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Biochimica et Biophysica Acta 1555 (2002) 14–20

BIOCHIMICA ET BIOPHYSICA ACTA
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Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention

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Received 22 April 2002; accepted 3 June 2002

Abstract

Despite more than 75 years of research by some of the greatest scientists in the world to conquer cancer, the clear winner is still cancer. This is reflected particularly by liver cancer that worldwide ranks fourth in terms of mortality with survival rates of no more than 3–5%. Significantly, one of the earliest discovered hallmarks of cancer had its roots in Bioenergetics as many tumors were found in the 1920s to exhibit a high glycolytic phenotype. Although research directed at unraveling the underlying basis and significance of this phenotype comprised the focus of cancer research for almost 50 years, these efforts declined greatly from 1970 to 1990 as research into the molecular and cell biology of this disease gained center stage. Certainly, this change was necessary as the new knowledge obtained about oncogenes, gene regulation, and programmed cell death once again placed Bioenergetics in the limelight of cancer research. Thus, we now have a much better molecular understanding of the high glycolytic phenotype of many cancers, the pivotal roles that Type II hexokinase-mitochondrial interactions play in this process to promote tumor cell growth and survival, and how this new knowledge can lead to improved therapies that may ultimately turn the tide on our losing war on cancer.

Keywords: Cancer; Mitochondria; Glycolysis; Hexokinase; Gene regulation; Drug targeting

1. Background: the high glycolytic cancer phenotype and its link to mitochondrial bound hexokinase

One of the most common, profound, intriguing, and insidious phenotypes of highly malignant tumors, known for more than seven decades, is their ability to metabolize glucose at high rates [1–6]. This is a characteristic of highly malignant animal and human tumors including those derived from brain, breast, colon, liver, lung, pancreas, stomach, and retina [1,2]. For each, a close correlation exists among the degree of differentiation, growth rate, and glucose metabolism, where the most poorly differentiated tumors exhibit the fastest growth and the highest glycolytic rate [3–5]. In fact, this unique phenotype is used clinically worldwide via positron emission tomography (PET) to detect the most malignant tumors, and in some cases even predict patient survival time [7–9]. Despite the commonality of the high glycolytic phenotype, and its widespread use clinically as a diagnostic tool, it has not been exploited as a major target for arresting or slowing the growth of cancer cells. However, this neglect may be short lived as the underlying molecular basis of the high glycolytic phenotype is now known to involve a number of genetic and biochemical events [6], one of the most important of which is the overexpression of a mitochondrial bound form of hexokinase [10–21], now clearly identified as Type II [18–21]. It is this hexokinase isoform that constitutes the basis of this minireview. Significantly, evidence is mounting that Type II hexokinase plays a pivotal role in highly malignant cancer cells in promoting cell growth and survival, and for this reason may represent an ideal target in aggressive tumors for therapeutic intervention.

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PII: S0005-2728(02)00248-7

2. Properties of Type II hexokinase: K_m, molecular weight, and overexpression in tumors

Hexokinases (ATP: D-hexose-6-phosphotransferases) catalyze the first step in the glycolytic pathway as indicated below:

Glucose + ATP⇆Glucose-6-Phosphate + ADP

There are four major isozymes of hexokinase, referred to as Types I, II, III and IV (glucokinase) (reviewed in Refs. [22–25]). Types I–III show a high affinity (K_m = 0.02–0.03 mM) for glucose, are product inhibited by glucose-6-phosphate (G-6-P), and have a molecular mass near 100 kDa. In contrast, the Type IV isozyme (glucokinase) has a much lower affinity for glucose (K_m = 5–8 mM), is insensitive to product inhibition by G-6-P, and has a molecular mass near 50 kDa. The cDNA encoding each hexokinase isozyme has been cloned and sequenced from its major tissues of origin [21,26–29], and as discussed in greater detail below, the sequences obtained implicate a "gene duplication" explanation for the 100-kDa Type-I–III hexokinases.

