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The Role of Phosphometabolites in Cell Proliferation, Energy Metabolism, and Tumor Therapy

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A common characteristic of tumor cells is the constant overexpression of glycolytic and glutaminolytic enzymes. In tumor cells the hyperactive hexokinase and the partly inactive pyruvate kinase lead to an expansion of all phosphometabolites from glucose 6-phosphate to phosphoenolpyruvate. In addition to the glycolytic phosphometabolites, synthesis of their metabolic derivatives such as P-ribose-PP, NADH, NADPH, UTP, CTP, and UDP-N-acetyl glucosamine is also enhanced during cell proliferation. Another phosphometabolite derived from P-ribose-PP, AMP, inhibits cell proliferation. The accumulation of AMP inhibits both P-ribose-PP-synthetase and the increase in concentration of phosphometabolites derived from P-ribose-PP. In cells with low glycerol 3-phosphate and malate-aspartate shuttle capacities the inhibition of the lactate dehydrogenase by low NADH levels leads to an inhibition of glycolytic ATP production. Several tumor-therapeutic drugs reduce NAD and NADH levels, thereby inhibiting glycolytic energy production. The role of AMP, NADH, and NADPH levels in the success of chemotherapeutic treatment is discussed.

KEY WORDS: Glycolysis; pyruvate kinase type M_2; glutaminolysis; hydrogen shuttles; phosphometabolites; AMP; NADH; NADPH; apoptosis; tumor therapy.

AEROBIC GLYCOLYSIS

One of the constants observed in studies of tumors is an altered carbohydrate metabolism (Eigenbrodt and Glossmann, 1980, Eigenbrodt *et al.*, 1985, 1992, 1994). A subject of intense investigation has been the high rate of lactate production in the presence of oxygen, so-called *aerobic glycolysis.* In differentiated tissues the inhibition of glycolysis by oxygen has been termed the *Pasteur effect.* This serves as a mechanism for adjusting the consumption of glucose in the presence of oxygen to match the energy requirements of the cells. Under anaerobic conditions in differentiated tissues 1 mole of glucose yields 2 moles of ATP compared to 38 moles of ATP produced in the presence of oxygen. Hence, respiration is the most economical mechanism for the synthesis of ATP from glucose. In tumor cells, a high glycolytic rate under aerobic conditions means that only 5% of the energy available from glucose is utilized. This apparently senseless waste of energy in tumor cells prompted Warburg to postulate a defect in respiratory function leading to an increased lactate production from glucose in the presence of oxygen (Warburg, 1956).

Under special cultivation conditions cells with a defect in respiratory ATP production can be selected if glucose, pyruvate, and uridine are supplemented (King and Attardi, 1989; Larm *et al.*, 1994). Uridine must be added because the mitochondrial dihydroorotate dehydrogenase is inactive in respiration-deficient cells (Löffler, 1989; Larm *et al.*, 1994). In glycolysis 1 mole of glucose is converted into 2 moles of lactate. This ratio of almost 2:1 between lactate production and glucose consumption indicates that all lactate produced must be derived from glucose. However, the ratio between lactate production and glucose consumption is always somewhat less than 2 since some of the

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glucose carbons are used for synthetic processes and do not pass through pyruvate kinase to pyruvate (Mazurek *et al.*, 1997). Therefore, cells without respiration need extracellular pyruvate to eliminate the cytosolic NADH produced by the glyceraldehyde 3-phosphate dehydrogenase reaction and synthetic processes (Groelke and Amos, 1984; Hugo *et al.*, 1992; Mazurek *et al.*, 1997). This pyruvate is excreted as lactate. However, in contradiction to the assumption of Warburg those cell lines that are defective in respiration are less tumorigenic than cell lines with active respiration (Soderberg *et al.*, 1980; Camara *et al.*, 1987; Medina *et al.*, 1990; Goossens *et al.*, 1996).

Data from previous reports suggest that there are many factors that contribute to the origin of aerobic glycolysis. The altered control of glycolysis by expression of certain isoenzymes is one important factor (Fig. 1) (Burke *et al.*, 1978; Carney *et al.*, 1982; Eigenbrodt *et al.*, 1983a, 1985; Vora *et al.*, 1985; Hue and Rider, 1987; Mäueler *et al.*, 1987; Skala *et al.*, 1987; Arora and Pedersen, 1988; Baumann *et al.*, 1988, Hennipman *et al.*, 1989; Schwartz, 1990; Freitas *et al.*, 1991; Staal and Rijksen, 1991; Ahn *et al.*, 1992; Steinberg *et al.*, 1992, 1994; Brinck *et al.*, 1994; Bannasch, 1996). Furthermore, the glycerol 3-phosphate shuttle is inactivated (López-Alarcón *et al.*, 1979; Sánchez-Jiménez *et al.*, 1985; Katz *et al.*, 1992; Brinck *et al.*, 1994). Consequently, transport of cytosolic hydrogen into the mitochondria is reduced, requiring tumor cells to reoxidize NADH cytosolically by lactate dehydrogenase (Goldberg and Colowick, 1965; Goldberg *et al.*, 1965; Groelke and Amos, 1984; Mazurek *et al.*, 1997). Inactivation of the glycerol 3-phosphate shuttle is induced by reduction or total loss of cytosolic glycerol 3-phosphate dehydrogenase, whereas mitochondrial glycerol 3-phosphate dehydrogenase activity varies greatly depending upon the type of tumor (Hilf *et al.*, 1973; Gerbracht *et al.*, 1988; MacDonald *et al.*, 1990; Brinck *et al.*, 1994). The malate–aspartate shuttle can be activated or inactivated according to the glycolytic flux rate and the expression of malate dehydrogenase isoenzymes (Mazurek *et al.*, 1996, 1997). Tumor cells with a high glycolytic flux rate show an overexpression of the mitochondrial isoenzyme of malate dehydrogenase (Balinski *et al.*, 1983; Muchi and Yamamoto, 1983; Mazurek *et al.*, 1996). The concentration of mitochondrial malate dehydrogenase can be so high that the precursor of the mitochondrial isoenzyme is retained in the cytosol, presumably by interaction with a protein referred to as annexin II (p36) (Mazurek *et al.*, 1996). At high glycolytic flux rates and high NADH levels this form allows the channeling of cytosolic hydrogen into the mitochondria.

GLUTAMINOLYSIS

In tumor cells, oxidation of pyruvate and acetyl CoA is reduced in favor of glutamine oxidation as a result of the expression of mitochondrial phosphate-dependent glutaminase and mitochondrial NAD(P)-dependent malate decarboxylase (Sauer *et al.*, 1980; McKeehan, 1982; Marchok *et al.*, 1984; Gerbracht *et al.*, 1990; Matsuno and Goto, 1992; Singer *et al.*, 1995). The conversion of glutamine to lactate is called, in analogy to glycolysis, *glutaminolysis* (McKeehan, 1982; Mazurek *et al.*, 1997). The main role of glutaminolysis is the regeneration of energy and the production of glutamate, citrate, and aspartate (Zielke *et al.*, 1978; Brand, 1985; Lanks *et al.*, 1986; Brand *et al.*, 1987; Lanks, 1987; Lanks and Li, 1988). Citrate is necessary for cytosolic acetyl CoA, isoprenoid, and fatty acid synthesis; aspartate for pyrimidine synthesis. Additionally, oxidation of acetyl CoA is attenuated, presumably by the inactivation of aconitase and mitochondrial isocitrate dehydrogenase. Both of these enzymes are inactivated by superoxide radicals ($\cdot O_2^-$) (Hornsby and Gill, 1981; Boitier *et al.*, 1995; Bouton *et al.*, 1996). Acetyl CoA oxidation, but not glutamine oxidation, is highly sensitive to oxygen radicals (Hornsby and Gill, 1981).

