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The Cause of Death in Non-metastasizing Sarcoma-bearing Mice*

A Study with Relevance for Tumor Treatment Experiments in Mice

GÖSTA SVANINGER, JOHAN GELIN and KENT LUNDHOLM†

Departments of Surgery, Institution I & II, University of Gothenburg, Sahlgrenska Hospital, Gothenburg, Sweden

Abstract—*Adult sarcoma-bearing mice were used to demonstrate whether hypoglycemia was the immediate cause of death in experimental animals with rapidly growing tumors without metastases. This kind of tumor model is representative of the majority of animal models used in experimental cancer research.*

Tumor-bearing animals died with severe hypoglycemia under all experimental conditions, while pair-killed controls were normoglycemic. Anorexia prevented tumor-bearing animals from attenuating the hypoglycemia by drinking glucose-containing water while completely starved control animals survived more than 14 days with glucose-containing water as the only energy source. Adrenalectomy shortened survival in tumor-bearing animals, but survival of adrenalectomized tumor-bearing animals could be normalized by daily injections of pharmacologic doses of hydrocortisone (25 mg/25 g body wt/day) but not by physiologic replacement (20 μg/25 g body wt/day). Injections of pharmacologic doses of hydrocortisone did not influence on survival or body composition in tumor-bearing animals with intact adrenals. Glucagon was without effect on either survival, tumor growth or body composition.

Based on the results in this study and in our previous reports we conclude that hypoglycemia is the cause of death in the majority of murine tumor models. This hypoglycemic theory is important, since any treatment modality in animal experiments that influences glucose metabolism in the host may indirectly change tumor growth and may thus be misinterpreted as a direct tumor effect.

INTRODUCTION

MALIGNANT TUMORS may kill their host either by invasive growth, metastatic spread, metabolic exhaustion or by a combination of these events [1–3]. In experimental cancer, it is a general finding that tumor-bearing animals may die from cachexia without signs of metastasis when the tumor comprises 10–25% of the body weight [4–7]. Therefore, in most experimental systems, death seems to be of metabolic origin, although this has never been directly confirmed. The limiting factor for survival in experimental cancer is an important consideration, since a large number of models with unknown cause of death are used world-wide for evaluation of tumor treatment efficacy.

In this study we report that sarcoma-bearing mice die from hypoglycemia; a tumor model which has many metabolic similarities with clinical cancer [8] and which is probably representative of a large number of murine experimental systems.

MATERIALS AND METHODS

Tumor and experimental model

Mice of both sexes of the inbred strain C57BL/6J were used. In all experiments tumor-bearing and control animals were sex-matched. A transplantable 3-methylcholanthrene-induced sarcoma (MCG 101) was used. This tumor does not metastasize and has a growth curve which has been reported elsewhere [9]. The tumor tissue was implanted subcutaneously in the flanks of the animals and control animals were sham-implanted. The animals were housed in a room with a 12 h light/dark cycle. During several years at our laboratory this tumor model has been used in evaluating the mechanism(s) behind tumor-induced cachexia [4,8–16]. The

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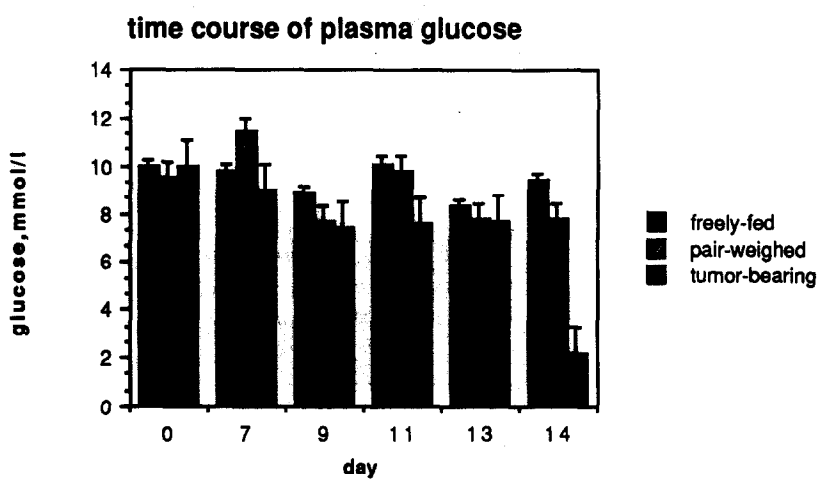
†Correspondence to: Kent Lundholm, Department of Surgery, Sahlgrenska Hospital, S-413 45 Gothenburg, Sweden.

