The cultures growing in pH 6.6 showed no significant affect in dehydrogenase activity by glucose reduction as also observed for cell growth.

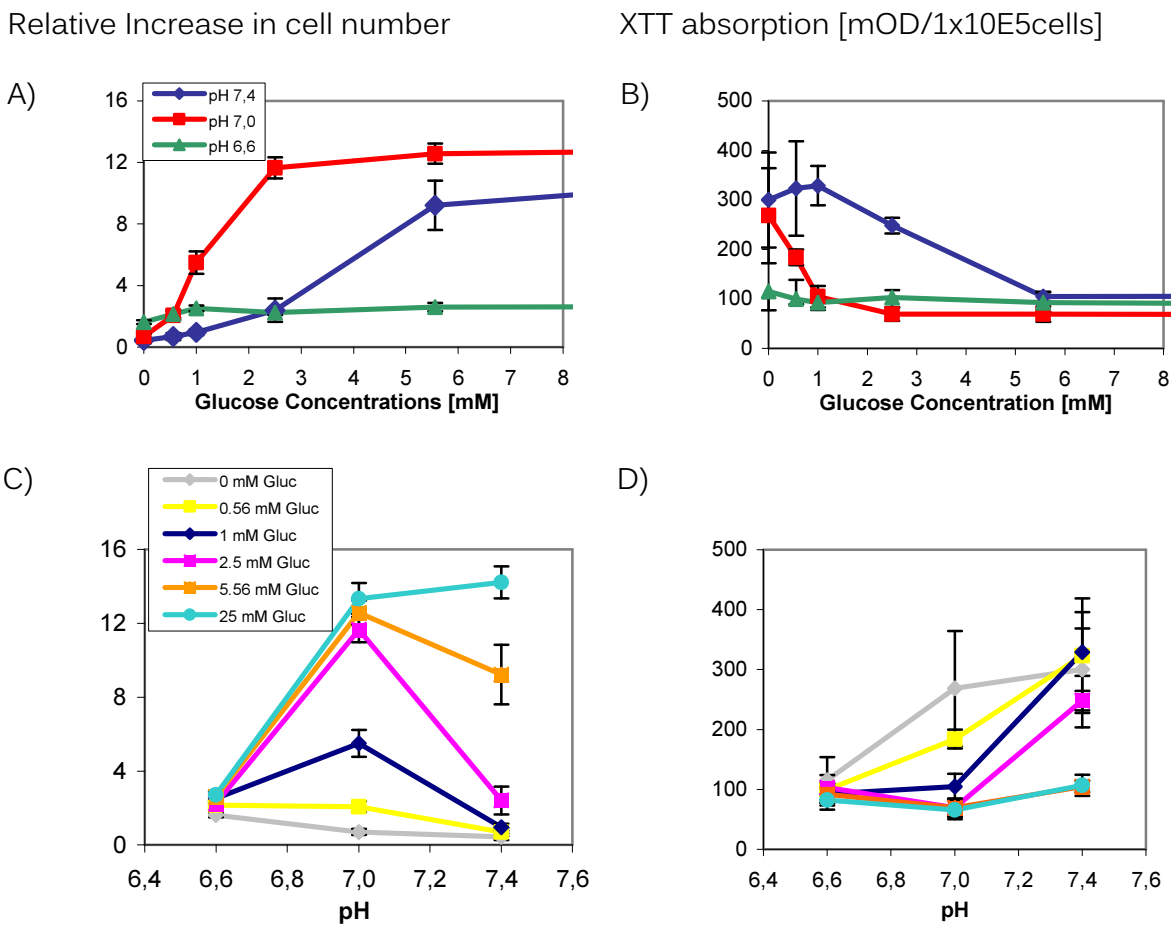


Fig. 3.6. Effect of pH and glucose deprivation on cell number and dehydrogenase activity of MCF-7 cells supplemented with 4mM glutamine. Cells were incubated for 5 days. A) Relative Increase in cell number at different glucose concentrations (25 mM glucose not shown). B) Dehydrogenase activity relative to cell number at different glucose concentrations (25 mM glucose not shown) C) Relative increase in cell number and D) dehydrogenase activity at different pH levels. Results are averages and ranges of three independent experiments.

Again, the curve progression of dehydrogenase activity and cell number are not comparable. The impression is conveyed that dehydrogenase activity rather indicates the mitochondrial activity than can be used synonymous with

the cell number. However, at pH 7.4 and 7.0 cell number and dehydrogenase activity were markedly dependent on glucose concentration, wereas at pH 6.6 cells appear to have a different survival strategy, for number and cell metabolism stay independent of glucose concentration.

3.2. Chemosensitivity testing in low growth medium

Doxorubicin and 2-deoxy-D-glucose were chosen as model agents for investigations on the influence of the metabolic state of MCF-7 cells on the chemosensitivity under reduced nutrient conditions.

3.2.1. Doxorubicin

Doxorubicin is a well- established agent in clinical breast cancer therapy, although its cytotoxic mechanism is not fully elucidated. However, cell culture experiments have shown that the antineoplastic effect of doxorubicin leads to apoptosis. The following experiments were performed to investigate changes in growth and mitochondrial activity of MCF-7 cells when treated with doxorubicin under low growth conditions.