The metabolic advantage of glutaminolysis lies in the consistently high glutamine levels in tissues and solid tumors (about 2 mM glutamine), and in the fact that some tumor cells can thus survive and proliferate under conditions of glucose limitation if oxygen is available (Reitzer *et al.*, 1979; Gerbracht *et al.*, 1988; Mazurek *et al.*, 1997). In addition, there are several reports that glutaminolysis and mitochondrial respiration are required for the development of transformation-specific morphological characteristics such as focus formation and growth in soft agar. If tumor cells are grown in the absence of glutamine or in a medium with reduced glutamine concentration (0.2 mM) a strong selection occurs for nontransformed cells with less tumorigenicity in nude mice (Rubin, 1990; Rubin *et al.*, 1990; Goossens *et al.*, 1996). Evidence has been obtained suggesting that peroxides produced during glutamine respiration might be essential for expression of the transformed phenotype. Overexpression of the enzyme manganese superoxide dismutase in MCF-7 cells reduces peroxide levels, inhibits transformation-

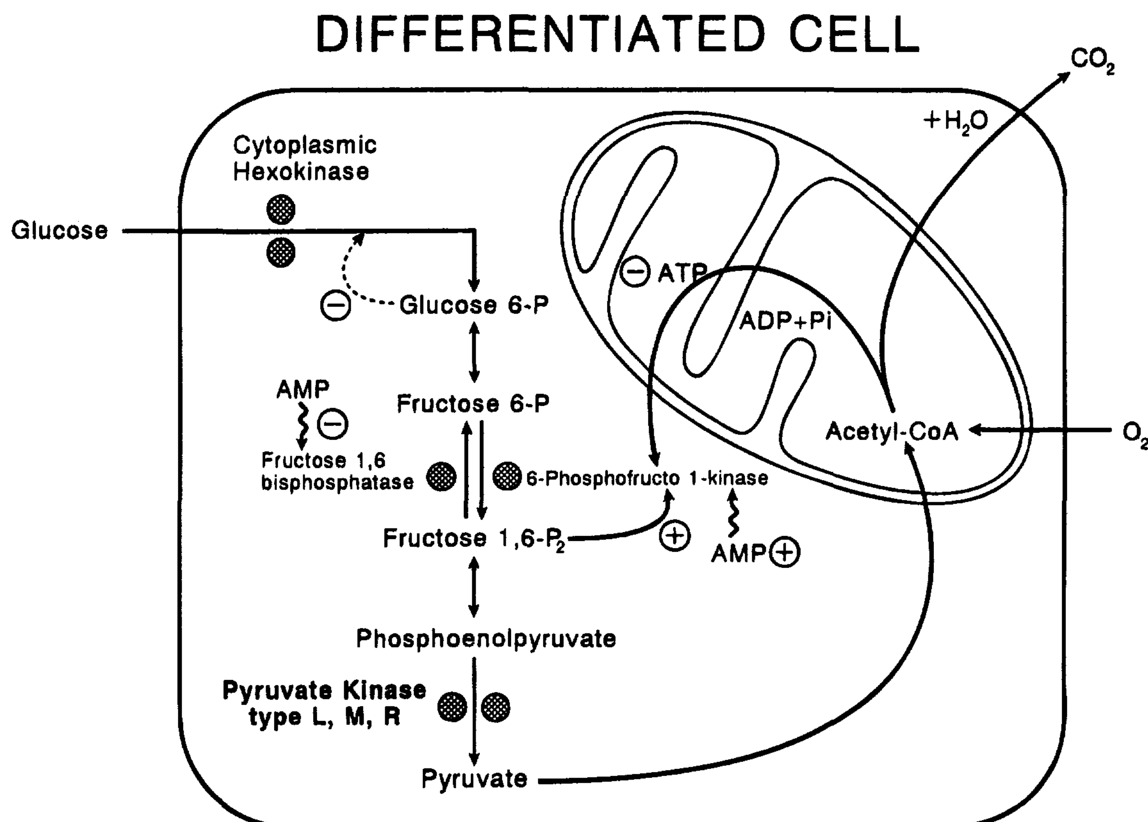

Fig. 1A. Regulation of glycolysis by oxygen in differentiated cells. With sufficient oxygen, differentiated cells adjust pyruvate production to their level of acetyl CoA consumption and energy requirements. The regulation occurs mainly as a result of the ATP inhibition of 6-phosphofructo 1-kinase, which leads to a decrease in the conversion rate of fructose 6-phosphate to fructose 1,6-bisphosphate. Steady-state levels of fructose 1,6-bisphosphate are low since this metabolite is rapidly converted to pyruvate as a result of the high rate of activity in the lower part of the glycolytic pathway, or to fructose 6-phosphate by fructose 1,6-bisphosphatase. Additionally the hydrogen of the cytosolic glyceraldehyde 3-phosphate dehydrogenase reaction is efficiently transported into the mitochondria by the highly active glycerol 3-phosphate and malate-aspartate shuttles. Therefore, fructose 1,6-bisphosphate does not accumulate due to the inhibition of the glyceraldehyde 3-phosphate dehydrogenase reaction. Low levels of fructose 1,6-bisphosphate are not sufficient to overcome the ATP inhibition of 6-phosphofructo 1-kinase. Glucose 6-phosphate, which accumulates as a result of the inhibition of 6-phosphofructo 1-kinase, in turn blocks its own synthesis at the level of hexokinase. Therefore, mitochondrial ATP production governs the glycolytic sequence mainly through 6-phosphofructo 1-kinase inhibition (Pasteur effect). Under anaerobic conditions, mitochondrial ATP production by oxidation of acetyl-CoA is blocked. ATP levels decrease and AMP levels increase. Thus, 6-phosphofructo 1-kinase is thereby deinhibited, and fructose 1,6-bisphosphatase is blocked by the increased AMP levels. Fructose 1,6-bisphosphate accumulates and further stimulates the 6-phosphofructo 1-kinase activity. The resulting reduction in glucose 6-phosphate levels enhances the hexokinase activity. The concerted effect of these various mechanisms is to provide the cells with the potential to utilize their total glycolytic capacity for ATP production under anoxic conditions. Lactate is thereby formed from pyruvate in order to reoxidize the NADH generated via the glyceraldehyde 3-phosphate dehydrogenase reaction.

specific morphology, and alters the requirement for pyruvate (Li *et al.*, 1995; Yan *et al.*, 1996).

THE ROLE OF GLYCOLYSIS AND GLUTAMINOLYSIS IN TUMOR FORMATION AND METASTASIS

Several lines of evidence have been obtained suggesting that neither of these two pathways is truly essential for tumor formation. Rather, both pathways represent opportunities for a metabolic strategy favorable for survival and growth under circumstances in which oxygen and nutrients are deficient (Eigenbrodt *et al.*, 1985, 1992). It is generally accepted that highly malignant tumor cells can grow under conditions of poor vascularization. Depending upon their distance from blood vessels, tumor cells can be starved for oxygen, glucose, or other nutrients (Vaupel *et al.*, 1987;