results presented in Tables 1–3 and Figs. 1 and 2 were obtained from separate experiments with groups of randomly allocated animals. Comparison between animal groups should therefore primarily be made within experiments. Variation in survival may for example occur among tumor-bearing animal groups being transplanted at different occasions with tumor tissue material originating from various tumor batches.

Adrenalectomy

Tumor-bearing animals with and without adrenals were used as study animals. Appropriate sham-operated and sham-transplanted control mice were used in all experiments. Adrenalectomy was performed through dorsal incisions. The animals had free access to saline in water (0.9% NaCl). Experiments started 14 days following adrenalectomy in order to allow full recovery from the operation.

In some experiments animals were given extra glucose in drinking water *ad libitum* (300 mg/ml drinking water). The animals were then supplied with fresh glucose in new bottles daily. In such experiments all animals had free access to glucose in one bottle and tap-water in another. The control animals had free access to tap-water in two bottles. All bottles were of the same size and shape. In additional experiments, tumor-bearing animals with intact adrenals were injected with glucagon (0.8 μg/25 g body wt/day). This dose was divided on two injections. The glucagon injection started on day 9 following tumor implantation. The glucagon dose corresponded to three times the recommended dose per body weight to treat insulin coma in



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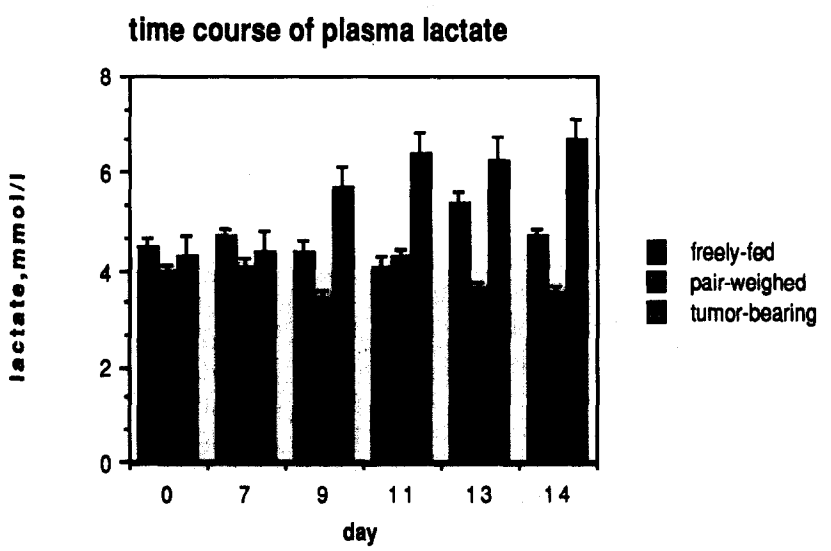

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Figs. 1 and 2. Tumor-bearing animals were killed on different days following tumor implantation and control animals (pair-weighed, freely fed) were pair-killed at the same time. Plasma glucose and lactate were measured. Mean values ± S.E.; five animals in each group at each time point.

