Pyruvate Inhibits Growth of Mammary Adenocarcinoma 13762 in Rats

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ABSTRACT

The growth of implanted mammary adenocarcinoma 13762 was measured in rats consuming a liquid diet (35% fat, 18% protein, 47% carbohydrate) supplemented with pyruvate (37.3 g/liter; n = 13) or maltose-dextrin (placebo; n = 13) for 21 days. Mean tumor diameter, measured on day 11, 14, 18, and 21 subsequent to tumor implantation, was 41, 32, 21, and 19% smaller in the pyruvate group (P < 0.05). When euthanized, tumor weight was also smaller in the pyruvate group: pyruvate = 15.0 ± 2.3 (SEM) g; placebo = 24.9 ± 3.2 g, P < 0.05. Visual inspection of organs suggested decreased lung metastases with pyruvate feeding (P < 0.05). Upon microscopic evaluation of organs, hepatic tumor was found only in the placebo group. We conclude that pyruvate inhibits implanted tumor growth in rats.

INTRODUCTION

We have previously shown that partial isoenzymatic substitution of dietary three-carbon compound energy (pyruvate) for six-carbon compound energy (glucose) will inhibit body fat deposition in rats (1), swine (2), and humans (3, 4), normalize plasma glycemia and hepatic gluconeogenesis in non-insulin-dependent diabetics (5), enhance arm and leg muscle glucose uptake and endurance capacity in normal males (6, 7), and enhance cardiac efficiency in dogs (8). The mechanisms by which pyruvate induces these varied physiologic responses are not established. Although it is doubtful that one metabolic change results in all of these responses, it would not be unreasonable to expect some common intermediate metabolic change subsequent to pyruvate consumption.

Salahudeen et al. (9) have recently shown that pyruvate can inhibit hydrogen peroxide-induced renal injury, possibly by reducing free radical production. Indeed, preliminary evidence from our laboratory suggests that pyruvate might function as a free radical inhibitor. Functioning as a free radical inhibitor, pyruvate might inhibit malignant growth. The present study was performed to evaluate the effect of pyruvate on growth of mammary adenocarcinoma in the rat model.

MATERIALS AND METHODS

Animal Model. Twenty-six (pyruvate, n = 13; placebo, n = 13) 140- to 150-g female Fischer 344 rats were pair fed for 35 days a liquid diet (35% fat, 18% protein, 47% carbohydrate, 0.0043 MJ/ml, No. 710127; DYETS, Inc., Bethlehem, PA) isoenergetically supplemented with 37.3 g of pyruvate (22.3 g of calcium pyruvate, 15.0 g of sodium pyruvate) or an isoenenergetic amount of maltose-dextrin. The electrolyte composition of the two diets was made equal by addition of calcium carbonate or sodium citrate to the placebo diet. Energy intake during this 35 days was 9.8 ± 0.2 (SEM) MJ in the pyruvate group and 10.1 ± 0.1 MJ in the placebo group (P = NS). Total pyruvate intake was 73 g. At this point, tumor cells were implanted on the back of the animals, and the growth in rats.

Tumor. The 13762 mammary adenocarcinoma, a tumor line maintained in the solid form by the National Surgical Adjuvant Breast Cancer Laboratory at the University of Pittsburgh, was used as the tumor model because of its reliable growth in the Fischer rat (10, 11). Fresh tumor, obtained immediately after sacrificing a subcutaneous tumor-bearing animal, was trimmed of necrotic debris, minced, and strained through a sieve to obtain a pure form of the tumor. Tissue culture medium 199 with 1 g of penicillin/liter and 80 g of streptomycin/liter was used to aid in staining the tumor cells and in preparation of the tumor brei. Cells were quantitated with a homocytometer, and viability was assessed by trypan blue exclusion. After anesthesia (0.3 ml of a 1:9 Numbtalsaline solution), the backs of the animals were shaved, and approximately 7 million viable cells (0.5 ml of tumor suspension) were injected intradermally in the lower midline between the L2-L3 spine. Because tumor growth was expected 10 days after inoculation (11), tumor length, width, and height were measured on day 11, 14, 18, and 21 after inoculation. Mean tumor diameter was calculated as

\[(\text{length} \times \text{width} \times \text{height})^{1/3}\]

On day 21 after tumor implantation, animals were euthanized. Tumor was excised and groin lymph nodes, liver, and lungs were visually evaluated for gross abnormalities and sections were sent for hematoxylin and eosin preparation.