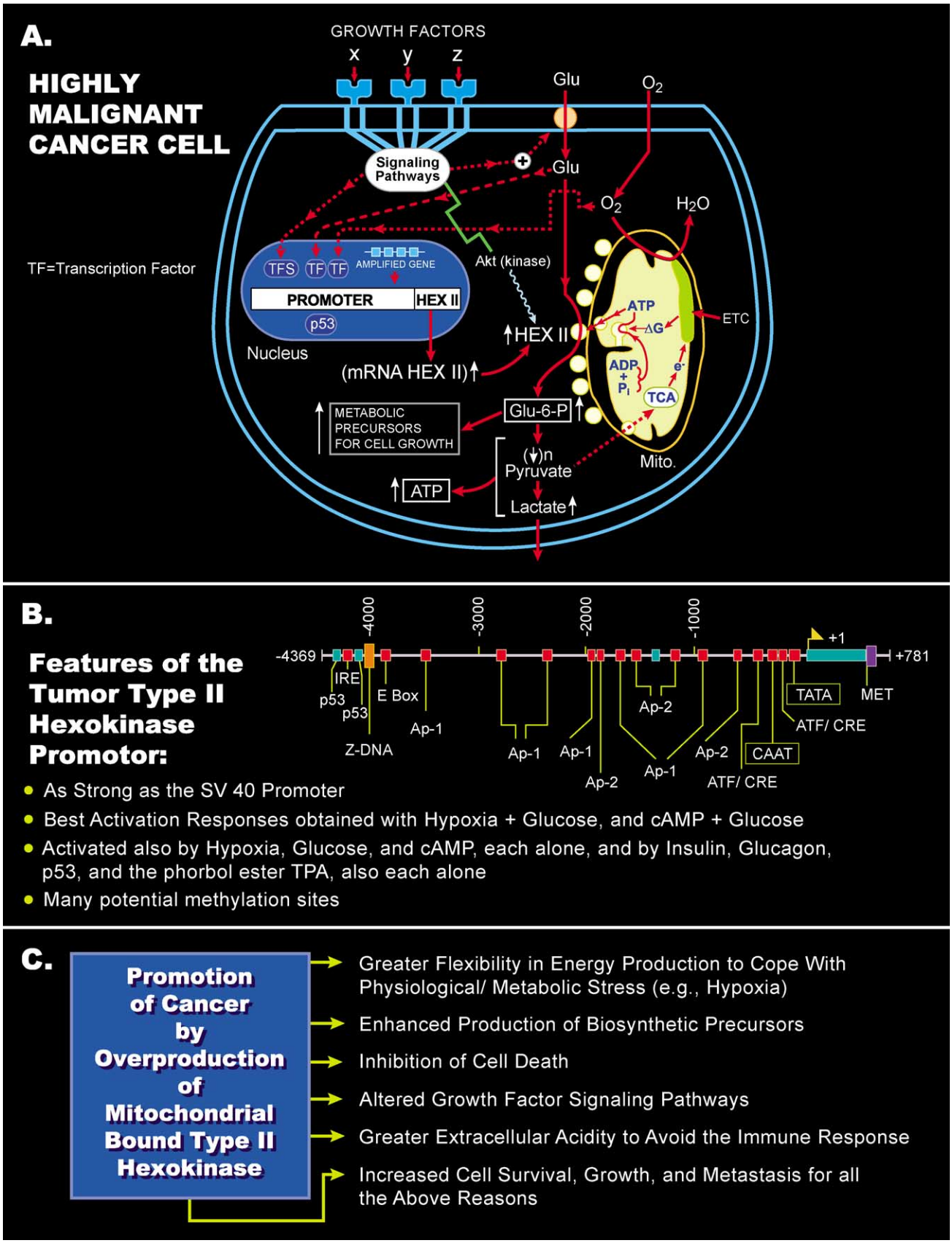
Hexokinase isozymes from tumors exhibiting the high glycolytic phenotype have been cloned and sequenced also [21,30]. Significantly, these sequences correspond to Type II hexokinase, the major isozyme overexpressed (>100-fold) in such tumors, and found normally in muscle and adipose tissue in low amounts. Type I hexokinase, found normally in brain, breast, kidney, and retina is present also in some highly glycolytic tumors [19,20,31–33], but at very low levels relative to the Type II isozyme. Possible exceptions are brain tumors [34,35] where the Type I enzyme may be higher. Nevertheless, recent work shows that the Type II enzyme, nearly absent in normal brain tissue, is expressed in significant amounts in gliomas [36]. The same pattern applies to many human breast cancers where 45% of all samples examined in a recent study contained Type II hexokinase [37]. Finally, although Type I hexokinase is also the predominant form of hexokinase in normal retina tissue, retinoblastoma and a retinoblastoma cell line express considerable amounts of the Type II isozyme [38]. Although more work needs to be done, there should be some concern if significant amounts of Type II hexokinase are found in a biopsy of a tumor derived from a tissue that normally does not express this isoform. It may mean that the tumor is already of the rapidly growing, highly glycolytic type or that it is on its way to becoming such.