Table 1. *Hypoglycemia and survival in tumor-bearing (TB) and control animals with and without adrenals*

	Spontaneous death		Pair-killed		
	TB with adrenals [6]	TB without adrenals [9]	TB with adrenals [9]	Controls with adrenals [6]	Controls without adrenals [9]
Initial body weight (g)	25.6 ± 0.5	25.2 ± 0.3	25.3 ± 0.3	24.9 ± 0.42	25.1 ± 0.3
Carcass dry weight (g)	5.23 ± 0.09*†	5.69 ± 0.08§	5.49 ± 0.11	7.6 ± 0.15	7.78 ± 0.11
Tumor dry weight (g)	0.93 ± 0.03*	0.40 ± 0.06	0.55 ± 0.10		
Tumor dry weight / Carcass dry weight	0.18 ± 0.01*	0.07 ± 0.01‡	0.10 ± 0.01		
P-Glucose close to death (mmol/l)	1.97 ± 0.21†	1.63 ± 0.18‡§	9.44 ± 0.41	9.36 ± 0.45	9.41 ± 0.42
Survival (days)					
mean	14.0 ± 0.4*	9.9 ± 0.7			
median	13.8	11.0			
range	(12.8–16.3)	(7.3–12.8)			

*$P < 0.01$ TB (spontaneous death) vs. TB without adrenals (spontaneous death).
†$P < 0.001$ TB (spontaneous death) vs. C with adrenals (pair-killed).
‡$P < 0.001$ TB without adrenals (spontaneous death) vs. TB (pair-killed).
§$P < 0.001$ TB without adrenals (spontaneous death) vs. C without adrenals (pair-killed).
Mean ± S.E.M. Number of animals within parentheses. Animals were pair-killed in relation to spontaneously dying TB without adrenals. Carcass dry weight represents final weight.

Table 2. *Glucose supplementation and survival in tumor-bearing mice (TB) with and without adrenals*

	Water/saline + extra glucose *ad libitum*			Water/saline *ad libitum*		
	TB with adrenals [18]		TB without adrenals [16]	TB with adrenals [18]		TB without adrenals [12]
Initial body weight (g)	24.7 ± 0.8		25.6 ± 0.9	24.8 ± 0.7		26.1 ± 0.8
Carcass dry weight (g)	5.15 ± 0.28		5.80 ± 0.21	5.00 ± 0.24	$P < 0.01$	5.90 ± 0.17
Carcass lipid content (g)	1.00 ± 0.16		0.89 ± 0.05	0.76 ± 0.15		0.89 ± 0.05
Lipid free dry weight (g)	4.11 ± 0.16	$P < 0.01$	5.00 ± 0.19	4.12 ± 0.13	$P < 0.01$	5.07 ± 0.16
Tumor dry weight (g)	1.01 ± 0.09	$P < 0.001$	0.41 ± 0.06	1.11 ± 0.13	$P < 0.001$	0.40 ± 0.06
Tumor dry weight / Carcass dry weight	0.20 ± 0.02	$P < 0.001$	0.07 ± 0.01	0.23 ± 0.03	$P < 0.001$	0.07 ± 0.01
P-Glucose, close to death (mmol/l)	3.65 ± 0.65	$P < 0.001$	0.74 ± 0.25	3.25 ± 0.45	$P < 0.001$	1.48 ± 0.53
Survival (days)						
mean	12.9 ± 0.4	$P < 0.001$	9.3 ± 0.4	14.0 ± 0.5	$P < 0.001$	9.0 ± 0.2
median	13.0	$P < 0.001$	9.5	14.0	$P < 0.001$	9.3
range	(10.8–15.3)		(7.0–10.8)	(10.7–16.3)		(7.8–9.5)

One group received extra glucose *ad libitum* in drinking water (300 mg/ml). Mean ± S.E.M. Number of animals within parentheses. Carcass dry weight represents final weight.