Gross Inspection. An arbitrary scoring system was used for gross evaluation of the tumor and organ specimens. Tumor was evaluated for necrosis and presence of fluid. Necrosis was scored as absent = 0, mild = 1, and extensive = 2. Fluid was scored as absent = 0, trace = 1, and large amount = 2. Groin lymph nodes were evaluated for gross architectural changes; liver and lungs were evaluated for metastases. Lymph nodes were scored as normal to slightly enlarged = 0, large and distorted = 1, or completely distorted with extensive adhesions = 2. Liver and lung were scored as no nodules = 0, 1 to 2 nodules = 1, or >2 nodules = 2.

Microscopic Evaluation. Sections of the right groin lymph node, right lobe of the liver, and a 0.5-cm² area of lung tissue with an obvious metastatic nodule were stained with hematoxylin and eosin. Lymph nodes were evaluated for presence or absence of tumor and necrosis. The liver was evaluated for presence or absence of tumor and for cellular necrosis. Lung sections were scored according to the number of metastatic lesions per 0.5 mm² section: 0, 1 to 10, >10. Metastatic lesions were scored according to the number of mitotic figures per high power field. Metastatic lesions were divided into groups with diameter less than or greater than 100 µm and mean diameter was recorded. Morphologic characteristics of metastatic lesions and surrounding lung tissue architecture were also recorded.

DNA Analysis. In paraffin sections of tumor, single- and double-strand DNA breaks were estimated with an in situ end-labeling technique (12). Biotinylated nucleotides were incorporated at breaks after addition of DNA polymerase (12). Specific for our technique was a pepsin digestion of 25 min duration and DNA polymerase I concentration of 400 units/ml. Using a 100 X Olympus oil-immersion lens, the number of dianmonobenzidine-positive nuclei or bodies in 10 fields was counted in solid nonnecrotic areas of tumor. Because a hallmark of apoptosis is cleavage of DNA into oligonucleosome fragments, the above score was also used as an estimate of apoptosis (12). Touch imprints were made from sections of the tumor. Samples were fixed in 10% neutral-buffered formalin for 30 min, rinsed in distilled water, and air dried. Slides were then stained for DNA with the Feulgen method (13). Two hundred nuclei were analyzed for each specimen (S phase or proliferation phase, G0-M phase or premitosis phase) using the Cell Analysis Systems 200 Image Cytometer (Elmhurst, III) utilizing a QDA software program. Normal DNA index for this instrument was 0.92 to 1.09. DNA index for all of our samples were within this range.

Statistics. Tumor growth differences between groups were analyzed with a univariate analysis of variance with repeated measures. For categorical vari-
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ables, differences were evaluated with the Fisher’s Exact test (14). All statistical assumptions were met. Differences were considered significant with P < 0.05.

This study was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

RESULTS

Mean tumor diameter is presented in Fig. 1. With pyruvate feeding, mean diameter was 41, 32, 21, and 19% smaller on day 11, 14, 18, and 21, respectively (P < 0.05). On day 21, tumor weight was 10 g less (40%) in the pyruvate group compared to the placebo group (P < 0.05; Fig. 2).

Gross appearance characteristics of the tumor, lymph nodes, liver, and lungs are presented in Table 1. In the pyruvate group the tumor had less fluid present, with a more solid appearance (P < 0.05). By visual inspection, lung metastases were decreased with pyruvate feeding (P < 0.05).

Microscopic evaluation of sections of the tumor revealed a moderate to poorly differentiated adenocarcinoma with extensive areas of necrosis in both groups of rats. Microscopic evaluations of lymph nodes, liver, and lung are summarized in Table 2. Lymph node tumor and necrosis tended to be greater in the pyruvate group, but differences were not significant. Hepatocyte necrosis (P < 0.05) and metastatic tumor (P = NS) were found only in the placebo group of animals. Mean diameter of lung metastases with diameter <50 μm was similar in both groups of animals (pyruvate = 36 ± 13 μm; placebo = 39 ± 7 μm; P = NS), but the mean diameter of metastases with diameter >50 μm was decreased in the pyruvate group (pyruvate = 225 ± 49 μm; placebo = 642 ± 108 μm; P < 0.05). Morphology and number of mitotic figures of lung metastases, and morphology of surrounding lung tissue, were similar in both groups of animals (data not shown).

The number of DNA breaks were decreased by 40% in the pyruvate group (Table 3; P < 0.05). DNA kinetic analysis of the tumor revealed the S phase and G2M phase values to be similar in both groups of animals (Table 3).

