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Glucose catabolism in the rabbit VX2 tumor model for liver cancer: characterization and targeting hexokinase

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Abstract

The rabbit VX2 tumor when implanted in the liver has proven convenient as a model for studying hepatocellular carcinomas. However, its metabolic properties have not been well studied. Significantly, studies described here show that the VX2 tumor exhibits a high glycolytic/high hexokinase phenotype that is retained following implantation and growth in rabbit liver. In addition, results of a limited screen show that the glycolytic rate is inhibited best by 2-deoxyglucose (2DOG) and 3-bromopyruvate (3BrPA), the former compound of which is phosphorylated by hexokinase but not further metabolized, while the latter directly inhibits hexokinase. Finally, when tested on hepatoma cells in culture both inhibitors facilitated cell death. These studies underscore the usefulness of the VX2 tumor model for the study of advanced liver cancer and for selecting anti-hepatoma agents. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Liver cancer; Tumor metabolism; Hexokinase; Drug targeting; 2-Deoxy Glucose; 3-Bromo Pyruvate

1. Introduction

Liver cancer, in particular hepatocellular carcinoma (hepatoma) remains one of the most common fatal cancers in the world [1]. The clinical prognosis is very poor with the medium survival time approaching 6 months. In recent years the VX2 tumor, an epidermoid rabbit tumor induced by the Shope papilloma virus [2], has shown promise as a model system for studying liver cancer [3,4]. This is because it grows well when implanted in the rabbit's liver where it takes on growth properties and a vascularization system similar to many human liver tumors [3,4]. Here, the tumor is fed through a hepatic artery while the liver is fed through the portal vein. Thus, it is possible via the method known as transcatheter chemoembolization [5,6] to deliver anticancer agents directly to the tumor via the hepatic artery [5,6]. In addition, it has been shown that when delivery is made using certain oils the mixture preferentially localizes in the tumor rather than in the surrounding liver tissue [3,4]. This is important as it may allow for the targeting of exceptionally potent cancer killing agents directly to the tumor for brief periods of time thus minimizing damage to the surrounding liver tissue and toxicity to the host.

With the above in mind, it is important to learn more about the energy metabolism of the VX2 tumor in order to determine to what extent it mimics that of rapidly growing hepatomas. These cancers are known to exhibit a high glucose catabolic rate [7,8],

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and where examined carefully, to contain elevated levels of hexokinase bound to their mitochondria [9–11]. Moreover, in the AS-30D hepatoma, the most extensively studied tumor in this class, it has been shown also that the gene for hexokinase is amplified [12] and that the mRNA levels are markedly elevated [13]. In experiments described below, we demonstrate clearly that the VX2 tumor does in fact exhibit a high glycolytic/high hexokinase phenotype, and that a large fraction of the total cell hexokinase is mitochondrially bound. In addition, we report the results of a limited screen for inhibitors of VX2 tumor glycolysis that may prove useful in future chemoembolization experiments designed to specifically target these agents to the tumor growing in the host liver, and, once there, arrest tumor cell growth.

2. Materials and methods

2.1. Materials

New Zealand white rabbits weighing 3.5–4.2 kg were obtained from Robinson Services Inc. The VX2 tumor, normally grown in the hind limb of these animals, was obtained locally from Dr John Hilton, Department of Oncology, Johns Hopkins University School of Medicine. AS-30D hepatoma cells, an established line, is maintained by Min Gyu Lee in the authors' laboratory. This is done by growth and passage of the cells in the peritoneal cavity of female Sprague–Dawley rats (Charles River Breeding Laboratories). The following agents were obtained from Sigma: D-glucose, 2-deoxyglucose, 2-fluoro-2-deoxyglucose, 6-fluoro-6-deoxyglucose, 3-O-methylglucose, 5-thio-D-glucose-6-phosphate, L-glucose, D-xylose, D-lyxose, 3-bromopyruvic acid, ATP, ADP, $NADP^+$, D-mannitol, Hepes, succinate, oligomycin, and bovine albumin. The lactic acid kit containing lactic dehydrogenase, NAD^+, hydrazine, and a glycine buffer, pH 9.2 was obtained also from Sigma. NaP_i, KP_i, and sucrose were obtained from J.T. Baker, and the Coomassie dye binding agent from Pierce. Glucose-6-phosphate dehydrogenase was obtained from Roche Molecular Biochemicals, and both the DMEM tissue culture medium and trypan blue were from Life Technologies Gibco BRL. The Clark oxygen electrode was purchased from Yellow Springs Instruments.