Table 3. Body composition and survival in tumor-bearing mice (TB) with and without adrenals. Animals were substituted with either hydrocortisone (20 μg/day, 2.5 mg/day) or saline

	TB without adrenals					TB with adrenals	
	NaCl		Hydrocortisone (20 μg/day)		Hydrocortisone (2.5 mg/day)	NaCl	Hydrocortisone (2.5 mg/day)
Initial body weight (g)	24.4 ± 0.3		24.4 ± 0.3		25.1 ± 0.5	26.9 ± 0.4	27.3 ± 0.5
Carcass dry weight (g)	4.73 ± 0.19		4.75 ± 0.13		4.48 ± 0.09	5.01 ± 0.09	5.26 ± 0.12
Carcass lipid content (g)	0.75 ± 0.03	$P < 0.05$	0.66 ± 0.02		0.65 ± 0.01	0.73 ± 0.03	0.72 ± 0.02
Carcass lipid free dry weight (g)	4.02 ± 0.18		4.07 ± 0.12		3.82 ± 0.08	4.26 ± 0.08	4.53 ± 0.11
Tumor dry weight (g)	0.52 ± 0.12		0.41 ± 0.03	$P < 0.001$	1.05 ± 0.10	1.52 ± 0.10	1.51 ± 0.12
Tumor dry weight / Carcass dry weight	0.11 ± 0.03		0.09 ± 0.01	$P < 0.001$	0.23 ± 0.02	0.30 ± 0.02	0.29 ± 0.02
Survival							
mean	11.1 ± 1.0		10.2 ± 0.3		17.6 ± 0.9	17.0 ± 0.9	18.0 ± 1.1
median	10.0		10.4	$P < 0.001$	17.4	17.3	17.3
range	(7.1–13.6)		(8.3–10.7)		(14.0–24.0)	(11.2–21.7)	(13.4–24.3)

Mean ± S.E.M. Eleven animals in each group.

humans. Sham injections to tumor-bearing controls consisted of saline.

Determination of body composition

Tumor dry weight, carcass dry weight, carcass lipid content and carcass lipid-free dry weight were determined as described elsewhere [4, 16].

Survival was studied in some experiments. When the animals showed pre-mortal signs such as shaggy fur, reduced interest in eating and depressed spontaneous movements, they were evaluated every 4th hour day and night. In this way the time point of death was measured with an accuracy of ±2 h. The same principle was used when pre-mortal plasma glucose concentration was measured. When the animals became extremely weak they were lightly anaesthesized with Pentobarbital in order to decrease the stress and blood was collected by cardiac puncture. Plasma glucose and lactate were measured by a kit from Boehringer-Mannheim, F.R.G.

Statistics

Time course changes and differences among animal groups were tested by analysis of variance (ANOVA). Values are presented as mean ± S.E. Differences between specific groups were compared using a multiple range test. $P < 0.05$ was regarded as the level of statistical significance. Survival time is presented as median and range, in addition. Median values were tested by Fischer's exact test.

EXPERIMENTAL GROUPS AND RESULTS

Experiment 1: hypoglycemia and survival (Table 1)

Figures 1 and 2 show the time course in plasma glucose and lactate in tumor-bearing mice compared to pair-weighed and freely fed controls. Plasma glucose decreased rapidly beyond 13 days following tumor implantation; otherwise no significant alteration was found among the groups. Plasma lactate was increased significantly from day 9 and on in tumor-bearing animals as shown in Fig. 2. Immediately before spontaneous death, plasma lactate decreased to levels between 2–3 mmol/l in tumor-bearing animals with or without adrenals.

Plasma glucose was measured close to death. Survival following tumor implantation and body composition at the time of death was also determined. Adrenalectomized tumor-bearing animals were used as the study group. Three groups of controls were used; tumor-bearing animals and non-tumor controls with intact adrenals and non-tumor controls without adrenals. For each adrenalectomized tumor-bearing animal dying spontaneously one animal from each control group was pair-killed.

