Low zinc intake suppressed N-methyl-N-nitrosourea-induced mammary tumorigenesis in Sprague–Dawley rats

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Zinc has been shown to be accumulated in N-methyl-N-nitrosourea (MNU)-induced rat mammary tumors. Zinc is required for cell proliferation and tumorigenesis is characterized by dysregulation of cell proliferation. An accumulation of zinc in mammary tumors perhaps indicates a reliance on zinc to sustain tumor growth. Limiting zinc supply by means such as reduced zinc intake should negatively modulate mammary tumorigenesis. Our objective was to determine the effects of zinc status on MNU-induced mammary tumorigenesis in sexually mature female rats. Twenty-one-day-old Sprague–Dawley rats were assigned to low-zinc (3 mg zinc/kg diet) or adequate-zinc (12 mg zinc/kg diet) ad libitum or pair-fed control group (n = 25–33 rats/group). On day 50 of age, all rats were intraperitoneally injected with MNU (50 mg/kg body wt) to induce mammary tumorigenesis. Rats were further maintained on their assigned diet until 14 weeks post-MNU injection. Total food intake and overall body weight gain were lower in low-zinc rats than in adequate-zinc control rats, but were similar to adequate-zinc pair-fed control rats. Plasma zinc concentration was lower in low-zinc rats than in adequate-zinc control rats, confirming moderately low-zinc status in low-zinc rats. Tumor incidence (46 versus 84 and 80%; P < 0.05) and tumor multiplicity (0.8 versus 5.0 and 2.6 tumors/rat; P < 0.05) and tumor number (28 versus 123 and 66 tumors) were reduced in low-zinc rats compared with that in adequate-zinc control rats, respectively. Tumor latency in low-zinc and adequate-zinc pair-fed control rats was not significantly different, but was longer than in adequate-zinc control rats (P < 0.05), suggesting that reduced food intake associated with low-zinc intake prolonged tumor latency. Tumor burden and size were not affected by zinc intake. Overall, our observations showed that moderately low-zinc status suppressed MNU-induced rat mammary tumorigenesis.

Introduction

Development of hypozincemia has been shown in some breast cancer patients (1–5). The degree of hypozincemia appears to be related to the cancer stage. For example, plasma and serum zinc concentrations are lower in metastatic breast cancer patients than patients with cancer localized to the breast (6,7) and in patients with advanced breast cancer than patients with benign and early stages of breast cancer (2). These observations suggest a link between zinc and mammary tumorigenesis.

Zinc is required for cell proliferation and growth, and tumorigenesis is characterized by dysregulation of cell proliferation. Increased cell proliferation during tumorigenesis generates a need for a continuous and sufficient supply of zinc to sustain tumor growth. Indeed, limiting zinc supply, as in the case of low-dietary zinc intake, suppresses the growth of Walker 256/M1 carcinosarcoma in rats (8) and Lewis lung carcinoma in mice, and increases the survival rate of mice with leukemia (9). In contrast, increasing zinc supply by switching from a low-zinc diet to a zinc-sufficient diet stimulates the growth of Walker 256/M1 carcinosarcoma (10). Besides, zinc intakes also influence the outcomes of several other types of cancer (11). The effects of zinc on tumorigenesis are not definite and are cancer-type dependent. For example, both dietary zinc deficiency and zinc supplementation inhibit the incidence of 3-methylcholanthrene-induced skin cancer (12) and 3′-methyl-4-dimethylaminoazobenzene-induced hepatoma (13) in rats. In contrast, dietary zinc deficiency enhances the incidence of methylbenzylnitrosamine-induced esophagus cancer (14,15) and forestomach cancer (16) in rats while zinc supplementation enhances N-ethyl-N-nitrosourea-induced rat brain cancer (17). However, it is not known whether limiting zinc supply would also suppress mammary tumorigenesis.

