Niacin Supplementation Decreases the Incidence of Alkylation-Induced Nonlymphocytic Leukemia in Long-Evans Rats

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Niacin deficiency impairs poly(ADP-ribose) formation and enhances ethylnitrosourea (ENU)-induced carcinogenesis. Previous experiments were compromised by rapid progression of cancer, and the current study was designed with half the number of ENU doses. Weanling male Long-Evans rats were fed niacin deficient (ND), pair-fed (PF) control (30 mg nicotinic acid/kg), or pharmaceutical niacin (NA; 4 g nicotinic acid/kg) diets. After 2 wk, rats were gavaged every other day with ENU [30 mg/kg body weight (bw)] or vehicle (6 doses). Four days after the last dose of ENU, all rats were switched to AIN-93M diet and mildly feed restricted to maintain a constant food intake per bw. Rats were monitored for termination criteria and assessed for cancer development. Total cancers developed more rapidly in rats on the ND diet compared to those receiving high dose supplements of NA ($P = 0.02$; Gehan’s generalized Wilcoxon test). Importantly, all of these differences occurred in the leukemias, especially the nonlymphocytic leukemia fraction ($P = 0.008$; Gehan’s generalized Wilcoxon test), with incidences of 36%, 17%, and 11% in ND, PF, and NA rats, respectively. Because nonlymphocytic leukemias represent the majority of secondary cancers, these data support the concept that niacin supplementation may help protect cancer patients from the deleterious side effects of chemotherapy.

INTRODUCTION

As the success rate in curing primary cancers has increased, the long-term consequences of therapy have become more apparent. Secondary, or treatment-related cancers, are induced by DNA damage from chemotherapy drugs and tend to occur in 5–15% of chemotherapy patients (1,2). Nonlymphocytic leukemias are the predominant secondary cancer in humans (1–6). The 2 main classes of drugs that cause secondary leukemias are topoisomerase inhibitors (3), such as etoposide, and alkylating agents (4), such as nitrogen mustard, cyclophosphamide, and the nitrosoureas. Alkylating agents tend to cause acute myeloid leukemia, peaking 4 to 6 yr after chemotherapy. Most leukemias (75–90%) show chromosomal abnormalities, with losses in chromosomes 5 and 7 being the most common.

Ethylnitrosourea (ENU) is a monofunctional alkylating agent that we have used to mimic the leukemogenic action of bifunctional nitrosoureas used in chemotherapy regimens (7–9). Like the majority of chemotherapy drugs, ENU does not require enzymatic metabolism to cause DNA alkylation, instead decomposing spontaneously to form an ethylating group that binds to nucleophilic sites on DNA (10). The ethylation of the oxygen and nitrogen residues on adenine, guanine, and cytosine lead to point mutations and cancer (11). Bifunctional nitrosoureas (e.g., 1,3-bis(2-chloroethyl)-1-nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) are more clinically effective in cancer therapy due to their bulky adducts and DNA cross-linking properties, but their induction of secondary leukemias is thought to be due to their alkylating activities (12,13). It is this aspect that we are modeling with the choice of ENU as a drug treatment.

Patients undergoing chemotherapy are usually nutritionally compromised, both from the effect of the disease process and the toxicity of the treatment. Niacin (vitamin B3) has been found to be deficient in a large portion of chemotherapy patients (14,15), and appears to be resistant to improvement through normal levels of supplementation (15). In some cases, chemotherapy patients develop clinical pellagra (16). Niacin is needed to form nicotinamide adenine dinucleotide (NAD$^+$) and NAD phosphate. These 2 pyridine nucleotides are critical redox cofactors in all areas of intermediary metabolism. However, NAD$^+$ is also a substrate for the formation of poly[adenosine diphosphate (ADP)-ribose] by poly(ADP-ribose) polymerase (PARP) enzymes, which play a variety of roles in genomic stability (17). PARP-1 forms the majority of poly(ADR-ribose) in response to DNA damage, but 16 other PARP-related gene sequences have...
been identified, and research on the function of the encoded proteins is very active (18).