Tumor-bearing mice with intact adrenals generally died around 2 weeks following tumor implan-

tation in accordance with our earlier results [4]. The tumor dry weight was then 0.93 ± 0.03 g. Adrenalectomized tumor-bearing mice had a median survival of 11 days following adrenalectomy. The tumor mass was then 0.40 ± 0.06 g, or 43% of the tumor mass in spontaneously dying tumor-bearing animals with intact adrenals. These animals had a median survival of 14 days. In pair-killed tumor-bearing animals with intact adrenals the tumor weight was of the same magnitude (0.55 ± 0.10 g).

Spontaneously dying animals with and without adrenals developed profound hypoglycemia close to death. All animals, that were pair-killed with adrenalectomized tumor-bearing animals, were normoglycemic. Also tumor-bearing animals with a tumor burden (0.55 ± 0.10 g) comparable to that of adrenalectomized animals (0.40 ± 0.06) were normoglycemic.

Experiment 2: *glucose substitution and survival* (*Table* 2)

Initial experiments were performed where survival and time course in body weight were studied in normal animals supplied with glucose in the drinking water as the only energy source. In these experiments, control animals were given water *ad libitum* only. These experiments were carried out in order to see how non-tumor animals survived and were affected when they have to choose between pure water and glucose-containing water in a starving situation.

In another set of experiments plasma glucose was measured close to spontaneous death in tumor-bearing animals with and without adrenals. Survival and body composition were also determined. Half of the animals in each group were given extra glucose in water *ad libitum* as described above. All these animals had also free access to standard food in pellets. These experiments were carried out to see whether the presence of glucose could overcome anorexia compared to ordinary food pellets in a condition with severe hypoglycemia. The complete starving control animals with intact adrenals and with only water supply had a survival time of 72 ± 3 h. The corresponding group of animals maintained on glucose in the drinking water as the only energy source lived, however, for more than 14 days. In such animals body weight declined from 24.5 ± 0.6 g to 16.0 ± 0.6 g during 14 days.

Tumor-bearing animals with free access to glucose in the drinking water seemed to prefer glucose during the initial phase of the tumor progression. From day 7, corresponding to the onset of anorexia in tumor-bearing animals [4] all animals spontaneously depressed their fluid intake to the same level. Tumor-bearing animals on extra glucose no longer preferred glucose-containing water. However, they did not completely avoid the glucose bottle. The average daily intake of glucose per animal was 1.06 g during days 1–7 and 0.27 g during days 8–12. The total fluid intake in this group was 4.4 ml/day (days 1–7) and 2.3 ml/day (days 8–12). The fluid intake in the other group was 3.0 ml/day (days 1–7) and 2.4 ml/day (days 8–12).

The supply of glucose-containing water had no impact on survival, body composition or tumor growth irrespective of whether tumor-bearing animals had intact adrenals or not. Even these animals displayed significantly depressed plasma glucose levels close to death indicating that both glucose production and glucose intake failed.

Experiments 3 *and* 4: *hydrocortisone and glucogen supplementation and survival* (*Table* 3)

Survival and body composition following tumor implantation were measured in adrenalectomized tumor-bearing animals injected with hydrocortisone (20 μg and 2.5 mg/day) or saline. Tumor-bearing animals with intact adrenals injected with hydrocortisone (2.5 mg/day) or saline were used as controls (Table 3). These experiments were carried out to see whether exogenous glucocorticoids could normalize survival in adrenalectomized tumor-bearing animals compared with tumor-bearing mice with intact adrenals.

Substitution with physiologic doses of hydrocortisone had no impact on survival or body composition compared to saline in adrenalectomized tumor-bearing animals. However, adrenalectomized tumor-bearing animals given substitution with pharmacologic doses of hydrocortisone had a 60–65% increased median survival to 17.4 days (Table 3). Adrenalectomized tumor-bearing animals given physiologic substitution doses of hydrocortisone or saline died with a tumor burden corresponding to 9–11% of the carcass weight. The dry tumor weight was 23% of the dry carcass weight in animals given pharmacologic doses of hydrocortisone (Table 3).