3. Mitochondrial binding of Type II hexokinase in tumors: facilitation of glycolysis and inhibition of cell death

Type II hexokinase binds to transmembrane channels formed by the protein called "porin" or "VDAC" [15] located within the outer mitochondrial membrane. This interaction (Fig. 1A) markedly reduces the enzyme's sensitivity to product inhibition by G-6-P [14], provides preferred access to mitochondrially generated ATP [39], and protects against proteolytic degradation [40]. As we described earlier [5,39,41,42], these combined properties, together with the high content of the enzyme in highly malignant tumors (>100-fold elevation), result in the rapid production of G-6-P. This key metabolic intermediate-precursor serves not only as a major carbon source for most biosynthetic pathways that are essential for the growth and rapid proliferation of tumors, but also as the initial substrate for glycolysis that generates ATP synthesis during its catabolism to lactate (Fig. 1A) (reviewed in Refs. [43,44]). Under aerobic conditions, as much as half the ATP produced in some tumor cells may be derived from glycolysis [2,45], in sharp contrast to normal cells where this value is usually less than 10% and oxidative phosphorylation is the predominant method for ATP generation. Under hypoxic (low oxygen tension) conditions near the core of solid tumors, the already high glycolytic rate may double [4] allowing at least some cells to not only survive but to undergo further mutations that increase their malignancy [46] and prepare them for metastasis [47].

Regarding the above, it is perhaps important to note that the extent to which a highly malignant tumor cell may generate its ATP from glycolysis is likely to depend on its local physiological/metabolic state. For example, if ample oxygen and mitochondrial substrates are available, it is likely that more ATP will be generated by mitochondrial oxidative phosphorylation than by glycolysis. However, if either of these staples is in short supply, for example, during hypoxia or during metastatic migration, more ATP will be generated by glycolysis than by mitochondrial oxidative phosphorylation, thus assuring tumor cell survival.

Interestingly, recent studies strongly indicate that highly malignant cancer cells employ Type II hexokinase not only to assure their survival during abrupt changes in physiological/metabolic state, but also to protect them against cell death, perhaps while the latter changes are taking place. Thus, mitochondrial binding of Type II hexokinase to the outer mitochondrial membrane has been shown to inhibit Bax-induced cytochrome *c* release and apoptosis in HeLa cells [48]. Other recent work suggests that both survival mechanisms noted above may be related to growth-factor-induced signaling pathways dependent on the serine/threonine kinase Akt/PKB, a major downstream effector of growth-factor-mediated cell survival [49–51]. Thus, growth factors acting through signaling pathways are believed to facilitate or enhance the binding of Type II hexokinase to the outer mitochondrial membrane, a view that had been suggested much earlier [52] but largely ignored. Perhaps growth-related signaling pathways that might normally control binding and debinding of Type II hexokinase in normal cells (e.g., muscle and adipose) have been altered in poorly differentiated cancer cells, such that this enzyme remains "locked" to the outer mitochondrial membrane thus enhancing cell survival, the malignant state, and the poten-


A.
HIGHLY MALIGNANT CANCER CELL
TF=Transcription Factor
GROWTH FACTORS
x y z
Glu
O_2
Signaling Pathways
Glu
O_2
H_2O
TFS TF TF
AMPLIFIED GENE
PROMOTER
HEX II
p53
Nucleus
Akt (kinase)
ATP
ΔG
ETC
ADP + Pi
e-
TCA
Mito.
↑HEX II
(mRNA HEX II)↑
METABOLIC PRECURSORS FOR CELL GROWTH
Glu-6-P
(↓)n Pyruvate
Lactate↑
↑ATP
B.
Features of the Tumor Type II Hexokinase Promotor:
-4000
-3000
-2000
-1000
+1
-4369
+781
IRE
p53
p53
Z-DNA
E Box
Ap-1
Ap-1
Ap-1
Ap-2
Ap-1
Ap-2
Ap-2
Ap-2
TATA
MET
ATF/ CRE
CAAT
ATF/ CRE
As Strong as the SV 40 Promoter
Best Activation Responses obtained with Hypoxia + Glucose, and cAMP + Glucose
Activated also by Hypoxia, Glucose, and cAMP, each alone, and by Insulin, Glucagon, p53, and the phorbol ester TPA, also each alone
Many potential methylation sites
C.
Promotion of Cancer by Overproduction of Mitochondrial Bound Type II Hexokinase
Greater Flexibility in Energy Production to Cope With Physiological/ Metabolic Stress (e.g., Hypoxia)
Enhanced Production of Biosynthetic Precursors
Inhibition of Cell Death
Altered Growth Factor Signaling Pathways
Greater Extracellular Acidity to Avoid the Immune Response
Increased Cell Survival, Growth, and Metastasis for all the Above Reasons