Cells were cultivated in DMEM + 10% FCS, DMEM + 1% FCS and low growth medium, doxorubicin was added in concentrations of 0.05 µM up to 0.2 µM. Fig. 3.7. A. shows that after 5 days of incubation, cells were almost completely inhibited in growth at all doxorubicin concentrations. The incubation time was shortend to 2 days to obtain the cultures in their metabolically active phase and to obtain a better impression of proliferation and metabolic changes

caused by this potent cytotoxic agent. Results showed a continuous decrease of cell number with increasing doxorubicin concentrations (Fig. 3.7. B.).
Even in these low doses, doxorubicin acts as a potent cytotoxic agent in all cell cultures. Low growth medium has no reinforcing or allevative effect on the cytotoxicity of doxorubicin, even though cells cultivated in 10% FCS showed overall highest cell numbers.

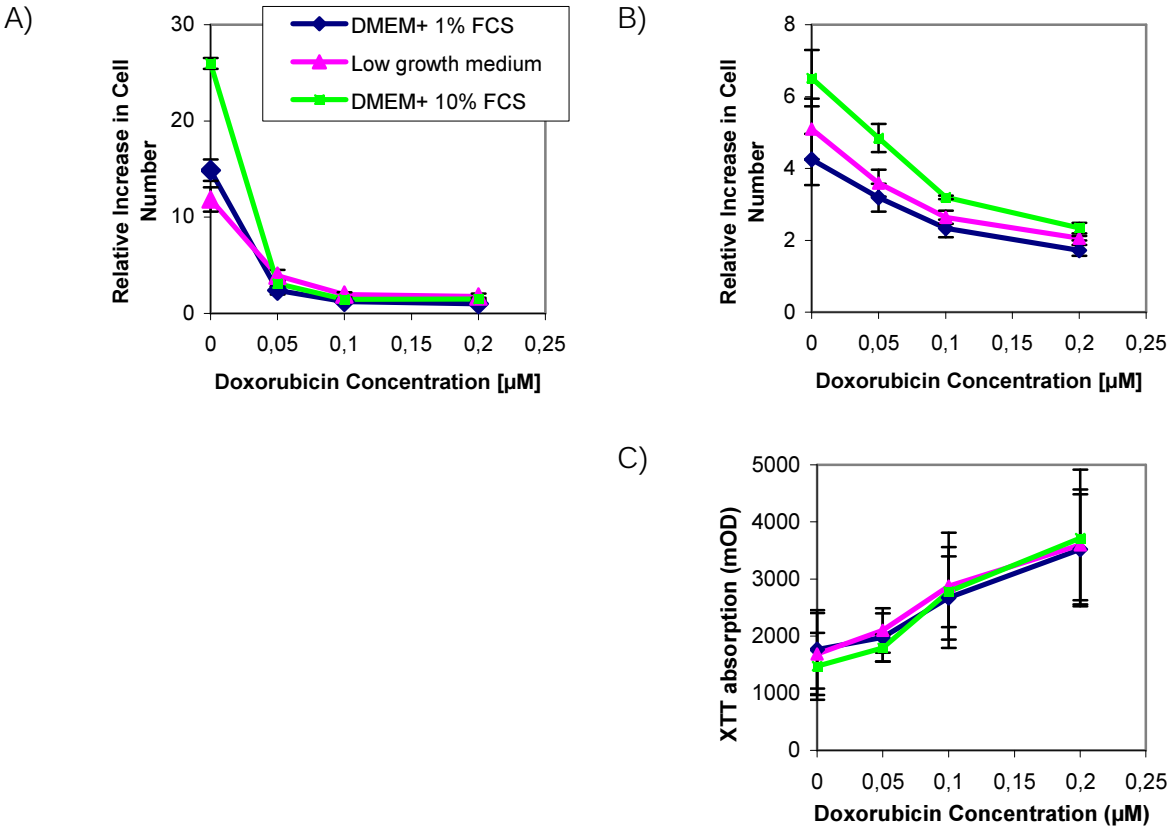


Fig. 3.7. Inhibitory effect of doxorubicin on MCF-7 cells growing in nutrient deprived medium on cell number after treatment with doxorubicin for 5 days A) and 2 days B). Dehydrogenase activity of MCF-7 cells growing in nutrient deprived medium and doxorubicin for 2 days C). Results are averages and ranges of three independent experiments.

However, cell growth in cultures treated with 0.2 µM doxorubicin reached a plateu regardless of the initial growth medium. Almost no cells survived treatment with doxorubicin over five days, independent of the doxorubicin dose or the culture medium. In contrast, dehydrogenase activity showed no significant difference between the three culture mediums (Fig. 3.7. C.). With rising doxorubicin concentrations an increase in dehydrogenase activity was observed.

These experiments confirmed that low concentrations of doxorubicin are potent to inhibit cell growth. After two days of treatment MCF-7 cells are found in a metabolic active phase, whereas after 5 days almost all cells are dead. To further investigate the metabolic changes and the mechanism of cell death, sensor chip measurements were performed.

3.2.2. 2-Deoxy-D-glucose

To test whether the standard dose of 10 mM 2d-glucose is sufficient for competetive inhibition in this experimental cell culture system, MCF-7 cells were grown in various glucose concentrations. Since a significant inhibitory effect was observed at glucose concentrations up to 25 mM (Fig. 3.8.) this 2d-glucose concentration was used for further experiments .