Hydrocortisone in pharmacologic doses to tumor-bearing mice with intact adrenals had no impact at all on survival or body composition compared to sham-injections with saline.

Tumor-bearing animals with intact adrenals were given exogenous glucagon to evaluate whether glucagon beside hydrocortisone could prolong survival. Injections of glucagon had no impact at all on either survival or body composition (results not shown).

DISCUSSION

We have recently evaluated whether gluconeogenetic precursors or lipid stores are limiting factors for survival of sarcoma-bearing mice [16]. In that study we concluded that the amount of endogenous substrates is not limiting for the survival, since complete starvation of terminal tumor-bearing mice

could further mobilize significant amounts of both protein and lipids at the time when the animals were expected to die spontaneously due to the tumor and anorexia. Therefore, we speculated whether a circulatory collapse was the immediate cause of death in tumor-bearing mice. Perhaps an insufficient blood volume could not keep up with the expanding vascular compartment in the exponentially growing tumor. More recent experiments in our laboratory have, however, also pointed to the possibility that additional factors may be involved. The present study demonstrates that sarcoma-bearing mice are dying with severe hypoglycemia, which is thus the likely immediate cause of death. The results also show that adrenalectomy reduces further the survival time by one third in tumor-bearing mice. This difference in survival corresponds to 100% difference in tumor burden (Tables 1 and 3).

Alterations in glucose metabolism have often been reported in association with cancer disease both in experimental animals and humans [17–26], although hypoglycemia is not a frequent finding in humans with cancer. On the contrary, patients with cancer often show a tendency to hyperglycemia in combination with insulin resistance [27, 28]. Experimental cancer is almost invariably associated with hypoglycemia in advanced stages [17–19], also in combination with insulin resistance [13]. We have earlier suggested that this unusual combination in tumor-bearing animals may be due to a limited capacity for gluconeogenesis in combination with a large tumor burden [4].

Shapot *et al.* [17] studied different tumor systems and reported that gluconeogenesis was shown to counterbalance the tendency towards hypoglycemia that was seen along tumor progression. However, a significant negative correlation has been reported between tumor burden and serum glucose levels in others work [18, 19]. When the tumor to body weight ratio exceeded 10–15% a tendency to hypoglycemia was seen [19]. It has earlier been shown that tumors have high glycolytic capacity and predominantly utilizes glucose as fuel [29, 30], although non-carbohydrates may also be used in the absence of glucose [31, 32]. In the present study we found a trend to decreased glucose from day 9 to 13 with rapid decrease around 14 days following tumor implantation, which was the time point for median survival in two of three experiments.

Many tumors are associated with pronounced anorexia despite hypoglycemic reactions that forced our control animals to choose glucose-containing water in order to survive starvation. However, tumor-bearing animals with available extra glucose no longer preferred glucose during the anorectic period. This suggests that tumor-bearing mice may have an altered sensation of taste during the anorectic period or that the set point for hypoglycemia is altered, since even severe hypoglycemia could not overcome the anorexia. An additional factor may be that the thirst sensation is also attenuated in tumor-bearing animals, since they decreased their fluid intake following onset of the anorexia. There is not an absolute or relative lack of water in the sarcoma-bearing mice [4], since these animals contain increased amounts of water which may be one factor counteracting thirst sensation. Such an effect may have prevented the animals from an adequate glucose intake to counteract the hypoglycemia. If so, depression of the thirst sensation may even be stronger than the sensation of hypoglycemia.