Recently, we have observed an accumulation of zinc in N-methyl-N-nitrosourea (MNU)-induced rat mammary tumors (18). This zinc accumulation was accompanied by an increased mRNA level of ZnT-1 (a transporter involved in zinc efflux), and increased mRNA and protein levels of metallothionein (a putative zinc storage protein) in MNU-induced rat mammary tumors compared with those in normal mammary glands. Considering the role of zinc in cell proliferation, zinc accumulation in MNU-induced rat mammary tumors can be part of an integrated process to ensure sufficient zinc supply to sustain tumor growth.

The relationship between dietary zinc intake and mammary tumorigenesis is not well studied. Mills et al. (19) reported that low zinc intake inhibited mammary tumor growth in male rats implanted with mammary adenocarcinoma. It is known that mammary tumorigenesis is estrogen dependent (20,21). Implanted mammary adenocarcinoma in male rats excludes the effects of ovarian hormones on the growth of mammary tumors. Moreover, although implanted mammary tumors provide a model for studying the growth of established mammary tumors, it does not provide information on how dietary zinc intake influences the development of mammary tumors. Using MNU-induced rat mammary tumorigenesis as a model, we have observed that marginally low-zinc intake exerts some suppressive effects on MNU-induced mammary tumorigenesis in weaning rats (22). However, development of mammary tumors in weaning rats lacks the influence of hormones such
as estrogen and progesterone, which are important to mammary tumorigenesis (20,21). In addition, the feeding regimen began on the same day that mammary tumorigenesis was induced. Because it takes several weeks to develop marginal zinc-deficient status (23), these rats had not yet developed marginal zinc-deficient status at the time that the mammary tumorigenesis was induced.

The objective of this study was to determine the effects of zinc status on MNU-induced mammary tumorigenesis in sexually mature female rats. In this study, MNU-induced mammary tumorigenesis was assessed using tumor incidence, tumor number, tumor weight, tumor burden, tumor multiplicity and tumor latency. Our observations showed that a moderately low-zinc intake suppressed MNU-induced rat mammary tumorigenesis as indicated by a lower tumor incidence, tumor number and tumor multiplicity.

Materials and methods

Dietary treatments and animals

Twenty-one-day old female Sprague-Dawley rats (UBC Animal Care Centre) with an average body weight of 49 g were assigned to a low-zinc or an adequate-zinc group. Two diets, the low-zinc diet (3 mg zinc/kg diet) and adequate-zinc diet (12 mg zinc/kg diet), were formulated and prepared as reported previously (22). The low-zinc diet was designed to produce moderately low-zinc status (22,23). The adequate-zinc diet was designed to meet the recommended dietary zinc intake for rats (24). Rats in the low-zinc and adequate-zinc groups were fed their respective diet ad libitum. Low-zinc intake suppresses food intake (22). To overcome this potential confounding factor, an adequate-zinc pair-fed control group was included. Rats in the adequate-zinc pair-fed control group were given the adequate-zinc diet at the same amount consumed by the low-zinc rats, on an individual basis, during the previous 24 h. Thus, there was a total of three dietary treatment groups: the low-zinc and adequate-zinc pair-fed and adequate-zinc ad libitum control groups. On day 1 of the experiment, each adequate-zinc pair-fed control rat was given 5 g of the diet. All rats were maintained on their assigned diet for a total of 18 weeks with 25 rats/group, except the low-zinc group in which there was a total of 33 rats. The rats were individually housed in stainless steel cages in a temperature and humidity regulated room with a 12 h light/dark cycle and with free access to double deionized water. All animals were cared for in compliance with the Canadian Council of Animal Care’s Guide.

Induction of mammary tumorigenesis

All rats were fed their assigned diets for 29 days. When the rats were 50 days old, all rats were injected intraperitoneally with MNU (Sigma, Oakville, Ontario) at a dose of 50 mg/kg body wt. MNU was prepared and administered as described earlier (25). Briefly, MNU was dissolved in cold saline (4°C, 0.9% NaCl) containing 0.05% acetic acid with a pH of 4 to increase its stability. Upon preparation, the MNU solution was kept on ice and protected from light. After injection, the rats were anesthetized and mammary tumors were extracted. Beginning 6 weeks post-MNU injection, all rats were palpated weekly to monitor the development of mammary tumors. After 14 weeks post-MNU injection, the rats were anesthetized and mammary tumors were extracted and evaluated histologically. The presence of the tumors was confirmed by checking against the palpation records at necropsy. Tumors detected prior to necropsy were counted as palpable tumors. Some tumors were very small in size and discovered during necropsy. These tumors were counted as non-palpable tumors. Total tumor number was the sum of the palpable and non-palpable tumors. Each tumor was individually weighed during necropsy.