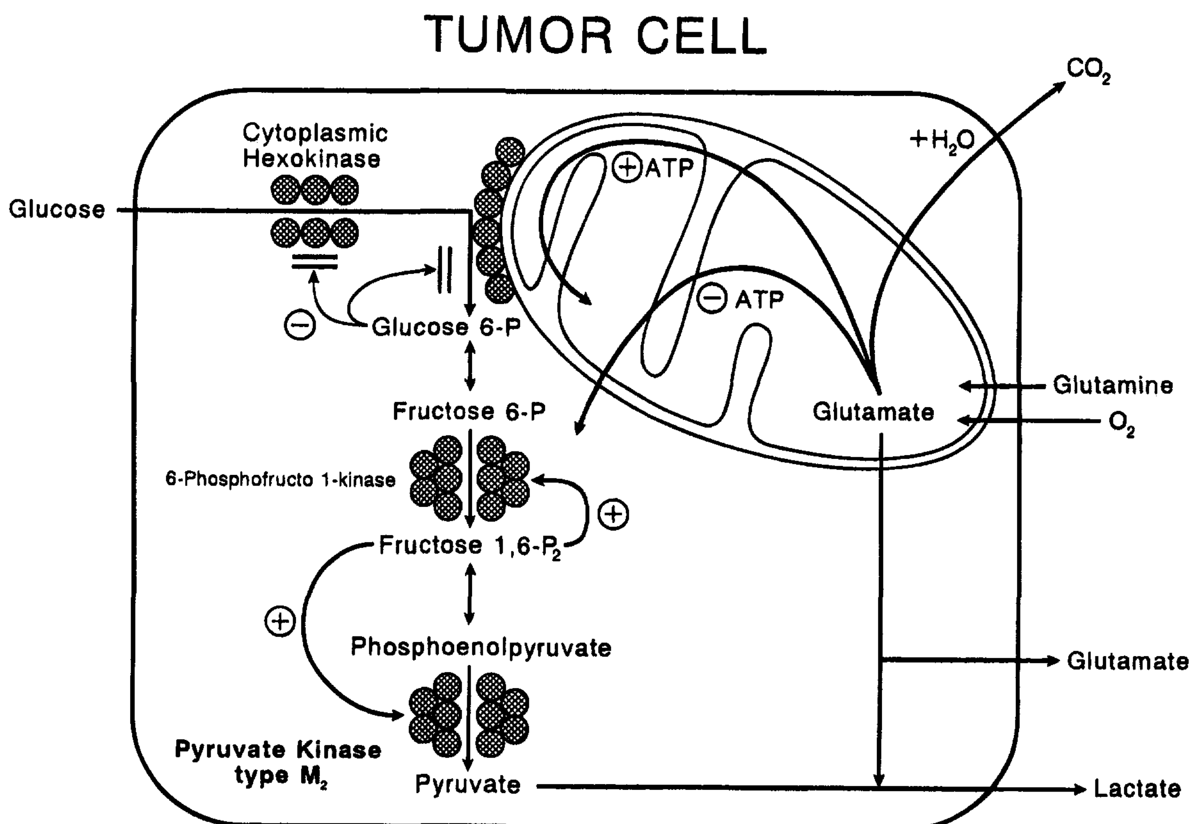

Fig. 1B. Regulation of glycolysis in tumor cells at high glucose concentrations. In tumor cells, enhanced activities of hexokinase (the mitochondrial bound form), 6-phosphofructo 1-kinase and pyruvate kinase ensure a high glycolytic capacity. Pyruvate kinase of the isoenzyme type M_2 is in the inactive dimeric form (tumor type). The fructose 1,6-bisphosphate formed is only slowly converted to pyruvate, until fructose 1,6-bisphosphate levels exceed a concentration necessary to overcome the inhibition of pyruvate kinase type M_2. The accumulated fructose 1,6-bisphosphate, thereby, overrides the mitochondrial control of glycolysis. The fully activated mitochondrial bound hexokinase (not inhibited by glucose 6-phosphate), together with deinhibited 6-phosphofructo 1-kinase and pyruvate kinase, results in a drastic increase in levels of the glycolytic intermediates together with a high glycolytic rate. Since fructose 1,6-bisphosphatase activity is sharply reduced in tumor cells, the fructose 1,6-bisphosphate levels and aerobic glycolysis rates remain permanently elevated. Tumor cells, in contrast to normal cells, can constantly use their full glycolytic capacity regardless of oxygen tension.

Kallinowski *et al.*, 1989; Eigenbrodt *et al.*, 1994). Therefore, a high glycolytic and glutaminolytic rate enables tumor cells to survive and grow under conditions of poor vascularization. Such abilities are not necessary for normal proliferating cells growing in an organized tissue with well-developed vascularization. The advantage of glycolytic ATP production is that oxygen is not required. Therefore, a high rate of glycolytic activity assures the survival of tumor cells in hypoxic areas. Furthermore, it allows the proliferation of tumor cells under low oxygen tension when uridine and pyruvate are available (Fig. 1) (King and Attardi, 1989; Löffler, 1989; Larm *et al.*, 1994).

An important function of glycolysis for tumor formation is its role in cell migration (Beckner *et al.*, 1990). Glutaminolysis is not essential for migration. As a consequence, migration itself activates glycolytic, but not glutaminolytic flux (Beckner *et al.*, 1990; Bereiterhahn *et al.*, 1995). On the other hand, migration is inhibited by substances like oxamate that inhibit the glycolytic enzymes such as lactate dehydrogenase (Beckner *et al.*, 1990). Therefore, activity of some glycolytic enzymes and mobility of cells is directly linked (Epner *et al.*, 1993). This may result from the binding of certain glycolytic enzymes directly to actin (Koppitz *et al.*, 1986; Wang *et al.*, 1996). For example aldolase type C, which is overexpressed during tumor formation, binds to actin, but aldolase types A and B do not. Since migration of tumor cells is one factor that is crucial for metastasis, it is not surprising that

aldolase C, glyceraldehyde 3-phosphate dehydrogenase, and pyruvate kinase type M_2 activities show a positive correlation with metastasis (Hennipman *et al.*, 1989; Board *et al.*, 1990; Epner *et al.*, 1993; Eigenbrodt *et al.*, 1994).

THE ROLE OF AEROBIC GLYCOLYSIS IN THE EXPANSION OF PHOSPHOMETABOLITE POOLS

A high glycolytic rate is not always linked to cell proliferation. There are several cell lines that are able to grow in a medium with 5 mM galactose or with low glucose supply (0.5 mM) without producing lactate via glycolysis, and yet these cells are able to proliferate (Reitzer *et al.*, 1979; Mazurek *et al.*, 1997). Similarly, cells with low glucose 6-phosphate isomerase activities fail to exhibit aerobic glycolysis (Pouyssegur *et al.*, 1980). Investigations with labeled glucose and galactose have shown that the carbons of the two carbohydrates can be used to either synthesize nucleotides, phospholipids, and complex carbohydrates or they can pass through pyruvate kinase to pyruvate and lactate for energy production (Reitzer *et al.*, 1979; Zielke *et al.*, 1980; Eigenbrodt *et al.*, 1992; Mazurek *et al.*, 1997). Under glucose starvation, energy is not produced by glycolysis but by pyruvate oxidation or by conversion of glutamine to lactate (Zielke *et al.*, 1978; Mazurek *et al.*, 1997). Pyruvate kinase, regulating the exit of the glycolytic pathway, determines the relative amount of glucose that is channeled into synthetic processes or used for glycolytic ATP production. For this purpose proliferating cells express a certain isoenzyme of pyruvate kinase termed type M_2, that can occur both in an active tetrameric and a less active dimeric form (Fig. 1B) (Eigenbrodt *et al.*, 1977, 1992; Ibsen *et al.*, 1981; Oude Weernink *et al.*, 1991, 1992). Tumor cells generally overexpress the dimeric form. Therefore, a variety of different tumor types can be detected by monoclonal antibodies specific for the dimeric form of pyruvate kinase type M_2 (tumor M_2-PK) (Scheefers-Borchel *et al.*, 1994; Oremek *et al.*, 1995; Petri *et al.*, 1996; Wechsel *et al.*, 1997). The switch between the two forms is primarily regulated by amino acids synthesized from glycolytic intermediates, e.g., L-serine and L-alanine and the glycolytic phosphometabolite fructose 1,6-bisphosphate (Eigenbrodt *et al.*, 1977, 1983b). Fructose 1,6-bisphosphate leads to a tetramerization of pyruvate kinase type M_2 and a strong increase in the phosphoenolpyruvate affinity of the enzyme (Eigenbrodt *et al.*, 1992). L-Serine allosterically increases the phosphoenolpyruvate affinity of pyruvate kinase and reduces the amount of fructose 1,6-bisphosphate necessary for tetramerization (Eigenbrodt *et al.*, 1992). L-Alanine lowers the phosphoenolpyruvate affinity, and in the presence of alanine larger amounts of fructose 1,6-bisphosphate are necessary for tetramerization of the enzyme. In addition to L-alanine, other amino acids such as L-cysteine, L-methionine, L-phenylalanine, L-valine, L-leucine, L-isoleucine, and L-proline also inhibit pyruvate kinase type M_2 (Schering *et al.*, 1982; Kedryna *et al.*, 1983, 1990; Eigenbrodt *et al.*, 1985; Collet *et al.*, 1996). Pyruvate kinase type M_2 can also be phosphorylated in serine and tyrosine, which serves to stabilize the dimeric form of the molecule (Eigenbrodt *et al.*, 1977; Presek *et al.*, 1980, 1988; Oude Weernink *et al.*, 1991, 1992). The exact role of phosphorylation is still under investigation. At first glance it is surprising that in tumor cells the shift observed in pyruvate kinase from the tetrameric form with strong affinity for phosphoenolpyruvate to the dimeric form with weaker phosphoenolpyruvate affinity correlates with an increased aerobic glycolysis (Eigenbrodt *et al.*, 1992). This unexpected correlation has been reported in RSV transformed fibroblasts, human glioma cell lines, and Ehrlich ascites tumor cells (Glaser *et al.*, 1980; Presek *et al.*, 1980, 1988; Oude Weernink *et al.*, 1991, 1992; Ashizawa *et al.*, 1992; Eigenbrodt *et al.*, 1992). There are several lines of evidence suggesting that increased aerobic glycolysis is caused by the special features and interaction of 6-phosphofructo 1-kinase and pyruvate kinase type M_2 (Cumme *et al.*, 1981; Termonia and Ross, 1981; Hess, 1983). Normally, the glycolytic flux rate is down-regulated by ATP produced by mitochondrial respiration. The main control step is 6-phosphofructo 1-kinase, which is inhibited by mitochondrial ATP (Tejwani, 1978).