2.2. Processing the VX2 tumor for biochemical analyses

In one set of studies the VX2 tumors, which had grown in the hind limb of New Zealand white rabbits for 4 weeks, were excised, cut into 1 g pieces (ca. $10 \times 10 \times 10$ mm) with a razor blade, and then subjected to assays described below for monitoring both glycolytic and hexokinase activities. In a second set of studies VX2 tumors, which had grown in the hind limb of a New Zealand white rabbit for about 2 weeks, were excised, broken into small chunks (<0.1 g), and then implanted into the livers of a number of other rabbits. Following implantation, VX2 tumors rapidly developed in the livers of each animal. They were excised at different times ranging from 2 to 5.7 weeks and also subjected to the assays described below for monitoring glycolytic and hexokinase activities, as well as an assay for monitoring mitochondrial respiration. In all cases the exterior surface of the tumor was shaved to remove any remaining normal tissue. In addition, special care was taken to assure that only the viable portion of the tumor located near or on the surface was removed for analyses. The preparation of the animals for surgery, and the surgical and implantation procedures, have been described in detail elsewhere [4]. These procedures were approved by the Animal Care and Use Committee at the Johns Hopkins University School of Medicine and conducted according to their guidelines.

2.3. Assay for glycolytic activity

Glycolysis was assayed by monitoring the formation of lactic acid following the addition of glucose to a medium containing VX2 tumor slices. Specifically, freshly excised tumors were washed 3 times at 4°C in 20 ml Chance Hess Medium containing 6.2 mM KCl, 154 mM NaCl, and 11 mM NaP_i, pH 7.4. Slices, 1 g each, were then prior incubated for 30 min in a Lab-Line incubator-shaker at 37°C in 2 ml of the same medium while shaking at 50 rev/min. Glucose was then added to give a final concentration of 6 mM, after which the incubation/shaking process was continued with 0.2 ml samples being removed every 30 min for up to 2 h. Then, samples were removed for