Mammary tumorigenesis was assessed by fastening tumor incidence, tumor numbers, tumor size, tumor burden, tumor multiplicity and tumor latency. Tumor incidence was the percentage of tumor-bearing rats in each dietary treatment group. Tumor incidence and tumor numbers were assessed using both palpable and total tumors. Tumor size was assessed by tumor weight. Tumor burden was calculated as the percentage of total tumor weight in each tumor-bearing rat relative to the final host body weight, which was the final body weight less the total tumor weight. Tumor multiplicity was calculated as the average number of total tumors per rat in the group. Tumor latency was the average time that the first mammary tumor was palpated in a tumor-bearing rat in each dietary treatment group.

Statistical analysis

The difference between the means was analyzed by one-way analysis of variance followed by Tukey-Kramer HSD procedure for food intake, body weight gain and plasma zinc concentration. Tumor multiplicity, tumor burden, tumor latency, tumor volume and tumor weight were analyzed by Kruskal-Wallis procedure. Tumor incidence was analyzed by the χ² test. The observations were considered statistically significantly different if the probability level of observed differences between the means was <0.05. Statistical analyses were performed using JMP software (The SAS Institute, Cary, NC).

Results

Body zinc status

Body zinc status was assessed by body weight gain, total food intake and plasma zinc concentration (Table I). Comparing with the adequate-zinc ad libitum control rats, the body weight gain in the low-zinc and adequate-zinc pair-fed control rats was reduced throughout the feeding trial (P < 0.05; Figure 1) with an overall reduction of 8 and 9% in the low-zinc and adequate-zinc pair-fed control rats, respectively. Body weight gain in the low-zinc rats was also reduced between week 2 and week 9 of the feeding trial compared with the adequate-zinc pair-fed control rats (P < 0.05), but was not significantly different for the remaining 9 weeks of the feeding trial. The growth rate was significantly higher during the first half of the feeding trial (from the beginning to week 9) than during the second half of the feeding trial (from week 10 to week 18) regardless of dietary zinc intake (2.0 versus 1.7 g/rat/day for the low-zinc rats, 2.3 versus 1.2 g/rat/day for the adequate-zinc pair-fed control rats and 3.2 versus 0.7 g/rat/day for the adequate-zinc ad libitum control rats; P < 0.05). During the first half of the feeding trial, the growth rate was the highest in the adequate-zinc ad libitum control rats followed sequentially by the adequate-zinc pair-fed control rats and the low-zinc rats.

<table>
<thead>
<tr>
<th>Table I. Effects of dietary zinc intake on body zinc status in rats injected with MNUa</th>
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</thead>
<tbody>
<tr>
<td><strong>Dietary treatments</strong></td>
</tr>
<tr>
<td>Body wt gain (g/rat)</td>
</tr>
<tr>
<td>Total food intake (g/rat)</td>
</tr>
<tr>
<td>Plasma zinc (μg/ml)</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM (n = 33 rats for the low-zinc group and 25 rats for the adequate-zinc pair-fed and ad libitum control groups).

Significantly different from the adequate-zinc ad libitum control group (P < 0.05).

Significantly different from adequate-zinc pair-fed control group (P < 0.05).
Low zinc intake suppressed mammary tumorigenesis

The effect of dietary zinc intake on tumor incidence was shown in Figure 3. The cumulative incidence of palpable tumors was significantly lower in the low-zinc rats than in the adequate-zinc ad libitum control rats starting from week 8 post-MNU-injection to the end of the feeding trial (P < 0.05). Cumulative incidence of palpable tumor in the low-zinc rats was similar to that in the adequate-zinc pair-fed control rats until week 10 post-MNU-injection. Starting from week 11 post-MNU-injection, cumulative incidence of palpable tumors was significantly lower in the adequate-zinc pair-fed control rats than in the adequate-zinc ad libitum control rats from week 8 to week 10 post-MNU-injection (P < 0.05), but was similar thereafter.