Mitochondrial control of 6-phosphofructo 1-kinase is released by a high level of fructose 1,6-bisphosphate and fructose 2,6-bisphosphate (Fig. 1) (Tejwani, 1978; Hue and Rider, 1987). Due to the strong activity of mitochondrial-bound hexokinase in tumor cells in the presence of a sufficient glucose supply, all phosphometabolites above pyruvate kinase accumulate until the levels of fructose 1,6-bisphosphate and fructose 2,6-bisphosphate are high enough to activate 6-phosphofructo 1-kinase and to shift pyruvate kinase to the active tetrameric state (Fig. 1) (Glaser *et al.*, 1980; Hue and Rider, 1987; Gauthier *et al.*, 1989; Ashizawa *et al.*, 1992; Eigenbrodt *et al.*, 1992). The

flow of phosphoenolpyruvate through pyruvate kinase is then strongly increased and the mass of lactate is derived from glucose (Fig. 1). The shift between the dimeric and the tetrameric form of pyruvate kinase type M_2 ensures the supply of phosphometabolites for synthetic processes, although the passage through pyruvate kinase increases. Therefore, aerobic glycolysis is a prerequisite for the expansion of glycolytic phosphometabolite pools to ensure that sufficient glucose carbons are available for synthetic processes (Figs. 1 and 2). Only if sufficient synthetic products are available does cell proliferation proceed (Eigenbrodt *et al.*, 1992; Mazurek *et al.*, 1997). This feedback regulation guards the cells against the deleterious effects of growth hormones and transformation when nutrients such as glucose are limited (Eigenbrodt *et al.*, 1992). In proliferating cells under glucose limitation, glucose is primarily used in the synthesis of nucleic acids; therefore we have termed normal proliferating and tumor cells as "nucleogenic cells" to distinguish them from cells that are mainly "lipogenic," such as adipocytes, "gluconeogenic," such as liver and proximal kidney cells, or cells which have other central metabolic functions such as neurotransmitter producing neurons (Figs. 1 and 2) (Eigenbrodt *et al.*, 1985).

In tumor cells the glycolytic enzymes are constantly overexpressed, thus providing an unlimited supply of precursors for nucleic acid synthesis (Eigenbrodt *et al.*, 1983a, 1994; Brand *et al.*, 1986; Resnick *et al.*, 1986; Schmidt-Ullrich *et al.*, 1986; Board *et al.*, 1990; Chernova *et al.*, 1995). Normal proliferating cells are "potentially nucleogenic" in response to the action of growth factors (Eigenbrodt *et al.*, 1994). In normal proliferating cells the glycolytic and glutaminolytic enzymes are up-regulated in the G1-phase of the cell cycle or by hypoxia (Diamond *et al.*, 1978; Hance *et al.*, 1980; Meienhofer *et al.*, 1983; Freerksen *et al.*, 1984; Tejwani *et al.*, 1985; Brand *et al.*, 1986; Nguyen and Keast, 1991; Stanton *et al.*, 1991; Arrick *et al.*, 1992; Netzker *et al.*, 1992; Hsu *et al.*, 1993). This up-regulation seems to be necessary for the expansion of phosphometabolite pools in the G1-phase of the cell cycle (Eigenbrodt *et al.*, 1994). In accordance with this, the overexpression of hexokinase type II or glucose 6-phosphate dehydrogenase increases the proliferation rate (Fanciulli *et al.*, 1994; Tian *et al.*, 1996).

The pyruvate kinase type M_2 activity measurable in the G1-phase of the cell cycle is 5–20-fold lower than the activity found in highly malignant tumor cells. It is unlikely that the low pyruvate kinase activity found in normal proliferating cells consumes all phosphometabolites below the glyceraldehyde 3-phosphate dehydrogenase reaction (Fig. 1B) (Gauthier *et al.*, 1989; Board *et al.*, 1990; Mazurek *et al.*, 1997). Therefore, it seems that the regulation of phosphometabolite pools in normal proliferating cells is more under the control of glucose 6-phosphate dehydrogenase and 6-phosphofructo 1-kinase activity than under the control of hexokinase and pyruvate kinase (Eigenbrodt *et al.*, 1992). This and the function of the tetramer–dimer shift in normal cell proliferation are still under investigation in our laboratory (Figs. 1B and 2).

PHOSPHOMETABOLITES AND THEIR SYNTHETIC PRODUCTS AS REGULATORS OF CELL PROLIFERATION

From the fact that growth factors and oncogene-dependent phosphorylation regulate glycolysis and phosphometabolite pools, one can assume that some phosphometabolites or synthetic products derived from the phosphometabolites, e.g., sugar phosphates, AMP, NADH, NADPH, and serine for sphinganine synthesis, regulate cell proliferation (Fig. 2) (Eigenbrodt *et al.*, 1994).

P-ribose PP

P-ribose-PP is formed from ribose 5-P. There are two sources of ribose 5-P. Ribose 5-P can be synthesized from glucose 6-phosphate with glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Alternatively, it can be synthesized or degraded to fructose 6-phosphate and glyceraldehyde 3-phosphate by the transketolase and transaldolase reaction, dependent upon the flow of carbons in the lower part of the glycolytic pathway (Eigenbrodt *et al.*, 1985). An increase in P-ribose-PP levels (10 fold) and availability have been reported after stimulation with mitogens in the G1-phase and after transformation with $pp60^{v\text{-}src}$ kinase (Hovi *et al.*, 1979; Smith and Buchanan, 1979; Eigenbrodt *et al.*, 1985; Ishijima *et al.*, 1988; Schöbitz *et al.*, 1991).

AMP

AMP is a synthetic product derived from P-ribose PP. In cells with a sufficient energy supply AMP is

immediately converted to ADP and ATP. Under energy limitation AMP accumulates due to the degradation of ATP (Weber *et al.*, 1971). AMP inhibits the enzyme P-ribose-PP synthetase and reduces NAD, NADH, and DNA synthesis (Henderson *et al.*, 1981; Hugo *et al.*, 1992; Mazurek *et al.*, 1997). The effect of AMP can be mimicked by extracellular AMP when sufficient nutrients and energy are available. AMP is degraded to adenosine by the 5′-ectonucleotidase. Adenosine is transported into the cells via an adenosine translocator and is phosphorylated to AMP by cytosolic adenosine kinase (Fig. 2) (Weisman *et al.*, 1984, 1988).

In differentiated tissues, the increased AMP levels lead to an activation of 6-phosphofructo 1-kinase and a strong stimulation of the glycolytic flux rate (Tejwani, 1978). In cell lines with a constantly high glycolytic flux rate such as MCF-7 cells (glucose/lactate = 1.7), AMP totally inhibits glycolysis. This is in contrast to MDA-MB-453 cells with low glycolytic flux rate (glucose/lactate = 0.6), where AMP slightly activates glycolysis. In both cell lines AMP activates 6-phosphofructo 1-kinase activity as it does in differentiated tissues (Fig. 1A) (Mazurek *et al.*, 1997). The difference in response to AMP is presumably caused by a different hydrogen shuttle mechanism. MDA-MB-453 cells, like undifferentiated tissues, have an active glycerol 3-phosphate shuttle due to a high rate of cytosolic NAD-dependent glycerol 3-phosphate dehydrogenase activity. This enzyme is not detectable in MCF-7 cells (Mazurek *et al.*, 1997).