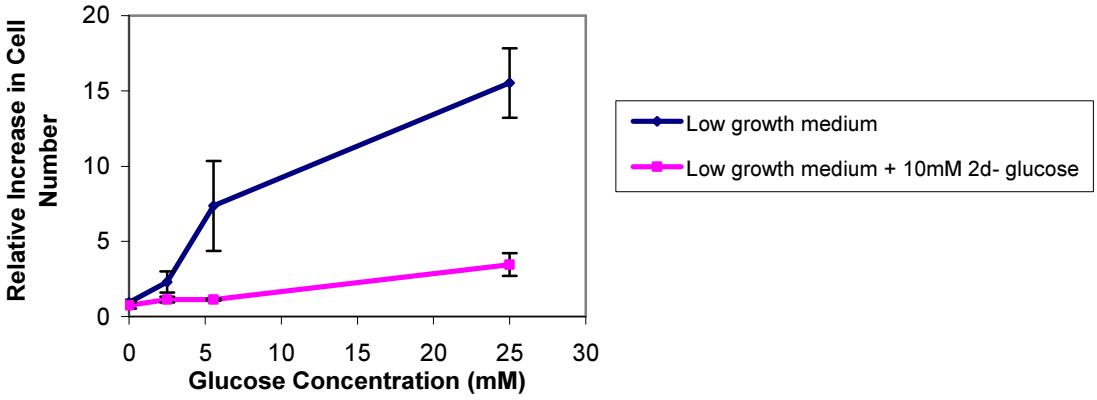


Fig. 3.8. Effect of 2d-glucose on cells growing in medium with variuos glucose concentrations. Cells were incubated for five days. Results are averages and ranges of three independent experiments.

The effect of 2d-glucose was investigated more closely at lower glucose levels of 5.56 mM and 0.56 mM. After five days of incubation, as expected, cultures growing in 5.56 mM glucose were more resistant to 2d-glucose than cells growing in the lower glucose concentration (Fig. 3.9. A). At 1 mM 2d-glucose did not appear to influence cell growth with 5.56 mM glucose. At higher concentrations of this cytotoxic agent, the cell number was continously diminished, until at 100 mM 2d-glucose no cell growth was observed.

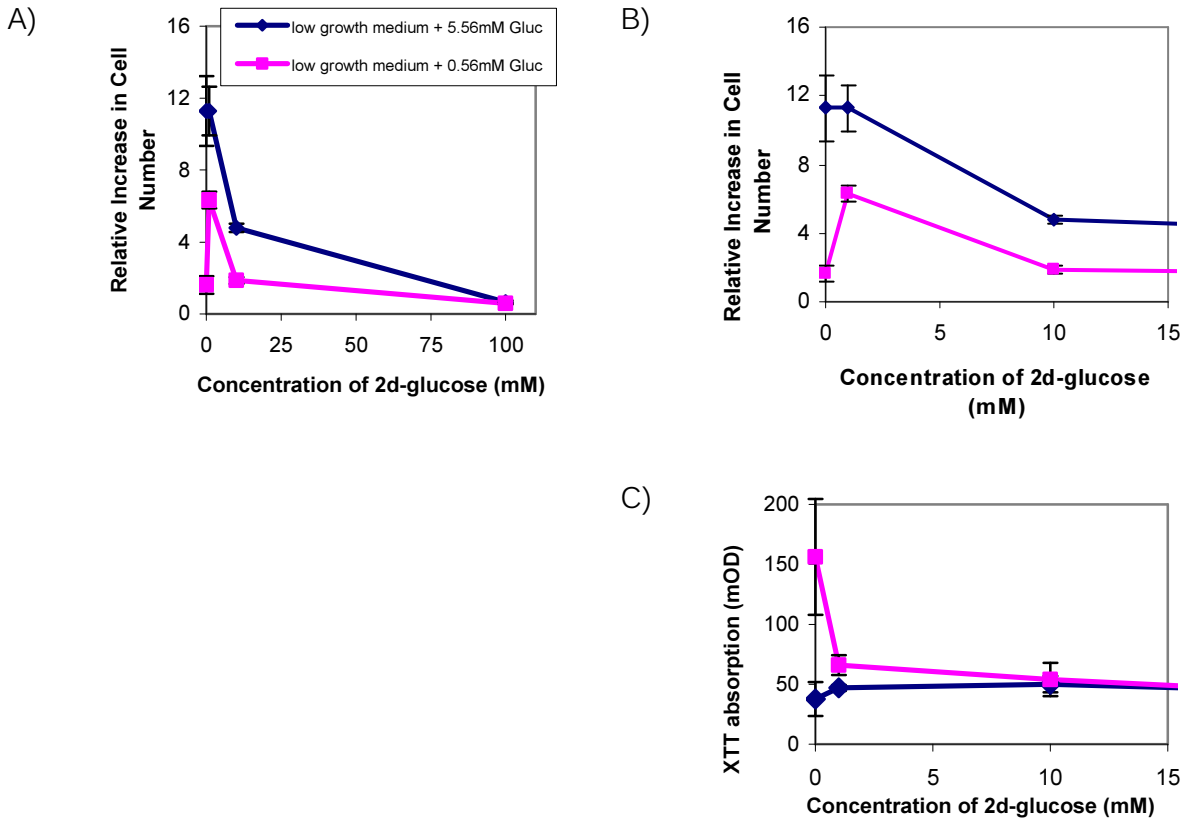


Fig. 3.9. Effect of 2d-glucose on MCF-7 cells under glucose deprived growing conditions (5.56 mM and 0.56 mM glucose). A) Relative increase in cell number after 5 days of incubation with a focus on low 2d-glucose concentrations (B) and its effect on dehydrogenase activity (C). Results are averages and ranges of three independent experiments.