tial for metastasis. Certainly, a very interesting story is now beginning to unfold with Type II hexokinase as a central player in the highly malignant cancer cell's capacity to survive a variety of stress situations.

4. Molecular and structural biology of Type II hexokinase

4.1. Chromosomal location

Interestingly, each of the four hexokinase isoforms is located on a different chromosome in both rats and human with Type II hexokinase, the subject of this review, being located on rat chromosome 4 and human chromosome 2. More specifically, in the rat, hexokinase Types I, II, III, and IV (glucokinase) have been assigned to chromosome bands 20q11, 4q34, 17q12, and 14q21, respectively [53]. In humans, Type II hexokinase has been mapped to the chromosome 2p13 locus [54], glucokinase to 7p22 [55] and 7p13 [56], Type I hexokinase to the short (10p11) or long arm (10q11) of chromosome 10 [57], and Type III hexokinase to 5q35 [58,59].

4.2. Gene duplication and three-dimensional structure

As indicated above, hexokinases Types I–III share two common properties; they have molecular weights of approximately 100 kDa and are sensitive to feedback inhibition by the product G-6-P. In contrast, Type IV hexokinase (glucokinase) has a molecular weight of approximately 50 kDa and is insensitive to inhibition by physiologically relevant concentrations of G-6-P. As such, mammalian glucokinase resembles hexokinases of yeast [60] that are insensitive to inhibition by G-6-P, and have molecular weights near 50 kDa. These observations implicate an evolutionary relationship, where the 100-kDa Types I–III mammalian hexokinases evolved from an ancestral 50-kDa enzyme similar to the yeast enzyme via a gene duplication and fusion event [61–64]. The "gene duplication" view has gained excellent support from studies where the cDNA corresponding to each hexokinase has been cloned and sequenced and the exon–intron structure mapped, for example, for the Type II hexokinase [63]. Thus, based on cDNA data, and the deduced primary sequence [21,26–28], each of the two 50-kDa halves (denoted N and C) of the Types I–III isozymes show close homology to each other, and to the 50-kDa hexokinase isozymes of yeast. Further support for the "gene duplication" view for the origin of mammalian hexokinases Types I–III has come from the elucidation of the atomic resolution structure of the Type I enzyme [65,66]. As predicted, both the N and C terminal halves, connected by a linker region, are very similar in three-dimensional space. For this reason, it is predicted also on the basis of homology arguments that the three-dimensional structure of each half of Type II hexokinase will be highly similar to the other.

Specifically, as it relates to this minireview, one of the most interesting findings that has emerged from a study of the two hexokinase halves (N and C) is that only those derived from the Type II isoform are both catalytic [67]. In the well-studied Type I isoform, the N half serves only a regulatory role while the C-terminal half exhibits both catalytic and regulatory roles [67–69]. Therefore, highly malignant cancer cells appear to have chosen wisely to overexpress the hexokinase isoform with the greatest catalytic potential.

4.3. Gene amplification, expression, and regulation

In highly malignant hepatomas expressing the high glycolytic phenotype, we know that mitochondrial bound Type II hexokinase activity is more than 100-fold greater than that found in liver hepatocytes. We know also from our studies [70] and that of others [19,20,71] that the Type II hexokinase mRNA levels are exceptionally high in such tumors. Identifying the factors involved has been of great interest to us, and, for this reason, we have initiated studies to determine to what extent the following events/factors are involved: (1) sequence differences between the normal and tumor promoters; (2) promoter activation; (3) gene amplification; (4) mRNA stability; and (5) changes in DNA methylation status.