The main difference in the response of these cell lines and of differentiated tissues to AMP must therefore be the result of their different shuttle systems. Due to the absence of the glycerol 3-phosphate shuttle in MCF-7 cells the mass of hydrogen produced in the cytosolic glyceraldehyde 3-phosphate dehydrogenase reaction must be excreted as lactate. The reduction of NAD and free NADH levels under AMP treatment attenuates the lactate dehydrogenase reaction. Thus, the generation of NAD in the cytosol is limited, and the glyceraldehyde 3-phosphate dehydrogenase reaction is inhibited. As a consequence, total glycolysis is inhibited in AMP arrested MCF-7 cells. Under these conditions energy is generated from an increased glutaminolytic flux rate (Hugo *et al.*, 1992). In cells with an active glycerol 3-phosphate shuttle such as MDA-MB-453 cells the mass of hydrogen produced in the glyceraldehyde 3-phosphate dehydrogenase reaction is transported into the mitochondria by the shuttle and is not excreted as lactate. An increase in AMP levels leads to a drop in NAD levels and reduces the flow of extracellular pyruvate to lactate by the lactate dehydrogenase reaction. However, the lowered NAD levels do not affect the glycerol 3-phosphate dehydrogenase reaction and the transport of hydrogen by the glycerol 3-phosphate shuttle into mitochondria. Therefore, the drop in NAD levels reduces the flow of extracellular pyruvate to lactate by the lactate dehydrogenase reaction but does not affect the glyceraldehyde 3-phosphate dehydrogenase reaction and glucose consumption in MDA-MB-453 cells. Due to the different enzyme make-up, these cells react differently to glucose limitation. MDA-MB-453 cells, but not MCF-7 cells, can grow in low-glucose medium (Mazurek *et al.*, 1997). All cell lines unable to grow under low glucose levels have a reduced capacity to transport cytosolic hydrogen into the mitochondria (Reitzer *et al.*, 1979; Maiti *et al.*, 1981; Whitfield *et al.*, 1981; Ohtsuka *et al.*, 1993).

NADH and NADPH

Evidence is beginning to appear to indicate that high NADH and NADPH levels may be essential for cell proliferation (Fig. 2) (Brand and Deckner, 1970; Schwartz *et al.*, 1974; Sun *et al.*, 1984, 1985; Wice *et al.*, 1985; Schwartz *et al.*, 1988; Chatterjee *et al.*, 1989; Ryll and Wagner, 1992; Mazurek *et al.*, 1997). Cytosolic NADPH is an important co-substrate of isoprenoid, cholesterol, and fatty acid synthesis (Ledda-Columbano *et al.*, 1985; Schwartz *et al.*, 1988; Eigenbrodt *et al.*, 1994; Bannasch, 1996). In AMP-treated cells cholesterol and triglyceride synthesis is inhibited (Hugo *et al.*, 1992). Certain substances other than NADH and NADPH that are also synthesized from P-ribose-PP have been linked to cell proliferation. These are CTP and UDP-N-acetylglucosamine, the levels of which are greatly reduced when cell proliferation is inhibited by AMP or glucose starvation (Wice *et al.*, 1985; Ryll and Wagner, 1992; Mazurek *et al.*, manuscript in preparation). Since there is a correlation with the P-tyrosine content in several cytosolic proteins, these phosphometabolites must either activate protein kinases or inhibit phosphatases and interfere with the protein kinase cascade that regulates cell proliferation (Mazurek *et al.*, 1997).

Fructose 1,6-bisphosphate

Fructose 1,6-bisphosphate is not a precursor for other biosynthetic pathways, but is a regulator of sev-

eral other enzymes. Due to its structural similarity to inositol 1,4,5-triphosphate, fructose 1,6-bisphosphate inhibits inositol 5-phosphatase, induces the release of inositol 1,4,5-triphosphate from aldolase C, and affects actin polymerization (Koppitz *et al.*, 1986; Gaertner *et al.*, 1991; Wang *et al.*, 1996). In addition, fructose 1,6-bisphosphate and inositol 4,5-bisphosphate stimulate protein synthesis (Rana *et al.*, 1986; Rabinovitz, 1991; Singh and Wahba, 1995). In AMP-treated MCF-7 cells fructose 1,6-bisphosphate levels and protein synthesis remain elevated and there is an alteration in morphology, whereas DNA synthesis is totally inhibited (Hugo *et al.*, 1992). Therefore, protein synthesis and DNA synthesis can be unlinked.

Serine

Serine is formed from the glycolytic intermediates glycerate 3-phosphate and glutamine. Serine activates pyruvate kinase type M_2. The flow of 3-phosphoglycerate to serine and to purine synthesis is generally enhanced in tumor cells (Fig. 2) (Katunuma *et al.*, 1972; Snell, 1984). Serine is an essential precursor for glycine and active methyl groups used in the synthesis of purines and pyrimidines and in the methylation of adenosyl homocysteine (Eigenbrodt *et al.*, 1994). Modulation of pyruvate kinase activity increases the channeling of glycerate 3-phosphate to purine *de novo* synthesis and alters the flow of methyl groups to S-adenosyl methionine (Fister *et al.*, 1982; Gerbracht *et al.*, 1993). Inhibition of the enzyme serine hydroxymethyltransferase, which transfers the methyl groups to tetrahydrofolate, blocks cell proliferation in the G1-phase of the cell cycle (Balk *et al.*, 1973; Kaminskas and Nussey, 1978; Lin *et al.*, 1996; Girgis *et al.*, 1997). In addition, the condensation of serine with palmityl CoA creates sphinganine (Fig. 2) (Smith and Merill, Jr., 1995). Inhibition of sphinganine synthesis by cycloserine, a serine analog, blocks cell proliferation (Hannun, 1994). One of the next condensation products with palmityl CoA is dihydroceramide or ceramide. In contrast to its precursor sphinganine, ceramide inhibits cell proliferation and induces programmed cell death or apoptosis (Hannun, 1994). Therefore, the balance between the cytosolic serine levels and acyl CoA levels is an important factor in the regulation between cell proliferation or cell death. This might explain why free fatty acids induce cell death in normal proliferating cells and inhibition of *de novo* fatty acid synthesis is cytotoxic for tumor cells (Fig. 2) (Pizer *et al.*, 1996; Paumen *et al.*, 1997).

CARBOHYDRATE METABOLISM AND CHEMOTHERAPY

Tumor cells are characterized by high hexokinase and pyruvate kinase activities. Pyruvate kinase can switch between an active and a less active state dependent upon the cell's need for glycolytic energy production or for synthetic processes. Additionally, a sharp reduction in NAD-dependent glycerol 3-phosphate dehydrogenase and an alteration in the malate–aspartate shuttle take place in tumor cells. In consequence cytosolic redox equivalents must be excreted mainly as lactate; otherwise glycolysis would be blocked at the level of the NADH producing glyceraldehyde 3-phosphate dehydrogenase reaction (Fig. 3). Indeed if tumor cells are starved for glucose and pyruvate, a situation can be created in which refeeding with glucose is cytotoxic. Glucose starvation reduces fructose 1,6-bisphosphate levels and induces a shift to the inactive dimeric state of pyruvate kinase (Ashizawa *et al.*, 1992; Eigenbrodt *et al.*, 1994; Mazurek *et al.*, 1997). After glucose refeeding and as a result of the high hexokinase and 6-phosphofructo 1-kinase activities, glucose is immediately converted to fructose 1,6-bisphosphate with the consumption of 2 ATP per mole fructose 1,6-bisphosphate. The inactive pyruvate kinase, however, prevents the generation of glycolytic ATP and pyruvate. The lack of pyruvate inhibits the recycling of NAD and therefore inhibits the glyceraldehyde 3-phosphate dehydrogenase reaction, whereby fructose 1,6-bisphosphate accumulates to levels as high as 5000 μM and ATP drops to nearly zero values. This occurs within 10 minutes after glucose refeeding. Fructose 1,6-bisphosphate then activates pyruvate kinase; pyruvate and lactate accumulate and ATP is generated 20 minutes after glucose feeding (Glaser *et al.*, 1980). The drop in ATP levels seen upon glucose readdition to starved cells is most pronounced in malignant tumor cells (Medina *et al.*, 1990). The addition of pyruvate can prevent this drop in ATP levels. Inhibitors of lactate dehydrogenase, such as oxamate, repress the regeneration of ATP by pyruvate kinase resulting in the death of the cells (Fig. 3) (Goldberg and Colowick, 1965; Goldberg *et al.*, 1965). The same principle has been shown for glucose analogs that can be phosphorylated by hexokinase but cannot be further used as substrates in glycolysis (Kaplan *et al.*, 1990a,b; Eigenbrodt *et al.*, 1994; Board *et al.*, 1995). As discussed above, in cells with a limitation in shuttle systems, especially in the glycerol 3-phosphate shuttle, a reduction of NAD levels strongly reduces energy production by glycolysis due to the inhibition of lactate dehydrogenase. It