When cells were growing in medium with even lower glucose concentrations of 0.56 mM, cell number was poor even when no 2d-glucose was added (Fig. 3.9. A). Surprisingly, the addition of 1 mM 2d-glucose lead to a remarkable increase in cell number, which was attenuated with higher concentrations of 2d-glucose.

The cellular metabolic activity, represented by XTT absorption, was higher in cell cultures growing in 0.56 mM glucose than in 5.56 mM glucose. Adding 2d-

glucose to cultures growing in 5.56 mM glucose induced no significant change in dehydrogenase activity (Fig. 3.9. C.). Highest activity was observed in cultures growing in 0.56 mM glucose without 2d-glucose. Adding 2d-glucose lead to a significant decrease of dehydrogenase activity (Fig. 3.9. C.). Again, the highest dehydrogenase activity was observed in cultures with the least increase in cell number. These results once more confirm, that dehydrogenase activity and cell number provide two independent sets of information about the metabolic state of the cell.

The stimulation of cell growth by 2d-glucose under low nutrient conditions was not expected. The effect of increasing concentrations of 2d-glucose up to 5 mM was investigated in MCF-7 cells cultured in low-growth medium with 0.56 mM glucose and an extracellular pH between 6.6 and 7.4 (Fig. 3.10.). In medium with pH 7.0, cultures attained a higher cell number than those at 7.4 (Fig. 3.10. A.), cultures of a pH of 6.6 showed poorest proliferation. A growth promoting effect was obtained in cultures with 2d-glucose concentrations between 0.5 mM and 2 mM and pH 7.4 and 7.0., while at pH 6.6 cells were not affected (Fig. 3.10. A.).

Dehydrogenase measurements of cultures growing in 2d-glucose concentrations up to 0.5 mM exhibit high values, whereas dehydrogenase activity decreased when 2d-glucose concentrations increased at both, pH 7.4 and 7.0 (Fig. 3.10. B.). These results confirm the effect that was already observed in previous experiments (Fig. 3.6., Fig. 3.9.): High cell number, as here observed at 0.56 mM and 1 mM 2d-glucose, correlates with a low

dehydrogenase activity. 2d-glucose did not influence dehydrogenase activity or cell number in cultures growing at pH 6.6.

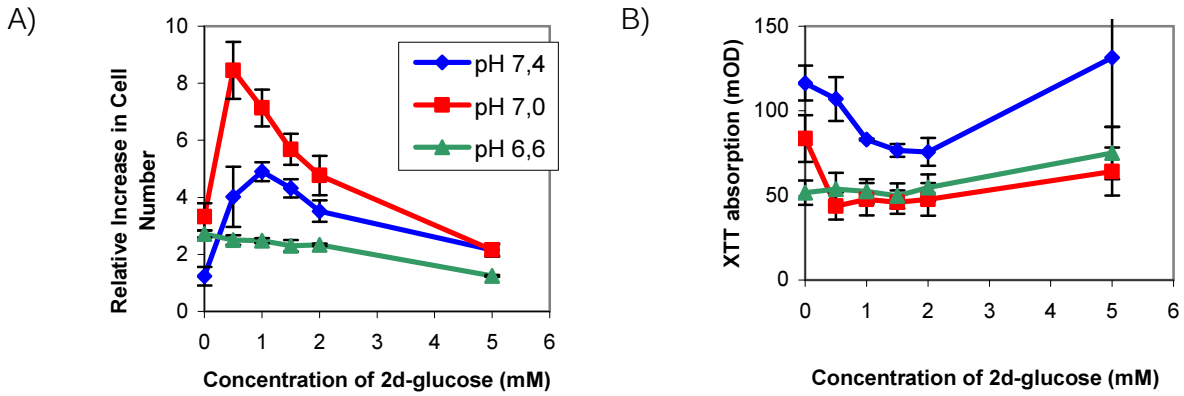


Fig. 3.10. Effect of low 2d-glucose concentrations between 0.5 mM and 5 mM on MCF-7 cells growing in medium with 0.56 mM glucose at pH 7.4, 7.0 and 6.6. Cells were incubated for 5 days. A) Relative increase in cell number. B) Dehydrogenase activity. Results are averages and ranges of three independent experiments.

3.3. Sensor chip measurements for metabolic monitoring

Sensor chips were developed for real-time monitoring of the cellular metabolism. MCF-7 cells were easily cultivated onto the sensor chips and showed a good cell growth with good attachment to the sensor plate.

The sensor chip system operated in conditions selected according to the cell culture results obtained in the previous chapters, results served to exemplify

and evaluate the application of this test system. The information obtained was compared with the standard metabolic test XTT.

3.3.1. Effect of doxorubicin

In standard cell cultures which were incubated with doxorubicin at concentrations up to 0.2 µM, a decrease of cell number after only two days was observed. For cell chip experiments, 1 µM doxorubicin was chosen to obtain an adequate and measurable effect in a shorter time.

After 6 hours of incubation with DME + 5%, a stable signal was obtained. The metabolic activity stayed constant during the next 24 hours. Upon addition of 1 µM doxorubicin a decrease in oxygen consumption was observed within two hours (Fig 3.11.), whereas acidification rate increased, reaching a maximum after 10 hours. Thereafter, acidification rate also decreased continuously, which is indicative for cell death. The experiment was stoped by adding hypotonic buffer and lysis solution.

These results show that rapid metabolic changes can be monitored by the sensor chip system. Furthermore, a metabolic change in MCF-7 cells was observed during the first 10 hours after treatment with doxorubicin, before cells underwent cell death.