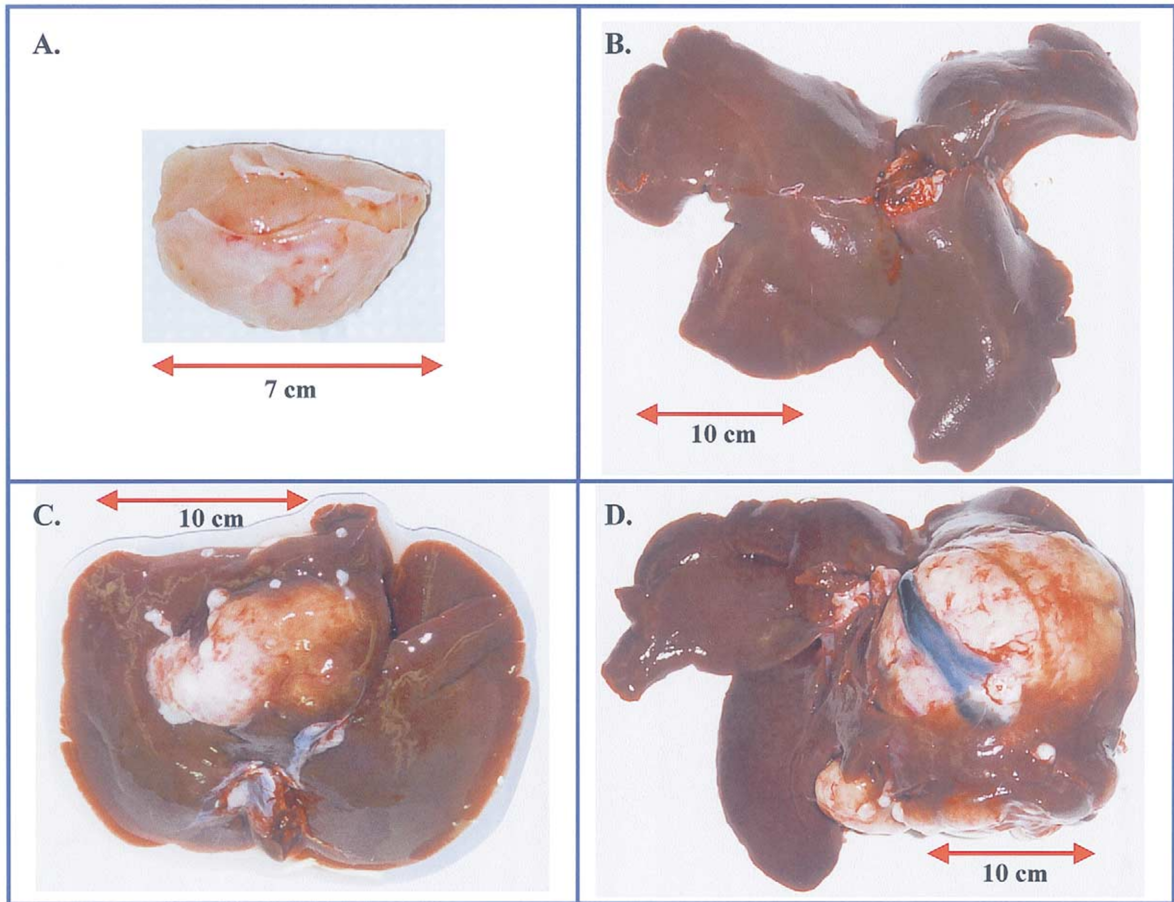

Fig. 1. Photographs of the VX2 tumor. (A) An excised VX2 tumor after 4 weeks growth in the rabbit hind limb. (B) Control liver isolated from a rabbit of the same age. (C,D) Livers harboring, respectively, VX2 tumors after 4 and 5.7 weeks of implantation.

analysis at two additional time points indicated Fig. 2. These samples were subjected to centrifugation at 14,000 rev/min in an Eppendorf Centrifuge (Model 5415C). Then, aliquots of the supernatant, 0.01 ml, were removed from each sample, diluted with 0.03 ml of water and subjected to lactic acid determination using the kit supplied by Sigma. The latter contains, in addition to lactic dehydrogenase and NAD^+, hydrazine, and glycine buffer, pH 9.2. The mixture was incubated for 30 min at 25°C after which the absorbance due to formation of NADH was determined at 340 nm using a Gilford Model 260 spectrophotometer. To assess the elevation of the glycolytic activity of the VX2 tumor over that of liver tissue, parallel studies were always carried out in an identical manner with 1 g liver slices derived from the host liver. Finally, in those cases where agents were tested for their capacity to inhibit glycolysis, these were introduced into the system at the prior incubation step with all subsequent steps being run in parallel with those described above for the VX2 tumor or liver alone.

2.4. Preparation of subcellular fractions for hexokinase and mitochondrial respiration assays

The freshly isolated liver or VX2 tumor tissues (15–20 g wet weight) was rinsed in three volumes of H-medium (210 mM D-mannitol, 70 mM sucrose, 2 mM Hepes, and 0.05% bovine albumin, pH 7.4) at 4°C and minced with a razor blade as finely as possi-

ble. A 30% (V/V) suspension was made using ice-cold H-medium in a Potter–Elvehjem glass homogenizer (55 ml capacity), and homogenization was achieved by applying four complete up and down strokes through the suspension with a rotating (~400 rpm), serrated, Teflon pestle attached to a motor. To remove cell debris and nuclei, the resultant homogenate was diluted to twice the initial volume and centrifuged at $630 \times g$ for 8 min at 4°C in a Sorvall RC-2B centrifuge using a GSA rotor. The supernatant was removed and then centrifuged at $6800 \times g$ for 15 min under the same conditions. The resultant supernatant was saved and referred to here as the cytosolic faction. The pellet was resuspended in the initial volume of H-medium and centrifuged twice at $9800 \times g$ for 15 min at 4°C. The washed pellet represents the mitochondrial fraction.

2.5. Assay for hexokinase activity

The assay coupled the glucose-6-phosphate formed in the hexokinase reaction to the glucose-6-phosphate dehydrogenase reaction [10]. Here, $NADP^+$, oxidizes glucose-6-phosphate to a γ-lactone while becoming reduced to NADPH, thus allowing the formation of the latter to be monitored spectrophotometrically at 340 nm. The final reaction mixture contained the following ingredients in a total volume of 1 ml at 25°C: 25 mM triethanolamine, pH 7.6, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0.45 mM NaCN, 0.005 mg/ml oligomycin, 0.014 mM DAPP [P^1,P^5-Di (adenosine-$5'$) pentaphosphate], 5 mM ATP, 3.3 units glucose-6-phosphate dehydrogenase, 1 mM $NADP^+$, 0.1–0.3 mg cytosolic or mitochondrial fraction, and concentrations of glucose as indicated in Figs. 1–4. Glucose was used to initiate the reaction.