We have previously shown that niacin deficiency causes chromosomal instability (19,20), and that niacin status influences the long-term development of cancers (8,9). Our previous long-term studies used 12 doses of ENU [30 mg/kg body weight (bw)] and were hampered by a very rapid development of cancer in all dietary groups as well as different basal diets for deficiency and supplementation models. This study was designed to examine deficient, adequate, and pharmacological intakes of niacin in the same dietary model, with exposure to half the number of ENU doses used previously to determine the role of niacin status in the response of bone marrow cells to alkylation injury as experienced during chemotherapy. The results showed that niacin status has a significant effect on leukemogenesis in this model, especially nonlymphocytic leukemias similar to those seen as secondary leukemias following chemotherapy in humans.

MATERIALS AND METHODS

Animals
Animal care was done using the standards of the Canadian Council on Animal Care and was approved by the Animal Care Committee at the University of Guelph. Male Long-Evans rats, 40 to 50 g (Charles River, St. Constant, Quebec, Ontario, Canada) were housed individually in wire bottom cages. They had free access to tap water and were maintained on a 12-h light–dark cycle. On arrival, animals were weighed and placed in weight-matched groups of three, and randomly assigned to the experimental diets: niacin deficient (ND; 0 mg added niacin/kg diet; \( n = 31 \)), niacin-adequate pair-fed (PF) control (30 mg added niacin/kg diet; \( n = 31 \)), or pharmacological levels of nicotinic acid (NA; \( n = 30; 4 \text{ g/kg added NA} \)). Feed intake was determined daily, and rats in the PF and NA groups were pair fed to their respective ND rat, that is, provided the exact amount of diet that the ND rat consumed the previous day. Long-Evans rats were selected because this strain develops more nonlymphocytic and fewer lymphocytic leukemias than other rat strains (21), making them a better model for the types of cancers that develop in human cancer patients.

Short-Term Study
To establish when the animals became deficient and determine the time course of supplementation effects, animals in a preliminary study were euthanized at 0, 1, 2, and 3 wk and blood NAD\(^+\) and bone marrow NAD\(^+\) determined. NAD\(^+\) concentrations were determined as previously described by the colorimetric change cycling assay with some modifications (21). Briefly, an aliquot of total resuspended bone marrow was added to concentrated perchloric acid (PCA) to yield a final concentration of 1N PCA. The supernatant was transferred to a clean tube and frozen at \(-20^\circ\text{C}\). The sample was thawed and adjusted to a pH of 6.5- and 7 with KOH. Standards and samples were then analyzed by enzyme cycling using alcohol dehydrogenase (22).

FIG. 1. Effect of niacin status on bone marrow nicotinamide adenine dinucleotide (NAD\(^+\)). Niacin-deficient (ND), pair fed control diet (PF) and nicotinic acid supplemented (NA) rats were euthanized after 1, 2, or 3 wk on experimental diets. Bone marrow cell NAD\(^+\) was extracted with perchloric acid and quantified by enzyme cycling. \( n = 6 \) for ND, PF, and NA. Values are mean ± standard error of the measurement.

Long-Term Study
Figure 1 shows that bone marrow NAD\(^+\) was decreased between 1 and 3 wk in the ND group, whereas the NA diet had the greatest effect at 1 wk, and then decreased over the next 2 wk, maintaining about a twofold increase above PF controls. Due to this response, the NA group received PF diet for the 1st wk and were then placed on the NA diet for 1 wk before the start of ENU treatment, whereas ND rats were fed the deficient diet for 2 wk before the first dose of ENU to allow the greatest impact of the diets on bone marrow NAD\(^+\) to coincide with the ENU exposure period.

Chemotherapy Protocol
Treatment with ENU (Sigma Chemical, St. Louis, MO) began after 2 wk on ND or PF diets and 1 wk on the NA diet. A total of 6 doses were administered over 10 days (qd2). Doses were administered 4 h following the start of the light cycle, and the order in which the animals received the doses was randomized. For their treatment, animals were weighed, lightly anesthetized in 2.5% halothane (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and gavaged with ENU (30 mg/kg bw) resuspended in water (pH 4.0). After the 6th dose of ENU, animals remained on their respective diets for 4 days. This was to ensure that DNA repair and mutation events were occurring in the ND-, PF-, or NA-supplemented metabolic environment. A total of 4 days following the last dose of ENU, animals were placed on a mildly restricted AIN-93M diet. Feed provision was restricted to the number of g/kg bw that the majority of rats would completely consume. As soon as a number of animals were found to be leaving food in their bowls, the feed/body constant was decreased. The constant started around 14 g/100 g bw per day and decreased as the rats matured. This protocol ensured that food intake and body composition would not play a role in the effects of diet on cancer development.
Cancer Protocol