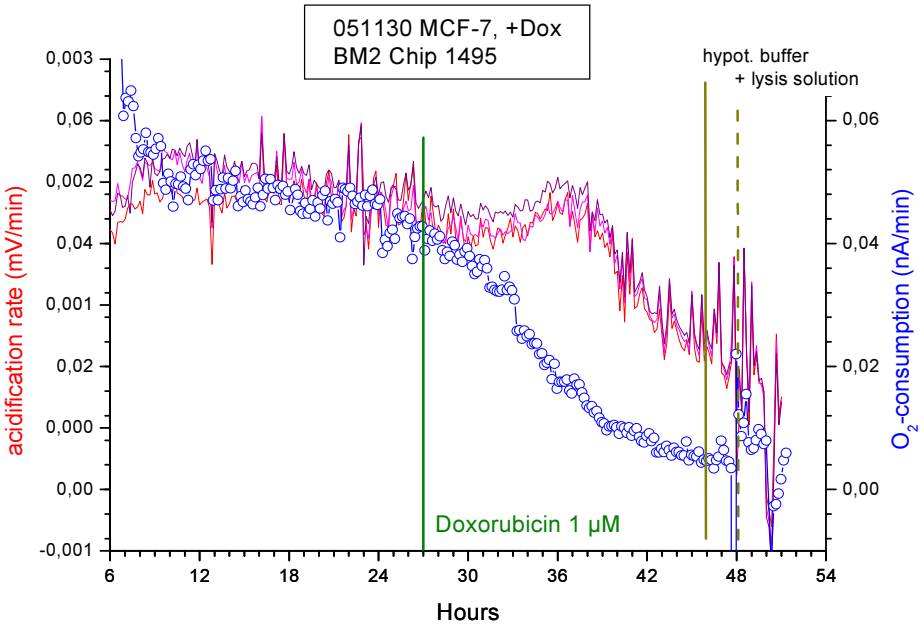


Fig. 3.11. Metabolic activity of MCF-7 cells growing in DME + 5% FCS measured with sensor chips. 1 µM Doxorubicin was added to the medium about 27 h after beginning of the measurement. At the end hypotonic buffer and lysis solution were added to terminate the experiment.

3.3.2. Effect of different growth conditions and 2-Deoxy-D-glucose

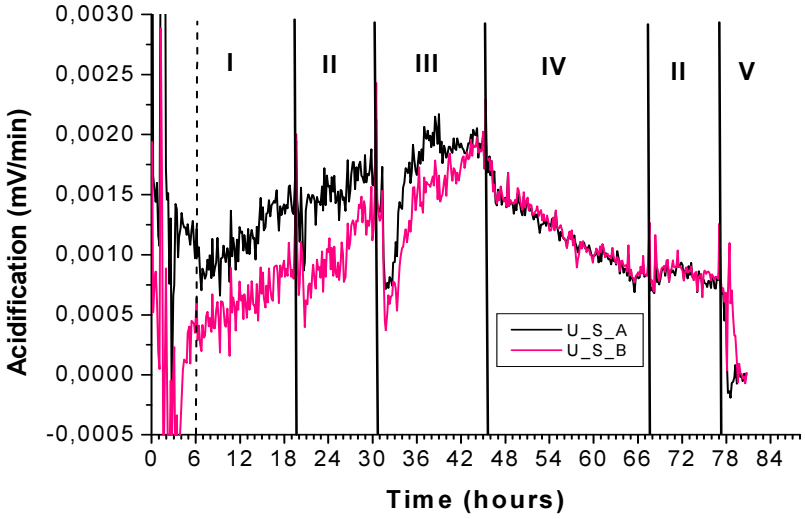
The effect of 2d-glucose on the rate of acidification and oxygen consumption was investigated according to previous conditions in cell culture (Fig. 3.8.). First, the effect of different growth media on cell metabolism was monitored with DMEM + 5% FCS (Fig. 3.12., (I)), before changing to the low growth medium (DMEM + 1% FCS + 50 nM Ins) at pH 7.4 (II). Acidification rate and oxygen consumption continued to increase. When the medium was then changed to

the low growth medium at pH 6.6 (III), there was a transient drop in cellular acidification, which rapidly increased again until it stayed stable at the same level as before. Similarly, oxygen consumption increased at a slightly higher rate and reached a stable level after 7 hours.

When 10 mM 2d-glucose was added to the medium at acidic conditions (IV), a continuous decrease in both acidification rate and oxygen consumption was observed. To test whether the cell could recover, the medium was changed back to the low growth medium at pH 7.4 (II). While acidification rate remained at a plateau value, oxygen consumption continued to decrease. The experiment was terminated by adding 0,2% Triton X to obtain the basic signal of non-viable cells.

The experiments showed that cells rapidly change their metabolic activity with a change in the extracellular milieu. After adding 2d-glucose, a continuous decrease in oxygen consumption and acidification rate indicated cell death, which was not reversible even after restoring low growth conditions.