In previous studies we have demonstrated that sarcoma-bearing mice have increased gluconeogenesis from both alanine, lactate and glycerol which is combined with elevated glucose turnover [20]. The liver tissue capacity for neosynthesis of glucose was, however, found to be largest for glycerol as the precursor [4], in contrast to findings in man who rather converts lactate to glucose at higher rates [23, 33]. In tumor-bearing animals, the liver tissue capacity was higher for both glycerol and alanine as the precursor at saturated substrate levels. The lactate conversion rate to glucose was doubled in the range of physiologic substrate levels [4]. Seen together, our previous and present results support that the lactate concentration increase in tumor-bearing mice (Fig. 2) is not due to either impaired enzyme capacity or depressed flux through hepatic pathways for gluconeogenesis. Rather, it seems as if the total capacity for glucose production becomes limited in combination with the lack of upregulation of carbohydrate intake. No indications are available that hepatic enzyme activities for glucose homeostasis are downregulated in our tumor-bearing animals [34].

Sarcoma-bearing mice also have increased adrenocortical activity confirmed as an increased excretion of corticosteroids in urine [15]. Hypoglycemia in such animals is therefore not explained by an absolute adrenal insufficiency, and the experiments with hydrocortisone supplementation to adrenal intact tumor-bearing mice rule out relative adrenal cortical insufficiency as the explanation. The extirpation of the adrenals markedly reduced survival probably due to the lack of corticosteroids, adrenaline or the combination of both. Tumor-bearing adrenalectomized animals had an average survival time of around 9–10 days following tumor implantation with a tumor weight of only 8–10% of the carcass weight. In such animals, replacement with physiologic doses of hydrocortisone did not prolong survival, but pharmacologic doses of hydrocortisone prolonged survival by more than 50% and the animals died with 100% larger tumors.

Therefore, it is obvious that the effect of cortisone in prolonging survival was not due to tumor growth inhibition. An induction of tumor cell differentiation leading to a lower glucose consumption in the tumor with secondary prolonged survival is unlikely, since the same large doses of cortisone to sham-operated tumor-bearing mice with adrenals did not show any tumor inhibitory effect. Even such animals displayed profound hypoglycemia close to death. We therefore conclude that the glucose demand from the tumor under all experimental conditions exceeds the gluconeogenetic capacity of the liver when the tumor has reached a certain mass. It is thus possible that a limiting step for survival in tumor-bearing animals may be their total hepatic enzymatic capacity for synthesis of glucose in relation to the tumor glucose consumption, which may be around 0.34 g glucose/day between 9 and 11 days following tumor implantation. This figure is derived from the elevated glucose turnover in tumor-bearing mice compared to freely fed controls [20]. Since pharmacologic doses of hydrocortisone to tumor-bearing animals with intact adrenals did not increase survival, it may be that the gluconeogenetic capacity already was maximally induced by endogenous glucocorticoids and other factors in these animals as was found for the activity of the hepatic enzyme tyrosine aminotransferase [15, 35].

Additional circumstantial evidence of a dependency for survival on the enzymatic capacity for gluconeogenesis and glycemia in tumor-bearing animals has been reported by others [18, 36, 37]. It has been demonstrated that hypoglycemia rapidly develops when the flux through the gluconeogenetic pathway is inhibited by 3-mercaptopicolinate [37, 38]. Injections of glucagon had no effect on survival in our tumor-bearing animals. This was, however, not expected to occur over several days, since tumor-bearing animals have low hepatic content of glycogen [11, 17, 31].

We conclude that hypoglycemia is the immediate cause of death in adult sarcoma-bearing mice. We also find it likely that hypoglycemia is the mechanism behind death in a majority of murine tumor models, particularly with solid tumors of considerable mass. This conclusion is important since experiments evaluating tumor treatment efficacy must take this fact into account. It is well recognized that tumor growth in experimental animals is highly dependent on the substrate supply [39–41]. Thus, by decreasing glucose flux or the flux of gluconeogenetic precursors, it is possible to indirectly attenuate tumor growth rates (experiments in progress). Such effects could then be misinterpreted as a direct tumor effect. This fact has not been accounted for in the vast literature of tumor treatment modalities where cytostatic and cytocidal drugs may well interact with the intermediary metabolism of glucose, fat and amino acids in the host as well as food intake (cytokines).

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