Animals were weighed and palpated twice a week. The termination criteria established in our Animal Care Protocol was either a 5% reduction from peak bw, morbid behavior, or a palpable tumor of 1 cm or more in diameter. Animals were anaesthetized under halothane and decapitated. Blood was collected in heparanized tubes. The hematocrit was determined, a cell count obtained to determine the number of red and white cells (Model Z2 Coulter Counter, Beckman Coulter, Burlington, Ontario, Canada), and several blood smears were made. A complete necropsy was performed, and any organs that appeared abnormal were collected and preserved in 10% buffered paraformaldehyde (Fisher Scientific, Ottawa, Ontario, Canada). Samples were embedded, stained with H&E and examined for neoplasms. Both femurs were excised and the condyles removed. An 18.5 gauge syringe was used to flush the bone marrow out into 5 ml of phosphate-buffered solution. Bone marrow smears were made using marrow collected from the tibia. Tissue sections and smears were interpreted by an experienced pathologist (R. Jacobs).

Statistics

During the gavage period, 3 ND rats died; 1 PF was euthanized due to an oral malocclusion; and 1 NA rat was euthanized due to lingering eye infection. These events were censored in all survival analyses. Survival was analyzed using life tables and Gehan’s generalized Wilcoxon test for multiple or pairwise comparisons. Life tables are better suited to smaller sample sizes than Kaplan Meier analysis, and Wilcoxon tests are suited to data in which the hazards are not proven to be proportional.

RESULTS AND DISCUSSION

Previous work by Boyonoski et al. (8) showed that niacin deficiency decreased the latency of cancer development following 12 doses of ENU. Conversely, niacin supplementation (combined analysis of nicotinic acid and nicotinamide) increased the latency of cancer development (9). Both studies were compromised by an extremely rapid cancer progression, which may have caused the dietary mediation of carcinogenesis to be less effective. In addition, niacin supplementation occurred in different basal diet, which led to anomalous rates of cancer development between the 2 models. With the idea that cancer patients tend to have poor dietary status, especially during chemotherapy, we wanted to test low, normal, and high levels of niacin status within the same basal dietary model to determine which level of niacin intake may be optimal to avoid bone marrow injury and secondary leukemias.

We also wanted to examine a less aggressive treatment to allow a longer latency of cancer progression. Switching from 12 to 6 doses of ENU worked to a certain extent in that the first animal was euthanized at 17 wk in this study as compared to 12 to 15 wk in the previous studies (8,9). In addition, the present experiment lasted about 12 wk longer than either of the previous studies. However, the latency of cancer development in this study was not doubled by decreasing the ENU exposure by half, and further reductions in exposure would be required to create a model in which cancer incidence was not 100% in all groups. A further reduction in ENU exposure might make the model even more sensitive to dietary intervention.

The experimental diets were only fed during the first 4 wk of the long-term protocol, and ENU exposure took place over the 10-day period ending 2 days before the end of the experimental diets. This design was based on data in Fig. 1, which showed that niacin deficiency-induced decreases in bone marrow NAD+ were established at 2 wk. In contrast, pharmacological dietary intake of niacin caused a rapid elevation of bone marrow NAD+, so the ND diet was started at Time 0, and the NA diet started at Week 1, with ENU treatment starting at Week 2, to time the greatest changes in bone marrow NAD+ with the ENU exposure. We have shown previously that these changes in bone marrow NAD+ are associated with dramatic changes in basal and DNA damage-induced poly(ADP-ribose) levels (8,9). Experimental diets were fed for 4 days after the last dose of ENU to allow DNA lesions to be resolved before rats were transferred to a control diet.

Figure 2 shows that the pair feeding protocol produced similar rates of growth in the different dietary groups. After ENU treatment, all animals were fed a marginally feed-restricted AIN-93M diet. The amount they were fed per 100 g bw was gradually adjusted over time to ensure that all rats were consuming an equivalent amount of food on a bw basis. This ensures
FIG. 3. A: Mortality curves for total cancer. Rats were gavaged with ethylnitrosourea (ENU) at 30 mg/kg body weight every other day for a total of 6 doses starting at 5 wk of age. Rats were maintained on different diets, niacin-deficient (ND), pair fed (PF), or nicotinic acid (NA) from the moment of their arrival (3 wk of age) until 4 days after their last dose of ENU (7 wk). Diet was significant within this model ($P = 0.05$, Gehan’s generalized Wilcoxon test for multiple comparisons); individually, ND and NA groups were different ($P = 0.02$), whereas ND and PF did not reach significance ($P = 0.08$, Gehan’s generalized Wilcoxon test, pairwise comparison). B: Mortality curves for nonleukemias. When leukemias are removed from the preceding data, there are no significant differences between ND, PF and NA survival curves.