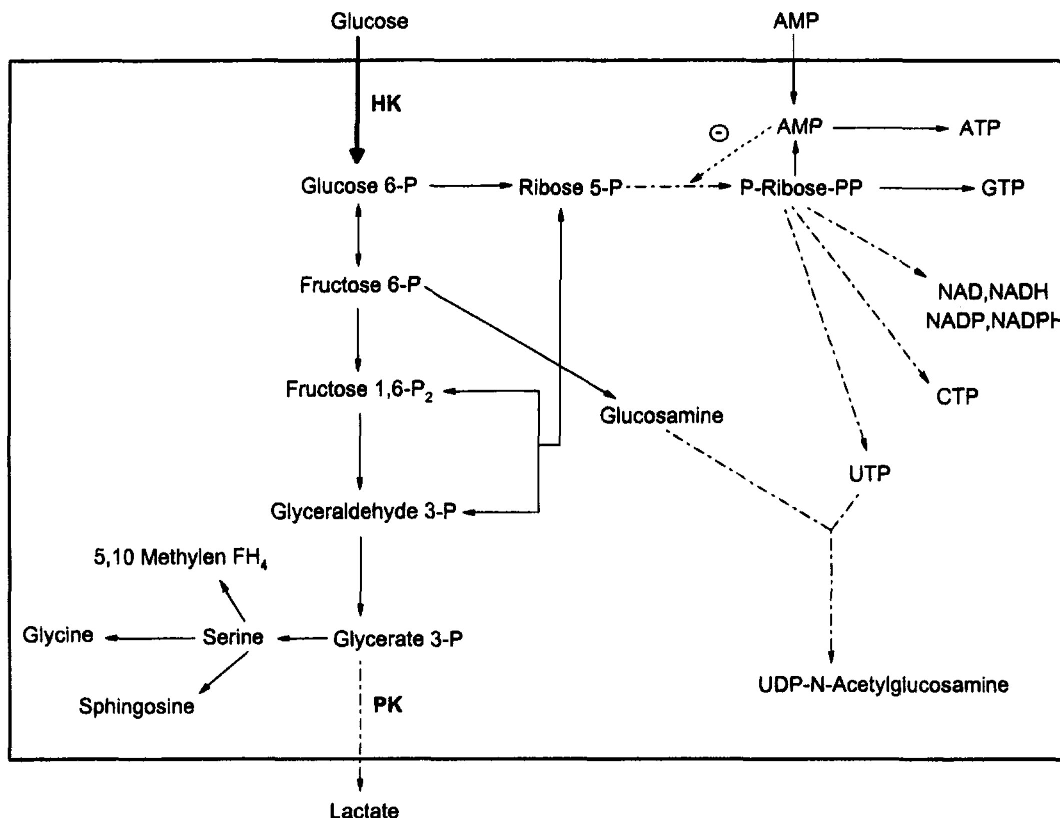

Fig. 2. The expansion of phosphometabolite pools between glucose 6-phosphate and phosphoenolpyruvate by a hyperactive hexokinase (HK) and a in partially inactive pyruvate kinase (PK). In addition to the increase in glycolytic phosphometabolites there is an increase in metabolites synthesized from fructose 6-phosphate and glucose 6-phosphate such as ribose 5-phosphate and P-ribose-PP. P-ribose-PP is the precursor for purine, pyrimidine, NAD, and NADP synthesis. Both the NAD, NADH and the NADP, NADPH levels are increased in tumor cells. AMP is a synthetic product derived from P-ribose-PP. In cells with sufficient energy supply AMP is immediately converted to ADP and ATP. Under energy limitation AMP accumulates due to the degradation of ATP. AMP inhibits the enzyme P-ribose-PP synthetase and reduces NAD and NADH and DNA synthesis. The effect of AMP can be mimicked by extracellular AMP when sufficient nutrients and energy are available. AMP is degraded to adenosine by the 5′-ectonucleotidase. Adenosine is transported into the cells via an adenosine translocator and is phosphorylated to AMP by cytosolic adenosine kinase. AMP lowers CTP and UDP-N-acetylglucosamine levels. The reduction of both metabolites correlates with a reduced growth rate in AMP-treated cells. Another important metabolite is serine synthesized from glycerate 3-phosphate. Serine is an essential precursor for glycine and active methyl groups used in the synthesis of purines and pyrimidines. The condensation of serine with palmityl CoA creates sphinganine. Inhibition of both pathways inhibits cell proliferation.

has been shown that several chemotherapeutic drugs that have DNA as a target depress NAD levels and affect glycolysis. These effects take place because DNA damage activates poly (ADP ribose) polymerase which is necessary for DNA repair (Fig. 3) (Kaminskas and Nussey, 1978; Skidmore *et al.*, 1979; Seto *et al.*, 1985; Berger *et al.*, 1986; Mol *et al.*, 1989; Hoshino *et al.*, 1990; Just and Holler, 1991; Marks and Fox, 1991). This process leads to a reduction in NAD levels and an inhibition of NAD-synthesis. The drop in NAD levels leads in turn to an inhibition of lactate dehydrogenase. It will be interesting to learn whether tumor cell lines react in different ways to chemotherapeutic drugs that reduce NAD levels such as MCF-7 and MDA-MB-453 cells (Fig. 3) (Mazurek *et al.*, 1997).

PROGRAMMED CELL DEATH AND METABOLISM

A new tumor therapeutic approach is to use the mechanism by which the immune system itself eliminates tumor cells. Activated immunocompetent cells release hormones, e.g., interleukin α, interferon, and