A)



B)

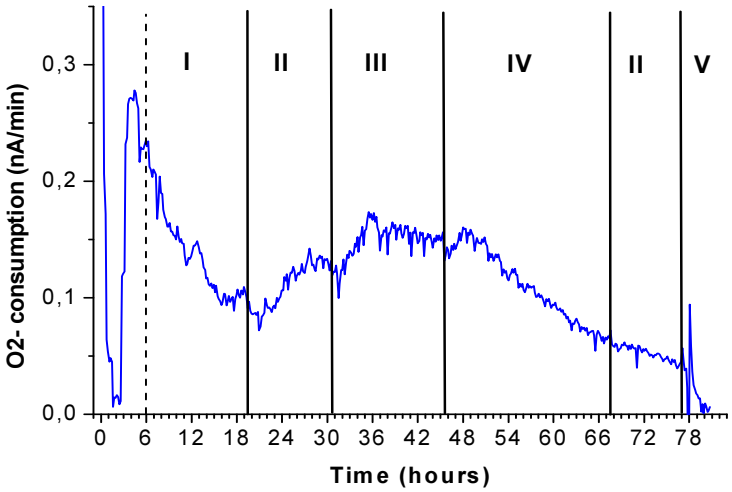


Fig. 3.12. Effect of starvation, medium acidification and 2d-glucose on acidification rate and oxygen consumption of MCF-7 cells grown on a microsensor chip.
A) Acidification rate. Results of two ISFET sensors of the same chip are shown. B) Oxygen consumption.
I) DMEM + 5% FCS; II) Low growth medium pH 7.4; III) Low growth medium pH 6.6; IV) Low growth medium + 10 mM 2-deoxy-D-glucose pH 6.6; V) addition of 0,2% Triton X;

4. Discussion

4.1. Sensor chip system

The underlying aim of this study was to investigate the metabolism of MCF-7 cells under diverse microenvironmental conditions and to gain data as reference for a potential establishment of the sensor chip system in the clinical routine. In this study, the goal of the sensor chip system was to monitor the effect of chemotherapeutics on tumor metabolism. In the future, an individual chemotherapy recommodation could be given by testing tumor biopsate tissue in such sensor chip systems and gaining precise metabolic information on the response rate. This approach would serve to individualize combinations of chemotherapeutic drugs and doses to improve response, minimize toxic side effects and thus lead to longer survival and higher quality of life in tumor patients.

The experiments in this study confirmed the potentials of a dynamic real-time monitoring of metabolic reaction of tumor cells in the sensor chip test system. It was possible to detect changes in the rate of acidification and oxygen consumption resulting from extracellular pH or reduced concentrations of FCS in the medium. Such metabolic changes can also be monitored in MCF-7 cells when treated with chemotherapeutics. This was exemplified in a decrease in acidification rate and oxygen consumption within hours after adding 2d-glucose or doxorubicin.

Other groups have reported similar approaches for real-time monitoring of metabolic changes resulting from chemotherapeutic treatment– albeit with other technological set-ups, other tumor cells or drugs (1, 28, 99, 100, 115, 148). For example, a recent publication showed the effect of doxorubicin on MCF-7 cells, using optochemical sensors with similar results for acidification rate and oxygen consumption (76). This shows that real-time measurements using sensor chips provide useful information on the metabolic activity.
However, it should be mentioned, that a number of problems were encountered during the experiments since the device which is based on electro-chemical sensors and was used in this study was still a prototype. These included breakdown of the sensor chip modules during measurements, leading to a limited reproducibility of the data. The electrotechnical and electrochemical dysfunctions which became apparent flowed into further technological developments, for example in optochemical sensors (148).

4.2. The use of tetrazolium salt-based assays

The tetrazolium salt-based assay was originally used to serve as reference for the sensor chip measurements. A decrease in specific dehydrogenase activity is indicative of a reduced metabolic activity of the cell. It likely would be expected that acidification rate and oxygen consumption measured by sensor chips is also reduced when cell metabolism is impaired. However, this correlation was not always observed: for example, when comparing the XTT experiments and the sensor chip measurements with doxorubicin an increase

in dehydrogenase activity was observed, while the rates of oxygen consumption and acidification (after an initial increase) decreased (Fig. 3.7. C and Fig. 3.11).

Moreover, when MCF-7 cells were exposed to pH 6.6, low activities of all three, acidification rate, oxygen consumption and dehydrogenase activity were measured (Fig. 3.6. B/D and Fig. 3.12. III). Due to these results it must be assumed that sensor chip measurements and dehydrogenase activity provide different types of metabolic information.

In contrast to many other results and reports, changes in specific dehydrogenase activity (i.e. per cell) do not correlate with the corresponding change in cell number. This effect was demonstrated with good reproducibility in all experiments that involved treatment with either limiting nutrient levels or chemotherapeutics as 2-deoxy-D-glucose or doxorubicin. The increase in dehydrogenase activity can be interpreted as enhanced metabolic activity of specific aspects of the cell. Knowing that the mitochondria play a central role in the initiation and process of apoptosis (119), the elevated dehydrogenase activity can also be an indicator of elevated mitochondrial activity to provide the cell with energy required in the initial state of apoptosis (54, 84).

It has to be emphasized that tetrazolium salt-based assays describe a metabolic activity of cells without giving precise information on the actual metabolic processes, on the rate of proliferation, or the toxicity of compounds, which is claimed in many commercial assay kits. A statement concerning cell proliferation or cell viability only on the basis of this test can not be made. The

appropriateness and the informational value of tetrazolium salt-based assays has to be reconsidered and evaluated for each cell system used.

4.3. Growth and metabolism of MCF-7 cells in a nutrient-deprived environment

As described above, most cell culture experiments with tumor cell lines are run in saturated nutrient conditions. For example, glucose concentration in DMEM (25 mM) would in vivo correspond to a blood glucose of 450 mg/dl. This pathologic concentration would be immediately treated with insulin when occuring in patients. The purpose of the first experiments was therefore, to develope culture conditions resembling more closely the in vivo tumor microenvironment.