Data is not presented past Week 17, as it would be skewed by the development of malignancies.

Fig. 3A illustrates the overall survival response, including all forms of cancer. The term survival is used for consistency with the literature, but these data do represent animals that were humanely killed according to specific health criteria (i.e., tumor size, weight loss, morbidity). Survival analyses showed that there was a significant effect of diet within this model ($P = 0.05$, Gehan’s generalized Wilcoxon test for multiple comparisons). Visual inspection of Fig. 3A suggests that niacin deficiency is deleterious in comparison with either adequate (PF group) or pharmacological intakes of niacin (NA group). Pairwise comparisons show the greatest statistical difference exists between ND and NA groups ($P = 0.02$, Gehan’s generalized Wilcoxon test), whereas the pairwise comparison between ND and PF groups did not quite reach significance ($P = 0.08$, Gehan’s
ALKYLATION-INDUCED NONLYMPHATIC LEUKEMIA

Figure 4. A: Development of total leukemias over time. *Indicates these 2 niacin-deficient (ND) rats each had 2 independent leukemias, a lymphocytic and a nonlymphocytic leukemia. They are shown separately in this figure to agree with data in Table 1, but in the survival analysis, they were treated as a single point. Niacin status in the multiple comparisons model was not significant, whereas the pairwise comparison between ND and pharmacological diets was significant ($P = 0.05$). B: Development of nonlymphocytic leukemias over time. The multiple comparisons model was highly significant ($P = 0.008$), and pairwise comparisons showed the ND diet to be significantly different from control and pharmacological niacin intake ($P = 0.01$ in both). PF, pair fed control diet; NA, nicotinic acid.

generalized Wilcoxon test). Table 1 shows the distribution of cancers in this model, and it is apparent that only about one-third are hematopoietic in origin. The majority of the cancers are epithelial cancers and stromal tumors. When the leukemia cases are removed, there are no significant effects of niacin status on the survival data of the nonhematopoietic cancers (Fig. 3B).

In humans, alkylating agents cause a high proportion of leukemias compared to other cancers, and the majority of these are nonlymphocytic in nature. Figure 4A and 4B show the development of total leukemias and nonlymphocytic leukemias, respectively, with other cancer and noncancer endpoints removed. With respect to total leukemias (Fig. 4A), niacin status in the multiple comparisons model was not significant, whereas the pairwise comparison between ND and NA diets was significant ($P = 0.05$). The comparison between ND and PF was not significant ($P = 0.22$). The nonlymphocytic leukemia data shows the greatest effect of niacin status (Fig. 4B). The multiple comparisons model was highly significant ($P = 0.008$), and pairwise comparisons showed the ND group to be significantly different from the PF and NA groups ($P = 0.01$ for both).
<table>
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<th>PF</th>
<th>NA</th>
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<td>19</td>
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<td>Lung (13) Lung with genitourinary (1) Intestinal (1) Pancreatic (1) Intestinal (1) Kidney (2) Lung with mammals (1)</td>
<td>Lung (15) Mammary (1) Thyroid (1) Lung with pancreas and kidney (1) Skin (1)</td>
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<tr>
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<td>Skin cyst (2) Squamous cell papilloma (1) Sebaceeous (2)</td>
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<td>Spindle cell (4) Kidney (5) Fibrosarcoma (2) Brain (1)</td>
<td>Spindle cell (2) Kidney (1) Fibrosarcoma (9)</td>
<td>Spindle cell (7) Kidney (6) Fibrosarcoma (3) Other (2)</td>
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<td>42</td>
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<td>Total leukemias (as % of malignancies)</td>
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<td>14%</td>
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<td>50%</td>
<td>37%</td>
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<tr>
<td>Nonlymphocytic leukemia incidence (as % of rats)</td>
<td>36%</td>
<td>17%</td>
<td>11%</td>
</tr>
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</table>

*Abbreviations are as follows: ND, niacin deficient; PF, pair fed; NA, nicotinic acid.*