2.6. Assay for mitochondrial respiration

Oxygen consumption rates were measured polarographically using a Clark oxygen electrode inserted into a 2.5 ml chamber equipped with a magnetic stirrer. The electrode was connected to a chart recorder calibrated between 0 and 100% saturation with atmospheric oxygen at 25°C. The loss of oxygen was monitored in a 2.1 ml system at 25°C containing 1 mg mitochondria, 0.5 mM EDTA, 2.0 mM Hepes, 220 mM D-mannitol, 70 mM sucrose, 2.5 mM KP_i, 2.6 mM $MgCl_2$, and 0.5 mg/ml bovine albumin, and, where indicated, 7.8 mM succinate (respiratory substrate), and 0.24 mM ADP (ATP synthesis substrate).

2.7. AS-30D hepatoma cells: culture conditions, and assessment of cell viability

AS-30D hepatoma cells grown in the peritoneal cavity of Sprague–Dawley female rats (see Section 2.1) were adapted to grow in tissue culture in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and counted in a Neubauer chamber after trypan blue dye addition (cell/dye volume = 1/1) by visualization under a Nikon inverted microscope.

2.8. Protein determination

Protein was determined using Pierce's Coomassie dye binding assay protocol.

3. Results

3.1. Description of the VX2 tumor prior to and after liver implantation

Photographs of VX2 tumors representative of those used in this study are presented in Fig. 1. When excised from the hind limb of the New Zealand white rabbit (donor) at 4 weeks the tumor is about 10 g in weight, flesh colored with some surface vascularization, and without any obvious signs of necrosis (Fig. 1A). When small chunks (<0.1 g) are implanted in the liver of a rabbit of similar size and age the tumor grows rapidly attaining a weight as high as 25 g in a 4 week period while retaining its solid, flesh colored features (Fig. 1C). Here too, there are no obvious signs of necrosis although an increased surface vascularization is apparent. At 5.7 weeks (Fig. 1D), the liver implanted VX2 tumor has become highly vascularized on its surface and more than doubled in size in the intervening 1.7 weeks. Much of the increased weight is due to fluid that has accumulated within the core of the tumor where it has become almost completely necrotic and taken on a mush-like texture. At this stage only the tissue near the surface of the