Reduced growth factor conditions (1% FCS) led to low, but sustained cell growth when supported by 50 nM insulin (Fig. 3.2.). No additional effect on cell growth is achieved by estradiol (Fig. 3.3.), even though MCF-7 are cells estrogen-positive. This observation can be explained by sufficiently activated IGF- receptors for growth stimulation by 50 mM insulin (86). In this case, further activation of cell growth by estradiol-dependent activation of the ERK pathway can not be achieved (44).

Glucose and glutamine concentrations were reduced in the culture medium to levels found in the interstitial compartment of solid tumors (72, 103). Poorer cell growth in cultures with 1 mM glucose compared to 2.5 mM glucose indicates a

depleted glucose supply after five days of cultivation, for after two days curves are still congruent (Fig. 3.5. A/B.). However, when glucose is depleted, cell metabolism is not influenced by the presence of glutamine since an increase in glutamine concentrations has no influence on cell number (Fig. 3.5. B.). Similar to the cell number, an effect on dehydrogenase activity can not be observed before day 5 (Fig. 3.5. C/D.). Changes in dehydrogenase activity indicate metabolic changes depending on the glutamine/glucose ratio on day 5 (Fig. 3.5. D.); however, these changes are not reflected in the cell number. Most cell culture experiments are performed at pH 7.4, even though there is now consensus that the microenvironment of tumor cells is likely to be more acidic than that of normal tissue (137, 143). No significant difference in cell number between tumor cells cultivated with physiological pH (7.4) and at pH of 7.0 was observed in DMEM with 25 mM glucose (Fig. 3.6. C). However, cultures growing in low glucose concentrations for five days had a higher cell number at pH 7.0 than at pH 7.4 (Fig. 3.6. A.). A markedly reduced growth activity of MCF-7 cells was observed in medium with a low extracellular pH of 6.6. It can be assumed that energy metabolism of MCF-7 cells growing at pH 6.6 is independent of glycolysis, for cell growth at these low pH levels was independent of glucose concentrations (Fig. 3.6. A.) and also did not show a significant sensitivity to 2-deoxy-D-glucose (Fig. 3.12. A.). As shown with sensor chip experiments, a rapid change in the acidification rate and O_2 consumption after reducing the extracellular pH indicate a modulation in cellular metabolic activity (Fig. 3.12. III). These results confim earlier reports postulating an

inhibition of glycolysis in other solid tumor cell lines at low pH (58, 125). However, the cell metabolism appears to have readapted in only a few hours, again showing an acidification rate as before. MCF-7 cells seem to cope with the adverse nutrient conditions by switching their metabolism and therefore survive under these marginal conditions. Such adaptation on chemotherapeutic treatment may be an important cause for failure of conventional therapies.

Taken together, these results indicate that the systematic combination of limiting microenvironmental parameters can conserve cell growth. Furthermore, cell cultivation in the commonly used oversaturated media could lead to results that are not representative for cells growing in tumor tissue and may thus lead to misinterpretations of the effects of chemotherapeutics.

4.4. Metabolism of MCF-7 cell under treatment with doxorubicin

Doxorubicin was chosen in this study since it is a well investigated chemotherapeutic drug with a high relevance in breast cancer treatment. To obtain MCF-7 cells in their metabolic active phase, the incubation time of experiments with doxorubicin was shortened to two days (Fig. 3.7. B.). Also, sensor chip measurements covered the first 20 hours after treatment with doxorubicin. The initial increase in acidification rate several hours after doxorubicin addition indicates a change in cell metabolism towards pathways

that produce more acidic metabolites, while at the same time oxygen consumption decreased. It can be assumed that, according to the known cytotoxic mechanisms of doxorubicin (102), mainly direct membrane effects and the induction of apoptosis cause these metabolic changes. Changes in tumor DNA due to doxorubicin would not be expected to effect cell metabolism within only a few hours. On the other hand, an elevated glucose metabolism under treatment with doxorubicin has been described in HL-1 cardiomyocytes (132). The increase in acidification rate can be explained by an elevated anaerobic glycolysis, which results in lactate production. Possibly, this metabolic pathway serves as ATP provider when apoptosis is induced. Further metabolic breakdown of glycolytic metabolites in the Krebs cycle is unlikely, since the decrease in oxygen consumption indicates impaired oxidative phosphorylation. Under treatment with doxorubicin, cell cultures showed an activation of dehydrogenase activity (Fig. 3.7. C), which suggests an activation of mitochondria, known to be required for the initiation of apoptosis. However, molecular processes of cells treated with doxorubicin are still not clear and have to be investigated in further experiments.

4.5. Metabolism of MCF-7 cells under treatment with 2-deoxy-D-glucose

Experiments performed with 2d-glucose were initially aimed at investigating the cell metabolism under glucose deprived conditions. After adding 10 mM

2d-glucose to the medium, an immediate reduction in the rates of acidification and O_2 consumption was measured using sensor chip measurements. This experiment underlines the ability of MCF-7 cells to adapt to metabolic changes within 1-2 hours. Therefore, these changes can not be due to changes in gene expression, but are more probably due to changes in enzyme activity. An enhancement in oxidative phosphorylation to compensate the inhibited glycolysis, as postulated (57, 82), was not confirmed by sensor chip measurements. An elevated oxygen consumption would be expected if oxidative phosphorylation was activated. However, treatment with 2d-glucose lead to a continuous decrease in oxygen consumption. Moreover, the decreasing acidification rate might be explained by the inhibition of glycolysis by 2d-glucose, and thus less lactic acid production.