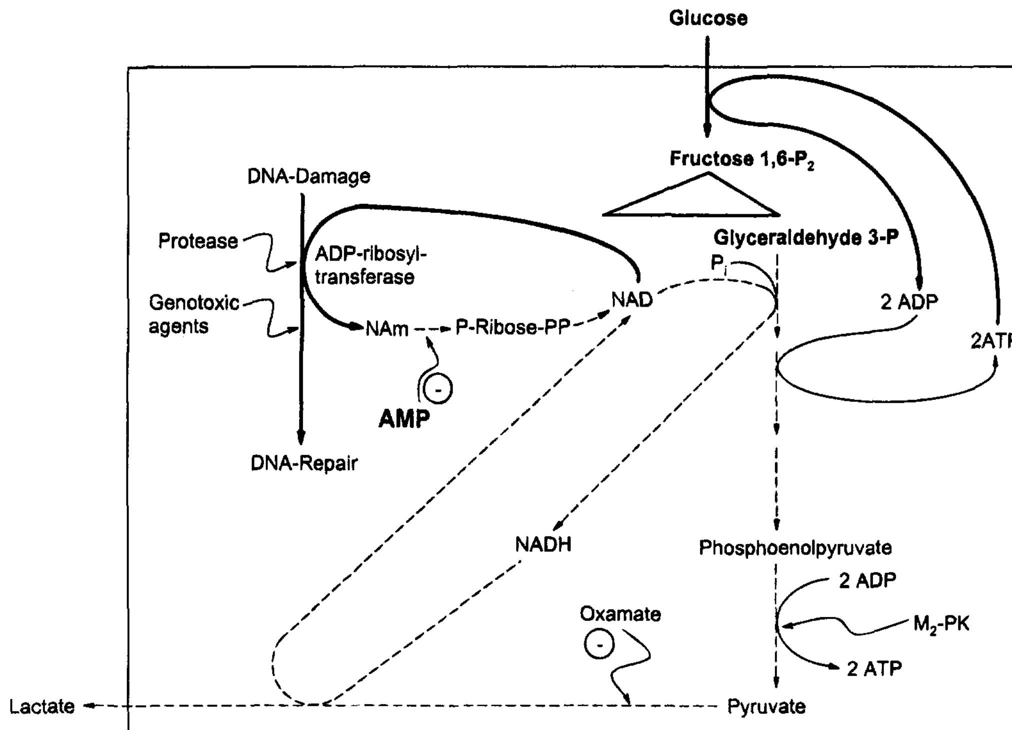

Fig. 3. The role of NADH regeneration in chemotherapy. Tumor cells are generally characterized by high hexokinase and 6-phosphofructo 1-kinase activities. Depending upon the cellular level of fructose 1,6-bisphosphate, pyruvate kinase is either inactive or active. The glycerol 3-phosphate shuttle and the malate-aspartate shuttle enzyme activites are limited. Therefore, cytosolic hydrogen derived from glyceraldehyde 3-phosphate dehydrogenase must be excreted as lactate. Under these conditions glucose can deplete the cell of ATP. Glucose is phosphorylated to fructose 1,6-bisphosphate by hexokinase and 6-phosphofructo 1-kinase, consuming ATP in the process. Due to the inactivated pyruvate kinase there is a lack of pyruvate. The glyceraldehyde 3-phosphate dehydrogenase reaction cannot proceed and ATP levels drop to near zero values. When fructose 1,6-bisphosphate reaches a certain level, pyruvate kinase is shifted to the active tetrameric state. In consequence, the pyruvate levels increase and ATP is re-synthesized. Inhibition of lactate dehydrogenase by oxamate or fluoropyruvate inhibits NADH regeneration by lactate dehydrogenase and inhibits glyceraldehyde 3-phosphate dehydrogenase, with the consequence that glucose becomes cytotoxic for the tumor cells. The same cytotoxic effect can be attained by lowering NAD levels. A reduction of NAD levels can be achieved by increasing AMP levels (Fig. 2). AMP inhibits the synthesis of P-ribose-PP from ribose 5-P by inhibition of P-ribose-PP-synthetase. This leads to a reduction in NAD levels. Another means of reducing NAD levels is by activating poly (ADP ribose) polymerase. This enzyme is activated either by genotoxic chemotherapeutic agents or by proteolysis. The enzyme splits NAD for the synthesis of poly (ADP ribose), thereby lowering NAD levels. In tumor cells with sufficient glycerol 3-phosphate or malate-aspartate shuttle capacities a reduction in NAD levels inhibits cell proliferation but is not cytotoxic, because the glycerol 3-phosphate shuttle, in particular, can transport hydrogen into the mitochondria at low cytosolic NAD levels.

TNF α that bind to specific receptors on tumor cells. Thus, the cells are killed by a regulated process which alternatively has been termed *programmed cell death or apoptosis* (Martin *et al.*, 1994a). Apoptosis can be induced by an overexpression of proteases that split the enzyme poly (ADP ribose) polymerase, thus increasing NAD turnover and affecting DNA repair (Fig. 3) (Armstrong *et al.*, 1994; Los *et al.*, 1995). The activation of endonucleases then leads to DNA fragmentation and cell death (Polzar *et al.*, 1993; Martin *et al.*, 1994a). Under physiological conditions cell death needs active glycolysis, respiration, and glutaminolysis (Berger *et al.*, 1987; Gaal *et al.*, 1987; Wong *et al.*, 1989; Dijkmans and Billiau, 1991; Schulze-Osthoff *et al.*, 1992; Hockenbery *et al.*, 1993; Garland and Halestrap, 1997). For example TNF α is only cytotoxic for cells with active glutaminolysis (Camussi *et al.*, 1991; Hockenbery *et al.*, 1993; Banki *et al.*, 1996; Goossens *et al.*, 1996). Overexpression of transaldolase lowers NADH and NADPH levels and the cells are less resistant to

several apoptotic stimuli including peroxides and TNF α (Fig. 4) (Banki *et al.*, 1996). Overexpression of glucose 6-phosphate dehydrogenase has the opposite effect on NADPH levels and on apoptosis (Gessner *et al.*, 1990; Ferretti *et al.*, 1993; Tian *et al.*, 1996). The role of NADPH may be explained by the fact that peroxides are detoxified by the use of NADPH (Fig. 4). There are several apoptotic stimuli that increase the rate of peroxide production (Schraufstätter *et al.*, 1986; Hyslop *et al.*, 1988; Wong *et al.*, 1989; Schulze-Osthoff *et al.*, 1992; Hockenbery *et al.*, 1993; Whitacre *et al.*, 1995). The role of NAD and NADH in this process is related to NAD as a substrate of poly (ADP ribose) polymerase. Activation of this enzyme lowers NAD levels (Berger *et al.*, 1986, 1987; Gaal *et al.*, 1987). In cells with low glycerol 3-phosphate shuttle systems the lowered NAD-levels inhibit glycolysis and energy regeneration at the levels of glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase (Fig. 3) (Mazurek *et al.*, 1997). ATP levels drop, thereby triggering apoptosis. In interferon and TNF α induced cell death there is at first a decrease in mitochondrial respiration leading to a reduction in ATP levels (Krippner *et al.*, 1996; Lewis *et al.*, 1996; Pastorino *et al.*, 1996). This explains why the response to apoptotic stimuli is regulated by the individual make-up of the various enzymes of glycolysis, the pentose phosphate pathway, glycerol 3-phosphate shut-