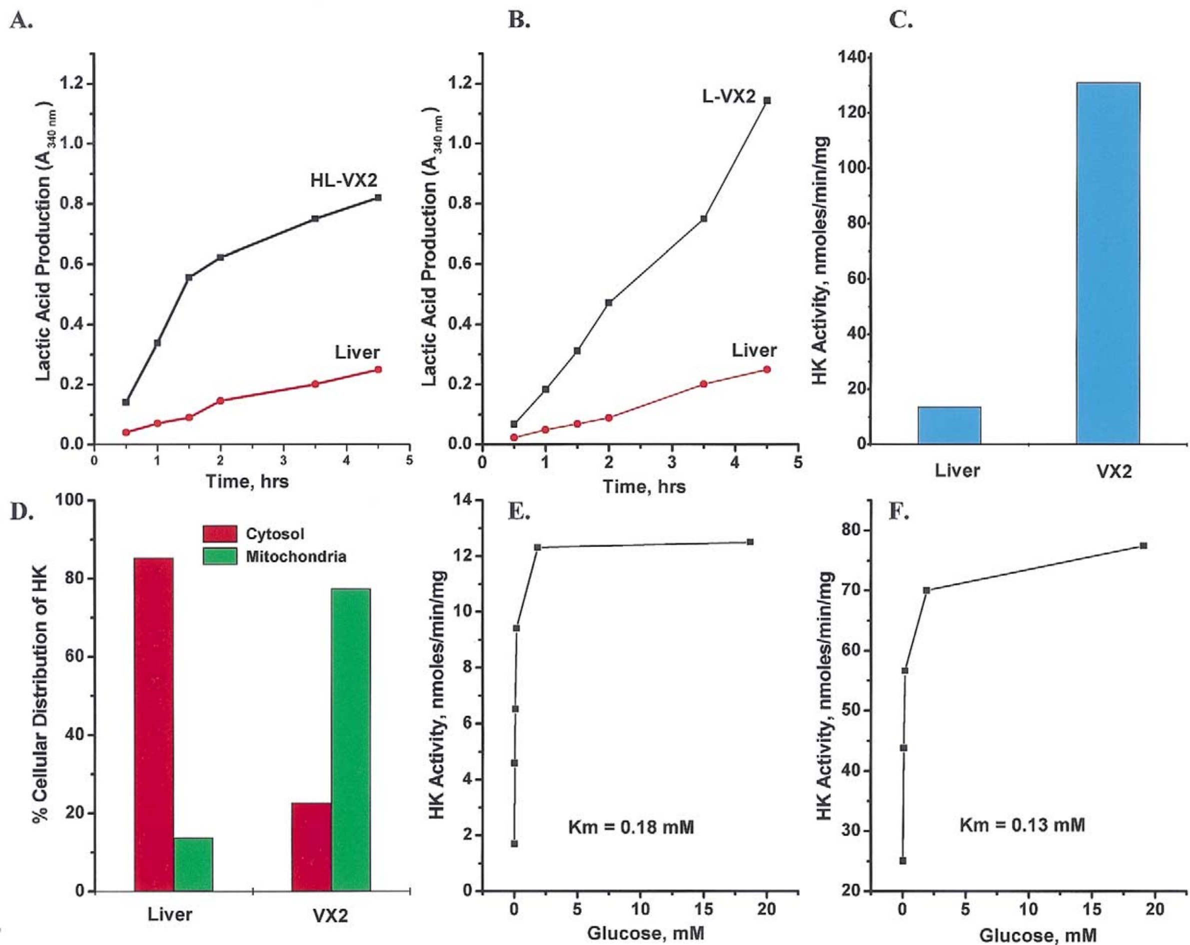

Fig. 2. (A,B), Lactic acid production by the VX2 tumors isolated, respectively, from the rabbit hind limb (HL-VX2) and rabbit liver (L-VX2). In both cases, slices of the tumors were incubated in the presence of 6 mM glucose for the times indicated and then assayed for lactic acid exactly as described in Section 2. Liver slices from the animals from which the tumors were obtained were subjected to the same assay. (C) Comparison of the activity of hexokinase per mg protein in liver and VX2 tumor tissues. The mean ± standard error are 13.7 ± 2.5 (liver) and 131 ± 10.1 (tumor). See Section 2 for assay. (D) Relative distribution of total hexokinase activity in the mitochondrial and cytosolic fractions of rabbit liver and VX2 tumor tissues. For liver, the mean ± standard error in % of total cellular distribution is 85.3 ± 2.6 (cytosol) and 13.7 ± 2.5 (mitochondria). For the VX2 tumor these values are 22.6 ± 6 (cytosol) and 77.4 ± 6 (mitochondria). The % of the total starting protein recovered in the mitochondrial fraction (~20–21%) was nearly the same for liver and tumor. (E,F) Michaelis–Menten kinetic constants (Km values for glucose) for hexokinase located, respectively, in the cytosolic and mitochondrial fractions of the VX2 tumor. The mean ± standard error is 0.18 ± 0.015 mM (cytosolic fraction) and 0.13 ± 0.02 mM (mitochondrial fraction).

tumor remains viable and can be used for biochemical studies. Tumors were not carried beyond 5.7 weeks.

3.2. Glycolytic capacity and hexokinase activity of the VX2 tumor prior to and after liver implantation

Results of experiments presented in Fig. 2A show that slices of the VX2 tumor isolated after 4 weeks of growth in the hind limb of a New Zealand white rabbit exhibit a glycolytic rate that is 7.4 fold higher than that observed for normal liver tissue derived from the same animal. Significantly, this enhanced glycolytic rate of the VX2 tumor relative to liver tissue is retained when small chunks of the tumor (<0.1 g)