An unexpected increase in growth was observed in MCF-7 cultures with limiting glucose concentrations (0.56 mM) and the addition of 0.5-1 mM 2d-glucose (Fig. 3.10.). This observation can not be explained by an incomplete inhibition of glycolysis, since in this case a further increase in cell number would be expected when 2d-glucose was further reduced. Appearently, there is an alternative metabolic route to gain energy with this extracellular glucose / 2d-glucose quotient. It can be postulated that collateral energy pathways allow a further metabolization of 2d-glucose-6-P, even though phosphohexoisomerase is inhibited, for example through the pentose phosphate pathway (Fig. 1.7.) (50, 107, 136). It is not quite clear whether oxidative phosphorylation is enhanced to maintain ATP production under these

conditions (82). Acetyl-CoA needed in the Krebs Cycle can also be obtained as metabolite from protein and fatty acid metabolism (90). However, all this can not explain why treatment with low concentrations of 2d-glucose supports and higher concentrations attenuates cell growth.
An other option might be that the cell is able to continue with gylcolysis by integration of e.g. dihydroacetonphosphate via the fatty acid metabolism. Inhibition of glycolysis leads to a new intracellular metabolic balance which triggers alternative energy providing pathways. Another possisble reason for the increase in cell number might be a stimulation of autophagic processes by low concentrations of 2d-glucose. Only little damage is induced, which can be repaired by this selfdigesting mechanism and thereby prevent apoptosis (Cap. 1.1.3.1.). Further experiments to determine metabolites of the potentially involved metabolic pathways are planed. The growth stimulating effect of 2d-glucose can not be explained with the present state of knowlege of metabolic mechanisms.

4.6. Conclusion and outlook

Using sensor chips measurements, cell metabolism can be monitored dynamically and in real-time. Conclusions on the changes in metabolic pathways can be drawn by observation of different phases of metabolic alteration, whereas conventional cell cultures solely reflect one point in time. However, numerous electrotechnical and electrochemical problems are still for

electrical engineers to solve before reliable and reproducible reference data can be collected.

In cell culture experiments influences of the microenvironment on cell metabolism and thus chemosensitivity were analyzed. In-vitro culture experiments performed in saturated growing conditions do not necessarily represent the cellular behaviour in-vivo. Interactions of nutrients and growth factors were observed at limiting nutrient concentrations, which had suprising influence on cell growth and energy metabolism. Without implying that the used experimental conditions were exactly those of a tumor microenvironment, it has to be admitted that certain effects are lost in media with saturated nutrient conditions. Tumor cells of one and the same type can exhibit a completely different growth behaviour, depending of the microenvironment that is offered. This leads even that far, that MCF-7 cells may have a better growth under treatment with low doses of the cytotoxic agent 2d-glucose than without it.

The more important it will be in the future to work with conditions that mimic the tumor microenvironment as well as possible to get a more authentic picture of the behaviour of malignant cells. Limiting nutrient conditions represent precarious conditions and should be included when testing chemotheraputic drugs. Further enzymatic tests will be useful to elucidate the functional state in terms of metabolic dynamics and are essential to complement the substantial data on gene expression of metabolic enzymes.

Furthermore, the results presented above suggest that the specific conclusion of dehydrogenase activity testing must be assessed for each experimental set-up. It was shown that XTT is not reliable as an equivalent for cell number, for the specific dehydrogenase activity is dependent on the cell vitality.

5. Summary

The purpose of my Medical Thesis was to study the metabolism of tumor cells under diverse microenvironmental conditions, to investigate its interrelation with chemotherapeutics and to gain data as reference for the potential establishment of the sensor chip system in the clinical use. In the experimental approach, a more tumor-like environment was mimicked by minimizing the concentration of nutrients and growth factors, and varying the pH of the growth medium. Under these conditions the metabolism of MCF-7 cells was systematically analyzed. Furthermore, chemotherapeutics were used to investigate the effect of microenvironmental parameters on the chemosensitivity. In addition to the one-time measurements of the cell culture, sensor chip measurements were performed to obtain a continuous monitoring of the cell metabolism during starvation and/ or cytotoxic treatment.

The results indicate that the systematic combination of limiting microenvironmental parameters can conserve cell growth. Furthermore, cell cultivation in the commonly used oversaturated media could lead to results that are not representative for cells growing in tumor tissue. The potentials of a dynamic real-time monitoring of metabolic reaction of tumor cells in the sensor chip test system was confirmed. The electrotechnical and electrochemical dysfunctions which became apparent flowed into further technological developments.

6. Acknowledgement

I am grateful to Prof. Dr. M. Schmitt for the opportunity to write my Medical Thesis under his supervision.

I am deeply grateful to my direct supervisor PD Dr. rer. nat. A. Otto for the design of this project, stimulating suggestions, continuous support and advice. She is a patient and a very inspiring discussion partner, and I thank her for critical reading of the manuscript.

I owe my most sincere gratitude to the head of the department of the Heinz Nixdorf- Lehrstuhl für Medizinische Elektronik Univ.- Prof. Dr. rer. nat. B. Wolf for the opportunity to perform my experiments at his department, which provided an excellent scientific environment with an outstanding equipment.

I like to sincerely thank Mrs. I. Szabados for training me in the techniques of laboratory work. Her positivity always lifted me up, even if nothing went as it should.

A very special thanks goes to Mr. Herbert Eger for the orthographic review.

Last but not least I want to thank my family, especially my mother, who provided me with encouragement and support which gave me the strengh to finish this thesis.

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