**Number of MNU-induced mammary tumors**

The effect of dietary zinc intake on tumor number was assessed by both total tumor and palpable tumor numbers (Table II). Total tumor number in the low-zinc rats was reduced by 77 and 58% compared with that in the adequate-zinc ad libitum and pair-fed control rats, respectively. Total tumor number in the adequate-zinc pair-fed control rats was reduced by 46% compared with that in the adequate-zinc and pair-fed control rats, respectively (P < 0.05). Both total tumor and palpable tumor incidences were similar between the adequate-zinc ad libitum and pair-fed control rats.

Incidence of MNU-induced mammary tumors

The effect of dietary zinc intake on tumor incidence was assessed by both total tumor and palpable tumor incidences (Table II). Total tumor incidence, which included both palpable and non-palpable tumors, in the low-zinc rats was reduced by 45 and 42% compared with that in the adequate-zinc ad libitum and pair-fed control rats, respectively (P < 0.05). Palpable tumor incidence in the low-zinc rats was reduced by 59 and 57% compared with that in the adequate-zinc ad libitum and pair-fed control rats, respectively. Among dietary treatment groups at each time point sharing a common letter are not significantly different (P < 0.05).

(P < 0.05), but the order was completely reversed during the second half of the feeding trial with the low-zinc rats showing the highest growth rate followed sequentially by the adequate-zinc pair-fed and ad libitum control rats (P < 0.05). Total food intake was reduced by 17 and 20% in the low-zinc and adequate-zinc pair-fed control rats, respectively, compared with that in the adequate-zinc ad libitum control rats (P < 0.05; Table I). Reduction in food intake was most severe during the first 6 weeks of the feeding trial and gradually eased over time (P < 0.05; Figure 2). By week 13, food intake was similar among the three dietary treatment groups. Food intake was similar between the low-zinc and the adequate-zinc pair-fed control rats throughout the feeding trial. Plasma zinc concentration in the low-zinc rats was ~50% of that in the adequate-zinc ad libitum and pair-fed control rats (P < 0.05), but was similar between the adequate-zinc ad libitum and pair-fed control rats.

**Fig. 1.** Effect of dietary zinc intake on body weight gain of rats treated with MNU. AZ-ad lib, adequate-zinc ad libitum control group; AZ-PF, adequate-zinc pair-fed control group; LZ, low-zinc group. Body weight gain among dietary treatment groups at each time point sharing a common letter are not significantly different (P < 0.05).

**Fig. 2.** Effect of dietary zinc intake on food intake in rats treated with MNU. AZ-ad lib, adequate-zinc ad libitum control group; AZ-PF, adequate-zinc pair-fed control group; LZ, low-zinc group. (a) Significantly different from the adequate-zinc pair-fed control and low-zinc (P < 0.05). (b) Significantly different from the low-zinc group (P < 0.05).
Table II. Effect of dietary zinc intake on the induction of mammary tumors by MNU

<table>
<thead>
<tr>
<th>Dietary treatments</th>
<th>Adequate-zinc ad libitum</th>
<th>Adequate-zinc pair-fed</th>
<th>Low-zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor incidence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palpable tumor (%)</td>
<td>80</td>
<td>76</td>
<td>33&lt;sup&gt;b, c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total tumor (%)</td>
<td>84</td>
<td>80</td>
<td>46&lt;sup&gt;b, c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tumor number</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palpable tumor</td>
<td>91</td>
<td>47</td>
<td>20</td>
</tr>
<tr>
<td>Total tumor</td>
<td>123</td>
<td>66</td>
<td>28</td>
</tr>
<tr>
<td>Tumor multiplicity</td>
<td>5.0 ± 0.9</td>
<td>2.6 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8 ± 0.2&lt;sup&gt;b, c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(number of tumor/rat)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor burden (%)</td>
<td>3.8 ± 1.3</td>
<td>1.2 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tumor latency (weeks)</td>
<td>9.3 ± 0.6</td>
<td>11.4 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.8 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tumor weight (g/tumor)</td>
<td>Mean</td>
<td>1.43 ± 0.36</td>
<td>0.72 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.31</td>
<td>0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values represent the mean ± SEM (n = 33 rats for the low-zinc group and 25 rats for the adequate-zinc pair-fed and ad libitum groups).