With regard to the above possibilities, initial studies rendered unlikely that normal/tumor promoter sequence differences are involved as isolation and sequencing of the Type II hexokinase promoter from normal hepatocytes and a highly glycolytic hepatoma (AS-30D) revealed a difference of no more than 1% [GenBank U19605, AY082375]. Interestingly, both promoters have well-defined TATA and CAAT boxes indicating precise positioning of transcription initiation for the Type II hexokinase mRNA transcript [70], and both are distinct in sequence from those obtained for hexokinases Types I, III, and IV [72–74]. Moreover, in contrast to normal/tumor promoter sequence differences,

Fig. 1. (A) Overview of those molecular events that lead to a marked overexpression of mitochondrial bound Type II hexokinase in many cancer cells and the resultant metabolic consequences. The marked overexpression of the enzyme involves both gene amplification and promoter activation. Other likely events involved are demethylation and mRNA stability. (B) Schematic of the tumor Type II hexokinase promoter region and a summary of some of its principal activators. The 4.3-kilobase pair promoter region shown contains well-defined TATA and CAAT boxes and potential response elements for numerous cellular factors including p53. The promoter is as strong as the SV 40 promoter and, in studies carried out so far, is activated best by hypoxic conditions plus glucose, and by cAMP plus glucose. (C) A summary of the different ways in which the overexpression of mitochondrial bound Type II hexokinase may promote cancer. In addition to providing an enhanced production of glucose-6-phosphate to accelerate biosynthesis for growth and glycolysis to combat hypoxic stress, the overproduced enzyme also inhibits cell death programs dependent on cytochrome *c* release from the mitochondria. Thus, the relative levels of Type II hexokinase in a tumor may provide a diagnostic index of its aggressiveness, or its potential to become aggressive.

activation of the Type II hexokinase promoter is likely to be a significant contributor to the overexpression of the Type II enzyme in tumors (Fig. 1B). Thus, reporter gene assays involving transfection of a highly malignant hepatoma cell line revealed that hypoxic conditions, glucose, insulin, glucagon, cAMP, p53, and the phorbol ester TPA [70,75–77] have a positive effect on transcription. Of these, the greatest activation observed to date (~ 7-fold) was obtained under hypoxic condition with glucose present and involved both the proximal and distal regions of the Type II hexokinase promoter [77]. This activation appears to involve also hypoxia inducible factor 1 (HIF-1) [78,79]. The second best activation response (5- to 6-fold) was obtained in the presence of dibutyryl cAMP and glucose [75], and requires further study to localize its site(s) of action within the promoter.

In addition to promoter activation, we have shown also that amplification (5- to 10-fold) of the Type II hexokinase gene may play a significant role in the overexpression of Type II hexokinase [80]. Finally, although we have preliminary evidence that mRNA stability and methylation/demethylation events are involved also in the high expression of Type II hexokinase, much more work is needed to assess their relative contribution.

5. Early and late events in tumor progression in relation to Type II hexokinase gene expression

In recent years, much attention has been given to the view that clinically diagnosed cancers in humans result from a series of apparently sequential genetic changes [81–84]. If we adhere strictly to this view of tumor progression, then it is only natural for us to inquire at what stage does the overexpression of an enzyme as seemingly important as Type II hexokinase occur. On the one hand, it is easy to rationalize that it is a late event. Support for this comes from studies conducted many years ago on the Morris rat hepatoma lines which showed that only those that had the most chromosomal aberrations (e.g., hepatoma 3924A) and grew the fastest exhibited a high glycolytic rate and a high hexokinase activity (reviewed in Ref. [5]). However, studies conducted somewhat later with a different system tend to lead one to either the opposite conclusion or certainly a modified conclusion. Thus, it has been shown that enhanced rates of glucose transport, utilization, and enhanced activities of membrane-bound hexokinase, with an increase in the Type II/Type I ratio, are early events during cellular transformation of chicken embryo fibroblasts using a temperature-sensitive Rous Sarcoma virus mutant (ts-68) [85,86]. The answer to this apparent paradox is not known, but when it is, it may provide further insight into the multiple roles of Type II hexokinase in highly malignant tumors and perhaps require some modification of currently accepted models to explain tumor progression.