Diet consumption and weight changes are presented in Table 4. Energy intake decreased by 20% within 1 week of tumor implantation, and by 40% after 3 weeks. Although total weight loss subsequent to tumor implantation was 14 g in both groups of animals, weight loss during the first week of feeding was decreased by 64% in the pyruvate group compared to the placebo group (P < 0.05).

DISCUSSION

In this evaluation of the growth of implanted mammary adenocarcinoma in the rat, oral consumption of pyruvate resulted in decreased tumor size, tumor weight, and metastases. The effect of pyruvate on tumor growth was probably within the first 10 days of implantation, because the rate of tumor growth was similar between our two study groups from day 10 to day 21 (slope of curves, Fig. 1). Two other findings suggest early effects of pyruvate in our tumor model. Tumor DNA kinetic analysis, done on the day of euthanasia (day 21), revealed no differential effect of pyruvate. This finding might not be unexpected, because the rate of tumor growth at this time point was similar in both groups of animals. Also, weight loss during the first 7 days of tumor growth was decreased in the pyruvate group, despite identical energy intake to that of the placebo group.

The question arises, however, whether the effect of pyruvate on tumor growth would have been different had energy intake (Table 3), and therefore pyruvate intake, remained constant throughout the 21 days subsequent to tumor implantation. But, despite decreasing pyruvate intake during the final 14 days of tumor growth, an effect of pyruvate on tumor growth persisted. Whether the effect of pyruvate on tumor growth is secondary to feeding during the first 10 days (or 21 days) posttumor implant and/or secondary to the 35 days of feeding prior to tumor implantation, cannot be established by the design of the present study.

The effect of pyruvate on tumor metastasis cannot be firmly established, as a constant intake of pyruvate could not be maintained because of decreased oral intake in our animals with time after tumor implantation. Nevertheless, pyruvate did seem to inhibit tumor metastasis. Visual inspection of intact organs for metastases can be markedly insensitive, and we utilized an arbitrary scoring system in this process. However, within these bounds, lung metastases were decreased in the pyruvate group. Microscopic analysis of organs indi-

![Fig. 1. Mean tumor diameter (L x H x W)^{1/3} (cm^{1/3}, mean ± SEM, n = 13 in each group) on day 11, 14, 18, and 21 after tumor implantation (P < 0.05 at each time point).](cancerres.aacrjournals.org)
cated that hepatic metastases and necrosis (right lobe) were completely absent in the pyruvate group, which was not the case in the placebo group of animals.

We selectively chose lung tissue that contained a tumor nodule (by visual inspection) for microscopic analysis to evaluate the morphologic characteristics of metastatic lesions between our two groups. Our findings of a similar number of microscopic lung metastases in both groups of animals, therefore, is not unexpected. On initial evaluation, lung metastatic lesions tended to group into those that were relatively small and those that were relatively large. Therefore, to evaluate differential effects of pyruvate on metastatic lesion size, we grouped the lesions into those with diameter less than or greater than 100 µm. Mean diameter of metastatic lesions with diameter >100 µm was decreased by 65% in the pyruvate group, but mean diameter of the smaller lesions was similar in both groups of animals. These data would suggest that pyruvate also inhibited metastatic lesion growth, as it inhibited primary tumor growth. The method of estimation of tumor metastases in our animal model can be subject to question. However, the combination of our macroscopic and microscopic evaluations does suggest that pyruvate might produce beneficial inhibitory effects on tumor metastases.

Free radicals might be involved in the induction and/or progression of malignancy (15), and known free radical scavengers have been implicated in cancer prevention (16–18). In agreement with others (9, 19), preliminary evidence in our laboratory suggests that pyruvate will scavenge free radicals. Most importantly, however, pyruvate might inhibit genesis of free radicals by inhibiting peroxisomal proliferation. Peroxisomal proliferation has been associated with increased hydroxyl radical production (20), the production of hepatic carcinoma (21), and has been observed in mammary adenocarcinomas (22). It has been suggested that increased lipid might stimulate peroxisomal proliferation (23). Pyruvate inhibits body lipid deposition (1–4). With the above, and the results of the present study, we can conjecture as to the mechanism whereas pyruvate inhibits malignancy growth. Pyruvate, by functioning as a free radical inhibitor and scavenger, might inhibit tumor growth by reducing DNA injury induced by oxidative stress. Our data indicate that DNA breaks were reduced by 40% by pyruvate. Pyruvate, by inhibiting lipid deposition, inhibits peroxisomal proliferation and free radical production. By oxidative decarboxylation of hydrogen peroxide (9), pyruvate scavenges free radicals. It would seem that pyruvate does not enhance programmed cell death, or apoptosis, as other antitumor agents (24). Cleavage of DNA is a hallmark of apoptosis (12). Our morphologic assessment of labeled DNA breaks can be used as an indirect estimate of apoptosis, and it would seem that programmed cell death is not increased by pyruvate.