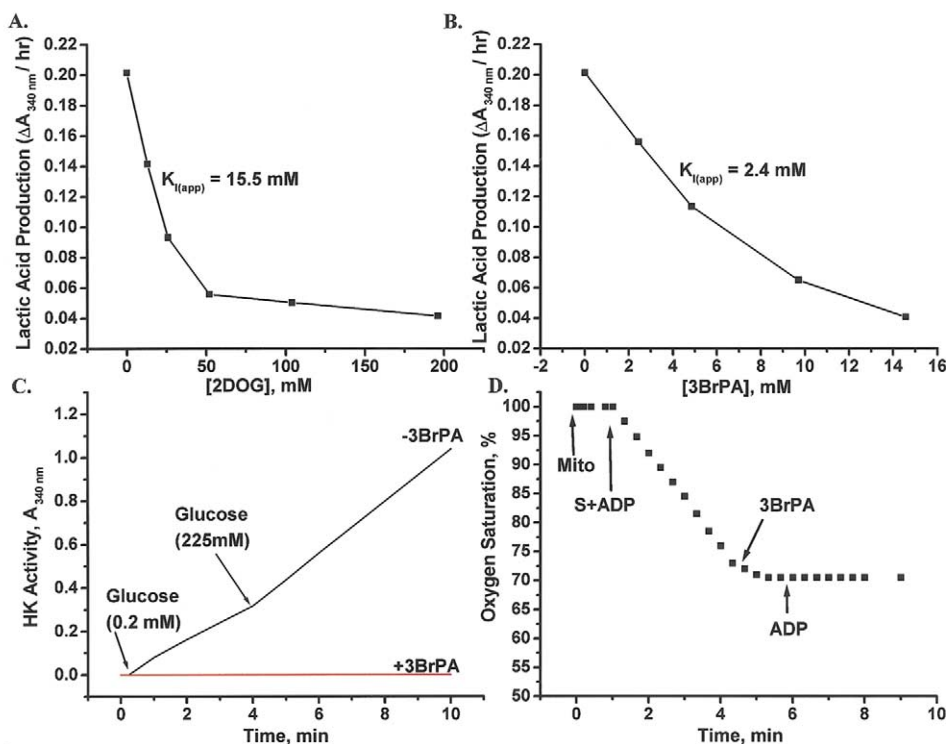

Fig. 3. (A,B) Comparison of the inhibitory effects, respectively, of 2DOG and 3BrPA on the glycolytic capacity of the isolated liver implanted VX2 tumor. In both cases various concentrations of 2DOG and 3BrPA were prior incubated for 30 min at 37°C with 1 g VX2 tumor slices. Glycolysis was monitored exactly as described in Section 2. Under the assay conditions used, 3BrPA was without effect on the activity of lactate dehydrogenase. C, The effect of 3BrPA on the activity of hexokinase bound to the mitochondrial fraction of the VX2 tumor. Hexokinase activity was monitored exactly as described in Section 2 in the presence and absence of 5 mM 3BrPA. Under the assay conditions used, 3BrPA was without effect on the activity of glucose-6-phosphate dehydrogenase. (D), The effect of 3BrPA on the ADP stimulated respiratory rate of rabbit VX2 tumor mitochondria. The procedures for preparing the mitochondria and assaying respiration are described in Section 2. Where indicated, 3BrPA was added to give a final concentration of 1.2 mM.

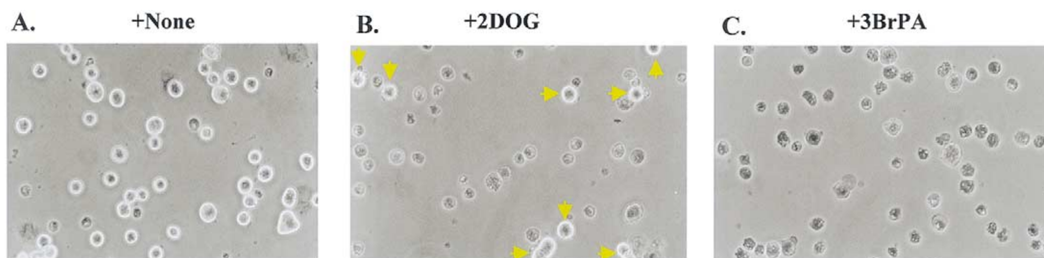

Fig. 4. Comparison of the effects of 2DOG and 3BrPA on the viability of rat hepatoma cells growing in tissue culture. Cells were maintained in tissue culture exactly as described in Section 2. Where indicated, 20 mM 2DOG (B) or 5 mM 3BrPA (C) were added to cells that had been growing for 24 h. After an additional 12 h, the viability of the cells was assessed as described in Section 2 using trypan blue. In (A) (Control) almost all cells in the field are viable as indicated by a dark nucleus and a surrounding bright appearing cytosol. The remaining viable cells in (B) (2DOG) showing this appearance are indicated by arrows. In (C) (3BrPA) there are no such cells as they have all taken up the trypan blue.

are implanted in the liver of another rabbit of similar size and age. Thus, Fig. 2B shows that the glycolytic rate of tumor slices derived from the liver implanted VX2 tumor after 4 weeks of growth is 8.3 fold higher than the glycolytic rate obtained with liver slices obtained from the same liver in which the tumor implant had been made. In data not presented here, this high glycolytic rate of the liver implanted VX2 tumor remained relatively constant through 5.7 weeks of growth.