<sup>b</sup>Significantly different from the adequate-zinc ad libitum control group (P < 0.05).

<sup>c</sup>Significantly different from the adequate-zinc pair-fed control group (P < 0.05).

ad libitum control rats. Palpable tumor number in the low-zinc rats was reduced by 78 and 57% compared with that in the adequate-zinc ad libitum and pair-fed control rats, respectively. Palpable tumor number in the pair-fed control rats was reduced by 48% compared with that in the adequate-zinc ad libitum control rats. The temporal pattern for the cumulative palpable tumor number was lower in the low-zinc rats than in both the adequate-zinc ad libitum and pair-fed control rats, and in the adequate-zinc pair-fed control rats than in the adequate-zinc ad libitum control rats throughout the feeding trial (Figure 4).

Multiplicity, burden, latency and size of MNU-induced mammary tumors

Tumor multiplicity in the low-zinc rats was reduced by 84 and 69% compared with that in the adequate-zinc ad libitum and pair-fed control rats, respectively (P < 0.05; Table II). Tumor multiplicity in the pair-fed control rats was reduced by 48% compared with the adequate-zinc ad libitum control rats (P < 0.05). Tumor burden in the low-zinc rats was reduced by 92% compared with that in the adequate-zinc ad libitum control rats (P < 0.05; Table II), but was similar between the low-zinc and adequate-zinc pair-fed control rats, and between the adequate-zinc pair-fed and ad libitum control rats. Tumor latency was significantly prolonged by 38 and 23% in the low-zinc and adequate-zinc pair-fed control rats, respectively, compared with that in the adequate-zinc ad libitum control rats.
Low zinc intake suppressed MNU-induced mammary tumorigenesis

As indicated by a lower tumor incidence, tumor number and tumor multiplicity, MNU-induced mammary tumorigenesis was reduced in the low-zinc rats compared with that in both the adequate-zinc ad libitum and pair-fed control rats. In this study, the adequate-zinc diet provided four times the amount of zinc as the low-zinc diet while all other nutrient and energy contents were the same in these two diets. Moreover, the low-zinc rats consumed a similar amount of food as the adequate-zinc pair-fed control rats. Reduced MNU-induced mammary tumorigenesis in the low-zinc rats, in comparison with the adequate-zinc pair-fed control rats, could be attributed to low-zinc intake per se.

MNU is an alkylating agent. The carcinogenicity of MNU is due to its ability to induce a G→A mutation in the second codon of the H-ras oncogene (33). Once the mutation occurs, the initiated cells could undergo cell death, DNA repair to form normal cells or, provided that there are enough nutrients required for growth, rapid cell proliferation. In theory, either increased death or decreased proliferation of the initiated cells or a combination of these two events could result in a lower tumor incidence. Zinc is critical to both cell death (e.g. apoptosis) and cell proliferation. The effects of zinc on apoptosis appear to be a function of zinc status. Zinc deprivation generally induces apoptosis (34-38) and zinc supplementation suppresses apoptosis (39-42). In addition, zinc status is also critical to DNA synthesis and cell proliferation. Zinc deprivation suppresses DNA synthesis and cell proliferation (43-47) while zinc repletion reactivates DNA synthesis and cell proliferation demonstrating the importance of zinc status in DNA synthesis and cell proliferation.