Both groups of animals lost 14 g of weight during the 21 days posttumor implant. However, the tumor mass, on the average, was 10 g greater in the placebo group of animals. Body mass loss, therefore, was 10 g or 40% less with pyruvate feeding. This effect of pyruvate was time variable, with the greatest effect being seen within the first 7 days posttumor implant. Whether this effect was predominantly secondary to effects on energy or tumor metabolism cannot be answered by the present study. It is entirely possible that pyruvate simply delayed tumor implantation and growth, and therefore, delayed the catabolic effects on body composition. However, we have previously shown pyruvate can differentially affect body composition, with enhancement of fat loss without nitrogen loss with hypoenergetic feeding (3, 4), and inhibition of fat gain but not nitrogen gain with hyperenergetic feeding (1, 2). This is the first study, however, that suggests pyruvate can inhibit body mass loss in a stressed state, in this case, under the catabolism of malignancy growth and metabolism. The results of the present study, in conjunction with our previous studies (1–4), seem to suggest that pyruvate might have selective effects on energy metabolism, all seeming to be dependent on the metabolic state of the host, and seeming to be beneficial to the host.

**Table 1** Gross characteristics of tumor, lymph nodes, liver, and lung

<table>
<thead>
<tr>
<th></th>
<th>Pyruvate</th>
<th>Placebo</th>
</tr>
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<tbody>
<tr>
<td>Tumor necrosis</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Tumor fluid</td>
<td>0.5 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Lymph node architecture</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Liver metastases</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lung metastases</td>
<td>1.1 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

*a* Scoring code: tumor necrosis, none = 0, mild = 1, extensive = 2; tumor fluid; none = 0, trace = 1, large amount = 2; lymph node architecture; normal to slightly enlarged node = 0, large distorted node = 1, completely distorted node, extensive adhesions = 2; lung metastases; none = 0, 1 to 2 nodules = 1, >2 nodules = 2.

*b* P < 0.05 vs. placebo.

**Table 2** Microscopic evaluation of lymph node, liver, and lung

<table>
<thead>
<tr>
<th></th>
<th>Percentage of animals with finding</th>
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<tbody>
<tr>
<td></td>
<td>Pyruvate</td>
</tr>
<tr>
<td>Lymph node tumor</td>
<td>85</td>
</tr>
<tr>
<td>Lymph node necrosis</td>
<td>77</td>
</tr>
<tr>
<td>Liver metastases</td>
<td>0</td>
</tr>
<tr>
<td>Liver cellular necrosis</td>
<td>0</td>
</tr>
<tr>
<td>Liver metastases (&lt;10)</td>
<td>23</td>
</tr>
<tr>
<td>Liver metastases (&gt;10)</td>
<td>62</td>
</tr>
<tr>
<td>Liver metastases (&gt;10)</td>
<td>15</td>
</tr>
</tbody>
</table>

*a* P < 0.05 vs. placebo.

**Table 3** DNA analysis

<table>
<thead>
<tr>
<th></th>
<th>Pyruvate</th>
<th>Placebo</th>
</tr>
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<tbody>
<tr>
<td>Breaks</td>
<td>8 ± 1</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>S phase (%)</td>
<td>26 ± 2</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>G0-M phase (%)</td>
<td>11 ± 2</td>
<td>15 ± 3</td>
</tr>
</tbody>
</table>

*a* Number of single and double-strand breaks in 10 fields of 100× oil-immersion objective.

*b* P < 0.05 vs. placebo.
which pyruvate affects tumor growth is secondary to an effect on free
been identified, to be added to the growing list which includes obesity,
tions into the time sequence of effects of pyruvate is required to
benefits on tumor metabolism. The hypothesis that the mechanism by
such as vitamin C and vitamin E, that influences malignancy metabo-
diabetes, hyperlipidemia, renal function, exercise capacity, and car-
lism and/or growth, and may be useful in understanding, preventing,
also, another area of metabolism, seemingly affected in a beneficial manner by the natural metabolite pyruvate, has
been identified, to be added to the growing list which includes obesity,
diabetes, hyperlipidemia, renal function, exercise capacity, and cardi-
metabolism, identified by ourselves and others (1-9, 19, 25, 26).
We conclude that pyruvate inhibits growth of mammary adenocar-
cinoma in the rat model.

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