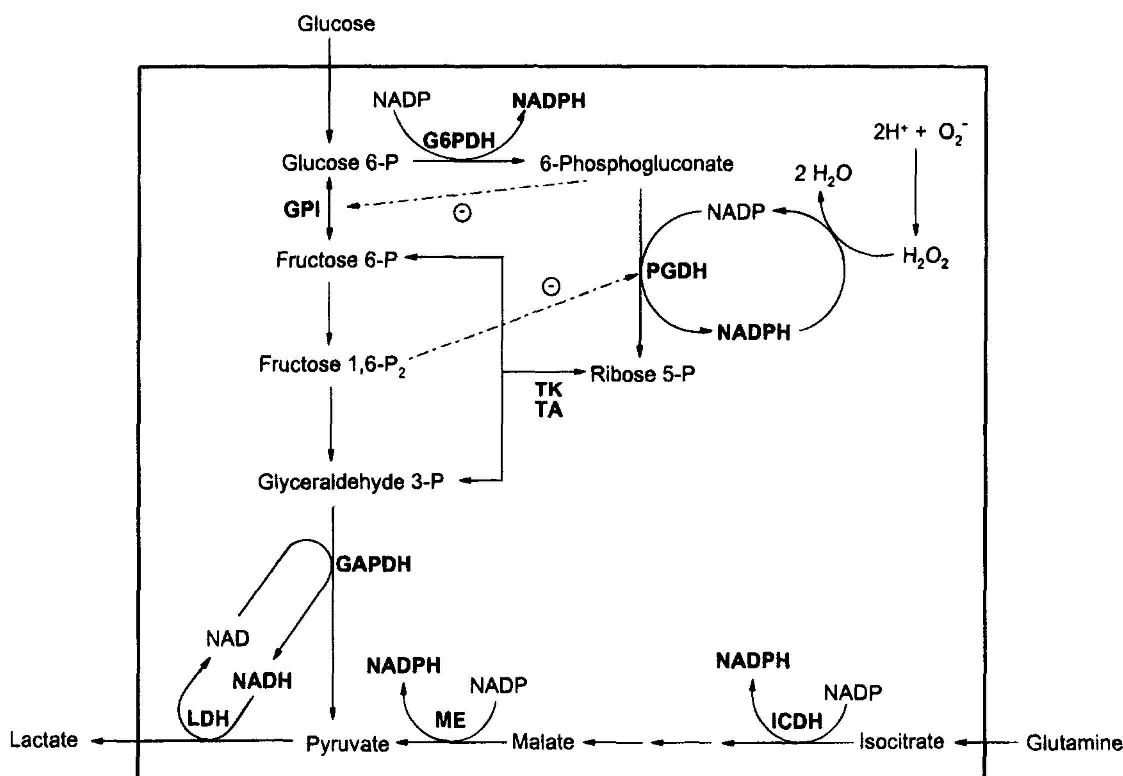

Fig. 4. The role of NADPH regeneration in chemotherapy. Cytosolic NADPH regeneration is an important factor for the success of chemotherapy. NADH regeneration is regulated by passage through the glycerol 3-phosphate and malate-aspartate shuttle as well as by the glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase reactions. Cytosolic NADPH regeneration is regulated by passage through the oxidative pentose phosphate pathway. A high rate of NADPH regeneration is necessary for the detoxification of several chemotherapeutic drugs and peroxides. Overexpression of glucose 6-phosphate dehydrogenase (G6PDH) increases NADPH regeneration and reduces the sensitivity to different apoptotic stimuli. The overexpression of transaldolase (TA) has the opposite effect. NADPH regeneration by the oxidative pentose phosphate pathway is inhibited by a high glycolytic flux rate, leading to high NADH levels and high fructose 1,6-bisphosphate levels. Fructose 1,6-bisphosphate inhibits 6-phosphogluconate dehydrogenase (PGDH) and NADPH regeneration. At the same time, 6-phosphogluconate inhibits glucose 6-phosphate isomerase (GPI) and glycolysis. Generation of NADPH by the cytosolic NADP-dependent malate decarboxylase (ME) and NADP-dependent isocitrate dehydrogenase (ICDH) is not restricted to these limitations. Overexpression of both these latter enzymes has been reported for several drug-resistant cell lines. (TK = transketolase; LDH = lactate dehydrogenase).

tle, malate–aspartate shuttle, and glutaminolysis (Fig. 4) (Dijkmans and Billiau, 1991; Banki *et al.*, 1996; Goossens *et al.*, 1996).

ENERGY METABOLISM AS A TARGET FOR TUMOR THERAPY

Tumor formation is characterized by an increase in glycolytic and glutaminolytic enzyme activities as well as by a reduction in pyruvate and fatty acid oxidation. In addition, the glycerol 3-phosphate shuttle and malate–aspartate shuttle activities are drastically reduced compared to differentiated tissues, whereas tumor cells that are capable of growing at low glucose levels have a low but sufficient glycerol 3-phosphate shuttle capacity (Mazurek *et al.*, 1997). One therapeutic approach is to inhibit both glycolysis and glutaminolysis by interfering with NAD metabolism. This can be achieved either by use of AMP analogs such as 4-methoxy- and 4-amino-8-(β-D-ribofuranosylamino)-pyrimidi-[5,4-d]pyrimidine, which inhibit NAD synthesis or by 6-aminonicotinamide which is incorporated into NAD and NADP, forming 6-amino-NAD and 6-amino-NADP (Ghose *et al.*, 1989; Street *et al.*, 1996). The accumulating 6-amino-NADP preferentially inhibits 6-phosphogluconate dehydrogenase. Thus, 6-phophogluconate accumulates, which inhibits glucose 6-phosphate-isomerase and glycolysis (Fig. 4) (Street *et al.*, 1996). Indeed, this type of therapeutic strategy has been successfully applied in an animal model and was shown not to produce drug resistance (Stolfi *et al.*, 1992; Martin *et al.*, 1994b; Ben-Horin *et al.*, 1995; Nord *et al.*, 1996).

Other interesting substrates are cyclocreatine, suramine, phenylacetate, aminooxyacetate, and lonidamine (Floridi *et al.*, 1981; Floridi and Lehninger, 1983; Samid *et al.*, 1992, 1993, 1994; Lillie *et al.*, 1993; Miller *et al.*, 1993; Fanciulli *et al.*, 1996; Miccadei *et al.*, 1996; Pulselli *et al.*, 1996). These compounds are not toxic even at high concentrations and inhibit tumor cell proliferation by interfering with energy metabolism, albeit by different mechanisms. Creatine and its analog cyclocreatine are phosphorylated by creatine kinase to creatine phosphate or cyclocreatine phosphate. In cells that stop proliferating in low-glucose medium, creatine phosphate can sustain one cycle of cell proliferation whereby it is converted into creatine (Barbehenn *et al.*, 1984). In cells that are capable of growth in low-glucose medium creatine phosphate levels remain relatively high (Becker and Schneider, 1989). Cyclocreatine phosphate is a poor substrate for creatine kinase and phosphorylates ADP less readily than creatine phosphate (Annesley and Walker, 1978; Turner and Walker, 1985). Under conditions in which there is a lack of energy AMP accumulates earlier in cells that contain cyclocreatine phosphate than in cells containing creatine phosphate (Fig. 3). Phenylacetate inhibits glutaminolysis because it is readily condensed with the γ aminò group of glutamine, thereby inhibiting glutamine consumption (Samid *et al.*, 1992, 1993, 1994). Aminooxyacetate is an inhibitor of glutamate oxaloacetate transaminase and inhibits glutaminolysis (Groelke and Amos, 1984; González-Mateos *et al.*, 1993). We have found that aminooxyacetate is cytotoxic for tumor cells able to grow under glucose limitation. Lonidamine alters mitochondrial glycerol 3-phosphate and malate respiration and leads to a release of the bound hexokinase from mitochondria (Floridi *et al.*, 1981; Floridi and Lehninger, 1983; Fanciulli *et al.*, 1996). Suramine inhibits cytosolic glycerol 3-phosphate dehydrogenase and malate dehydrogenase (Rago *et al.*, 1991). It will be interesting to discover by which means the altered hydrogen shuttle mechanism of tumor cells affects the actions of aminooxyacetate, suramine, and lonidamine. One important consideration in drugs that interfere with energy metabolism is the tumor-specific constellation of a superactive hexokinase and 6-phosphofructo 1-kinase together with an inactive dimeric pyruvate kinase. This leads to a rapid phosphorylation of glucose and glucose analogs and depletion of ATP. Since glucose remains available during therapy with glucose analogs, the accumulating fructose 1,6-bisphosphate activates pyruvate kinase and restores ATP levels, therefore limiting the effectiveness of this therapeutic strategy. This treatment could be improved, however, if it were possible to prevent the re-association of the inactive dimeric form of pyruvate kinase to the tetrameric form (Fig. 3) (Eigenbrodt *et al.*, 1994).

In addition to the substances mentioned above, various glucose analogs, glutamine analogs, and inhibitors of glycolysis and glutaminolysis are under investigation (Annesley and Walker, 1978; Mojena *et al.*, 1992; Castiglione *et al.*, 1993; Miller *et al.*, 1993; Floridi *et al.*, 1994; Rideout *et al.*, 1994; Sri-Pathmanathan *et al.*, 1994; Board *et al.*, 1995; Cornelissen *et al.*, 1995; Hamilton *et al.*, 1995; Modica-Napolitano *et al.*, 1996). Our studies of human breast tumors and colon carcinomas revealed a large variability in the glycolytic enzymes, glutaminolytic enzymes, the enzymes of the pentose phosphate pathway, and the

shuttle enzymes. Therefore, it is conceivable that each tumor reacts individually to the various drugs that interfere with energy metabolism. An improvement in therapy can only be attained if we learn how these enzymes modulate the response to the different drugs and we can achieve a true metabolite- and enzyme-guided tumor therapy (Fig. 4) (Fearon *et al.*, 1987; Lyon *et al.*, 1988; Gessner *et al.*, 1990; Ferretti *et al.*, 1993; Lillie *et al.*, 1993; Banki *et al.*, 1996).

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