There are many challenges in the choice and interpretation of animal models for complex human diseases. This is certainly true in the area of alkylation-induced cancers, which in human chemotherapy populations are predominantly nonlymphocytic leukemias. In rodent models, direct acting alkylating agents cause a greater spectrum of cancers, and in most strains of mice and rats, the leukemias are almost exclusively lymphocytic in nature. There are certain combinations of rat strains and alkylating agents that generate nonlymphocytic leukemias, the most specific of which appears to be the Donryu rat treated with butylnitrosourea (23), both of which are difficult to obtain. A more practical choice is the Long-Evans rat (24) treated with ENU, which we have adopted for our work in this area (8,9). The strength of this model is the development of alkylation-induced nonlymphocytic leukemias, but there are some general weaknesses and issues specific to this trial that deserve attention. In general, the main problem is the significant incidence of nonhematopoietic cancers. It is interesting that these are not influenced by niacin status, which is consistent with the susceptibility of the bone marrow to modification of NAD and poly(ADP-ribose) metabolism by dietary niacin (8,9) and the associated changes in genomic stability and cellular injury, which we have not observed in other tissues (25–27). To apply this data to the human condition, we would like to focus specifically on the nonlymphocytic leukemias of Fig. 4B. This raises obvious questions regarding the interaction of cancer processes in a survival model. Does the development of other cancers invalidate the data on nonlymphocytic leukemias? There are 2 points that are related and argue against this. First, the leukemias tend to develop earlier, and are therefore somewhat independent of the
development of other cancers. Second, the development of 1 type of cancer does not preclude the development of another. A number of rats developed more that 1 type of cancer, generally a leukemia and another type of cancer. To support these points, the average latency to leukemias was 28, 31, and 29 wk in ND, PF, and NA rats, respectively, whereas the average latency to nonleukemia cancers was 36, 38, and 39 wk, respectively. It is, however, likely that some rats were euthanized with a single nonhematopoietic cancer that would have eventually developed leukemia.

A decrease in ENU exposure from 12 to 6 doses has provided a less severe model in which the bone marrow is more sensitive to niacin status. The model is still more severe than intended, and we would probably decrease the ENU exposure again if we were to do further work in this model. Of human cancer patients, 5–15% may develop secondary leukemias (1,2). Our model displays an incidence of 15% nonlymphocytic leukemias in control rats, but the deficient rats, which may be a closer fit to human chemotherapy patients, had an incidence of 35%.

We have previously documented a striking degree of genomic instability in the niacin deficient bone marrow in this model, using micronuclei, sister chromatid exchange, single cell electrophoresis, and chromosomal aberrations as endpoints (18,19). The sensitivity of bone marrow cells to niacin deficiency is not seen in most other tissues that we have examined, including the lung (25) and liver (27), although the skin is also responsive to deficiency (28). This is consistent with our measurement of NAD and poly(ADP-ribose) levels in various tissues in our model of deficiency (8,29) in which bone marrow shows the greatest sensitivity to depletion. Mechanistically, we have shown that niacin deficiency impairs cell cycle arrest and apoptosis in response to ENU-induced injury (30). Pharmacological niacin intake enhanced ENU-induced apoptosis in bone marrow cells (30), perhaps through the poly(ADP-ribosyl)ation of apoptosis-inducing factor (31). If this effect also occurs in cancer cells, high dietary niacin may not only decrease the side effects of chemotherapy but also enhance the efficacy of tumor cell killing.

If the leukemia data, especially that of the nonlymphocytic form, is extrapolated to human populations, it suggests that niacin deficiency presents a particular risk to genotoxic side effects in the bone marrow and that niacin supplementation at pharmacological levels may be beneficial in protecting cancer patients from the long-term development of secondary malignancy. The overall statistics support the NA group as having the greatest level of protection relative to ND rats given that the statistical power was greatest for the ND/NA comparison for data on total cancers and total leukemias, although the NA and PF groups were equal in the nonlymphocytic leukemia data. The study would benefit from a greater number of rats in each group, but it was expensive and time consuming as conducted.

It has been shown that higher than normal levels of supplementation have been needed to enhance niacin status in human cancer patients (15), providing further rationale for the consideration of pharmacological dosing. However, additional animal-based experiments are needed to determine the effect of niacin supplementation on the efficacy of practical chemotherapy regimens against transplanted tumors before niacin supplementation of human cancer patients should be considered.

ACKNOWLEDGMENTS

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REFERENCES