More recently, we have shown zinc accumulation in MNU-induced rat mammary tumors (18). This zinc accumulation was coupled with an increased mRNA and protein levels of metallothionein, the main cellular zinc storage protein, and a reduced expression of ZnT-1, a zinc exporter. This altered expression of metallothionein and ZnT-1 suggests that zinc homeostasis might be altered in MNU-induced rat mammary tumors. Since adequate zinc supply is essential to cell proliferation and tumors are characterized by uncontrolled cell proliferation, zinc accumulation in MNU-induced mammary tumors probably is part of the mechanisms that ensure an adequate zinc supply to sustain tumorigenesis. Zinc is not stored in the body at an appreciable amount. Therefore, dietary zinc intake has a great impact on body zinc status. We speculate that zinc deficiency mediated increase in apoptosis and reduction in cell proliferation might collectively play a role in the inhibitory effects of low dietary zinc intake on MNU-induced rat mammary tumorigenesis.

Effects of reduced food intake associated with low-zinc intake on MNU-induced mammary tumorigenesis

In this study, cumulative incidence of palpable tumors was also reduced by 77% by week 10 post-MNU-injection in the adequate-zinc pair-fed rats compared with the adequate-zinc ad libitum control rats. In addition, tumor number, tumor multiplicity and tumor latency were also lower in the adequate-zinc pair-fed control rats than in the adequate-zinc ad libitum control rats. Reduction in these parameters used to assess MNU-induced mammary tumorigenesis in this study can be attributed to reduced food intake because the pair-fed control rats were fed the same adequate-zinc diet as the ad libitum.
control rats, but at a reduced amount. Reduced food intake represents a reduced intake of both energy and nutrients. However, it has been established that caloric restriction, but not the reduced nutrient intakes, inhibits carcinogenesis (48,49), including chemically induced mammary tumorigenesis in rats (50–52). Cohen et al. (50) reported that a 25% caloric restriction resulted in a significant reduction in the incidence of palpable tumors and total number of mammary tumors in MNU-treated rats. Gillette et al. (53) observed that a caloric restriction of 20%, which was similar to the food reduction (22%) in the adequate-zinc pair-fed control rats during the first 10 weeks post-MNU-injection observed in this study, resulted in a 71% reduction in tumor incidence. These observations were very similar to the results reported herein. It is interesting to note that, starting from week 11 to week 13 post-MNU-injection, the incidence of palpable tumors in the pair-fed control rats was the same as in the adequate-zinc ad libitum control rats. During the same period, food intake was not significantly different between the adequate-zinc pair-fed and ad libitum control rats. This lack of differences in both the palpable tumor incidence and food intake between the adequate-zinc pair-fed and ad libitum control rats from week 11 to week 13 post-MNU-injection to the end of the feeding trial further support the notion that reduced food intake associated with low-zinc intake also reduced the incidence of MNU-induced rat mammary tumorigenesis.

In summary, food intake and body weight gain were lower in the low-zinc rats than in the adequate-zinc ad libitum control rats, but were similar to those patterns in the adequate-zinc pair-fed control rats. Plasma zinc concentration was lower in the low-zinc rats than in the adequate-zinc ad libitum and pair-fed control rats, confirming the moderate zinc deficiency status in the low-zinc rats. Tumor incidence and tumor multiplicity were significantly reduced in the low-zinc rats compared with that in the adequate-zinc ad libitum and pair-fed control rats (P < 0.05). In addition, tumor number was also lower in the low-zinc rats than in the adequate-zinc ad libitum and pair-fed control rats. Tumor latency in the low-zinc rats was not different from that in the adequate-zinc pair-fed control rats, but was longer than in the adequate-zinc ad libitum control rats, indicating that reduced food intake associated with low-zinc intake prolonged tumor latency. Tumor burden and tumor size were not affected by zinc intake. Overall, our observations showed that moderately low-zinc status suppressed MNU-induced rat mammary tumorigenesis. Since zinc is important to cell proliferation and apoptosis, further studies are warranted to investigate the specific mechanistic role(s) of zinc in MNU-induced rat mammary tumorigenesis.

Acknowledgements

The authors would like to thank Drs K.Chang and V.LeMay for their assistance in the statistical analyses and B.Wu, Y.Tsukada and A.Gerber for their technical assistance. This study was supported by an operating grant from The Cancer Research Society Inc. (Z.Xu).

References


Received October 29, 2003; revised May 26, 2004; accepted June 10, 2004