Results presented in Fig. 2C show that the hexokinase activity is also markedly elevated (~ 9.5 fold) in the liver implanted VX2 tumor relative to the activity of this enzyme in the surrounding liver tissue. In addition, as shown in Fig. 2D, the subcellular distribution of this activity in the tumor (~70% in the mitochondrial fraction and ~30% in the cytosol) differs markedly from that in the surrounding liver tissue (~20% in the mitochondrial fraction and 80% in the cytosol). Finally, Fig. 2E,F show that the Km of the tumor hexokinase(s) for glucose is very low (0.11 mM, mitochondrial fraction and 0.19 mM, cytosolic fraction, mean value of three experiments) reflecting a high apparent affinity of the isoform(s) for glucose. This is in sharp contrast to liver where the Km for glucose of the major hexokinase isoform involved, i.e., glucokinase, is at least 5 mM in most reported studies [14].

These studies show clearly that the VX2 tumor, although of non-hepatic origin, exhibits glucose metabolic properties characteristic of many rapidly growing hepatomas [7–11], the biochemical hallmarks of which are a high glycolytic/high hexokinase phenotype, and binding of the hexokinase (low Km for glucose) to the mitochondrial fraction.

3.3. Identification of inhibitors of the glycolytic capacity of the isolated liver implanted VX2 tumor

A limited screen was carried out to identify inhibitors of the glycolytic capacity of the VX2 tumor with the purpose of selecting agents that might prove effective in arresting tumor cell growth. The screen included the following nine compounds: 2-deoxyglucose (2DOG), 2-fluoro-2-deoxyglucose, 6-fluoro-6-deoxyglucose, 3-*O*-methyl glucose, 5-thio-D-glucose-6-phosphate, L-glucose, D-xylose, D-lyxose, and 3-bromopyruvic acid (3BrPA). The screen was conducted by incubating VX2 tumor slices in a medium containing 6 mM glucose with 6 mM of each of these agents at 37°C for 5 h. The use of 6 mM glucose in the medium was used to mimic the maximal amount of glucose that might be in the blood in a real in vivo situation. Interestingly, in data not presented here, all glucose analogs or other sugars tested under these conditions proved to be ineffective as inhibitors of VX2 tumor glycolysis indicating the inability of these sugars to act as effective glycolytic inhibitors under the conditions specified. In sharp contrast, the pyruvate analog (3BrPA) almost completely inhibited glycolysis.

With the above preliminary data at hand, only two of the nine compounds screened were studied in more detail. One was 2DOG because under certain conditions it is known to inhibit glycolysis when it is phosphorylated by hexokinase to 2DOG-6-P which cannot be further metabolized [15]. The other compound tested in more detail was 3BrPA because of its effectiveness as a glycolytic inhibitor in the preliminary screen. Data presented in Fig. 3A show that 2DOG can inhibit glycolysis catalyzed by VX2 tumor slices provided it is used at concentrations of glucose much higher than those normally found in the blood, and provided it is prior incubated with the tumor slices in the absence of glucose. Here, it can be seen that half maximal inhibition of glycolysis requires about 15 mM 2DOG whereas maximal inhibition (70%) requires almost 50 mM. Results presented in Fig. 3B show that 3BrPA is a more effective inhibitor than 2DOG as it induces half maximal inhibition of the glycolytic activity of the VX2 tumor slices at a concentration of only 2.4 mM and complete inhibition at about 15 mM.

Additional experiments were undertaken to determine whether 3BrPA is also an inhibitor of the mitochondrial hexokinase of the VX2 tumor, as this enzyme is known to be markedly elevated in rapidly growing hepatomas [8–13], and where examined carefully, to be required for maintenance of the high glycolytic rate [9]. Results presented in Fig. 3C show that 5 mM 3BrPA inhibits completely glucose initiation of the hexokinase reaction in a system (see Section 2) containing among other ingredients VX2 tumor mitochondria, ATP, glucose-6-phosphate dehydrogenase, and $NADP^+$. (Although complete inhibition of hexokinase activity is achieved at a concentration of only 5 mM 3BrPA (Fig. 3C),

whereas a concentration near 15 mM is necessary to completely inhibit glycolysis (Fig. 3B), this is likely due to the fact that the former assay was conducted on a cell free extract whereas the latter was conducted on intact tumor tissue). Finally, in addition to these findings, was the very important discovery that 3BrPA (1.2 mM) also completely inhibits mitochondrial respiration (Fig. 3D), i.e. both the basal rate of respiration catalyzed by the respiratory substrate succinate, and the ADP stimulated rate of respiration normally associated with ATP synthesis by oxidative phosphorylation.