6. Prospects for therapeutic intervention targeted at mitochondrial bound Type II hexokinase

Considering the multiple roles that Type II hexokinase plays in highly malignant tumor cells, it clearly represents an attractive target for therapeutic intervention. Assuming that one knows via PET scanning and diagnostic test on biopsies that a given tumor is highly glycolytic and exhibits elevated levels of Type II hexokinase, then what agents can be administered that will inhibit this enzyme's activity or production, and how should such agents be delivered? We have been asking this question for the past few years. A strategy has now been developed and is currently being subjected to experimental test. Our focus has been on liver cancer for several reasons. First, hepatocellular carcinoma (hepatoma) is one of the most common fatal cancers in the world [87,88] and may soon reach epidemic levels due to increased viral induced hepatitis [89]. Secondly, liver is a common site for metastasis of other cancers, for example, colon cancer [90], and the resultant metastatic tumors that develop in the liver are frequently the cause of death. Third, there are numerous model systems, both animal and culture, for studying liver cancer.

Specifically, we are using two Type II hexokinase-related approaches to inhibit tumor cells. In the first, we screen for agents that inhibit both tumor glycolysis and mitochondrial oxidative phosphorylation. The identified compounds are then injected intraarterially [91] directly into a model tumor growing within the liver of a live animal. The logic of the approach is to completely and selectively inhibit total cell ATP synthesis fueling the tumor without harming surrounding normal tissue. One such agent, 3-bromopyruvate, a potent inhibitor of mitochondrial bound hexokinase that inhibits both glycolysis and mitochondrial oxidative phosphorylation is currently under study in our laboratory [92]. Significantly, in our most recent studies, we have shown that a single bolus injection (intraarterially) of 3-bromopyruvate directly into liver implanted rabbit tumors kills as many as 90% of the tumor cells without doing any apparent damage to surrounding liver tissue, other organs, or the general health and well being of the animal [93].

We are investigating also approaches that target only Type II hexokinase, for example, antisense RNA [94], the logic here being that Type II hexokinase may be so important for tumor survival that it is unnecessary to simultaneously inhibit oxidative phosphorylation. If properly packaged, such targeted antisense agents can also be delivered intraarterially. In this regard, it should be noted that many tumors are fed predominantly by arteries while normal tissues like liver are fed predominantly from the venous circulation, for example, the portal vein [95]. Therefore, intraarterial injection of a drug directly into a tumor is expected to find its target more quickly while minimizing its entry into the general circulation. In this way, toxic side effects are minimized or eliminated altogether.

7. Synopsis

In summary, much evidence has been obtained that demonstrates that Type II hexokinase is elevated in many tumors and bound to the outer mitochondrial membrane. This is not a characteristic of all tumors but is a characteristic of rapidly growing, poorly differentiated tumors that are highly malignant. If one believes strictly in the progression model for tumorigenesis, then one might expect the high glycolytic/high Type II hexokinase phenotype to be a property mainly of advanced stage and metastatic tumors. However, studies conducted in tissue culture show that the appearance of Type II hexokinase may in some cases also be an early event. Significantly, not one, but many events appear to contribute to the overexpression of the Type II hexokinase protein and the elevation of its total activity in highly malignant cancer cells (Fig. 1A). These include gene duplication, amplification, and perhaps demethylation, as well as promoter activation (Fig. 1B), mRNA stability, and binding to the mitochondria. The latter membrane binding event, that may be promoted by signal transduction pathways involving the serine/threonine kinase Akt/PKB, suppresses the enzyme's degradation, reduces product inhibition by G-6-P, and gives the enzyme preferred access to mitochondrially generated ATP. Mitochondrial binding of Type II hexokinase also prevents cell death, thus promoting cancer cell immortality. The net result of all of the above events is the development of a powerful cancer cell with specially endowed survival skills (Fig. 1C). It is highly resistant to abrupt changes in physiological/metabolic states, to death signals, and to the immune system. Such cells rapidly multiply and divide frequently forming an encapsulated army of cells (solid tumor), some of which via metastasis seek out new homes in other tissues, and ultimately via this aggressive behavior assure both the death of the host and their own death as well. As Type II hexokinase is a major player in promoting the growth and perhaps the metastasis of aggressive cancers, this enzyme and its gene represent ideal targets for therapy.

Acknowledgements

The authors acknowledge NIH grant CA 80118 to P.L.P. that supported much of our recent research described in this review. While part of this research was conducted, S.P.M. was a fellow of NIH grant T32 CA 67751 and A.R. was an awardee of the Deutsche Forschungsgemeinschaft. J.F.G. is a Johns Hopkins Gatewood Fellow supported by the Cardiovascular and Interventional Radiology Research Foundation.

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