3.4. Comparison of the relative capacities of 2DOG and 3BrPA to kill hepatoma cells expressing the high glycolytic/high hexokinase phenotype

The studies described above using the VX2 tumor model for liver cancer would predict that 2DOG and 3BrPA may have the capacity to kill hepatoma cells expressing the high glycolytic/high hexokinase phenotype. For this reason, both agents were tested for their capacity to inhibit the growth of AS-30D hepatoma cells, an established rat cell line known to exhibit a high glycolytic rate and to contain elevated levels of mitochondrial bound hexokinase. Significantly, comparison of control hepatoma cells (Fig. 4A) with those treated 12 h with 20 mM 2DOG (Fig. 4B) or with 5 mM 3BrPA (Fig. 4C) show that both 2DOG and 3BrPA have the capacity to induce cell death, with 3BrPA killing all cells in the population and 2DOG killing about 80%. Cell killing was assessed in these experiments by counting those cells in which trypan blue had entered.

4. Discussion

As indicated earlier, the VX2 tumor (Fig. 1A), although not of hepatic origin, has proven useful as a model for liver cancer particularly in the study of the delivery of anticancer agents via the process known as chemoembolization. Thus, when this tumor is implanted in the rabbit liver it grows rapidly (Fig. 1C,D) and develops a vascularization system similar to that of many human hepatomas. This allows potential anticancer agents to be delivered selectively to the tumor via the hepatic artery [3,4]. However, lack of specific knowledge about the VX2 tumor's metabolism has limited its current utility to the study of drug delivery to liver tumors, leaving its potential usefulness as a model system for also testing novel drugs for arresting the growth of liver tumors open to question.

Significantly, results of studies reported here show that the rabbit VX2 tumor exhibits glucose metabolic properties highly similar to rapidly growing hepatomas. Specifically, the VX2 tumor is shown to exhibit the high glycolytic/high hexokinase phenotype (Fig. 2A–C), and the latter enzyme is shown to be markedly elevated (Fig. 2C), to localize predominantly in the mitochondrial fraction (Fig. 2D), and to exhibit a low Km for glucose (Fig. 2E,F). As emphasized in earlier work, this phenotype, which is characteristic of many rapidly growing hepatomas [7–13], provides a mechanism for the rapid production of glucose-6-phosphate, which not only serves to fuel the glycolytic reaction pathway for the production of ATP, but serves also as a precursor for many biosynthetic processes essential for rapid tumor growth [16–18]. Noteworthy also is that the findings reported here are consistent with the earlier study of Oya et al. [19]showing that the uptake of a 2DOG analog and the activities of several key glycolytic enzymes are elevated in the VX2 tumor relative to liver tissue.

The above studies, suggested that the VX2 tumor growing in rabbit liver could be used not only to study the delivery of anticancer agents to the site of the tumor as previously [3,4], but could be used also to test the effectiveness of tumor glycolytic inhibitors as potential anti-hepatoma agents. For this reason, we carried out a limited screen for such inhibitors. Of the nine compounds tested in this study, two were found to be effective inhibitors of both glycolysis and the growth of hepatoma cells in culture. One of these, i.e., 2DOG, is a known glycolytic inhibitor which is not further metabolized when phophorylated by hexokinase [15], while the other, 3BrPA, was shown in this study to be both an inhibitor of tumor glycolysis (Fig. 3B) acting directly at the level of the hexokinase step (Fig. 3C), and also an inhibitor of the mitochondrial ATP synthetic machinery (Fig. 3D). Of these two inhibitors, 3BrPA was found also to completely kill hepatoma cells in tissue culture following a 12 h incubation period (Fig. 4C), whereas 2DOG, although effective in killing 80% of the cells in the same period of time (Fig. 4B), required a much higher concentration. Future studies will focus on

delivering these and related agents via chemoembolization procedures to the VX2 tumor growing in the rabbit liver, and assessing their capacity to inhibit tumor growth.

Acknowledgements

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