Acta Radiologica Oncology 25 (1986) Fasc. 4–6

FROM THE DEPARTMENT OF MOLECULAR ECOGENETICS, WALLENBERG LABORATORY, UNIVERSITY OF LUND, AND THE DEPARTMENTS OF ONCOLOGY AND PATHOLOGY, UNIVERSITY HOSPITAL, S-22185 LUND, SWEDEN.

RADIOSENSITIZING EFFECTS OF NICOTINAMIDE ON A C3H MOUSE MAMMARY ADENOCARCINOMA

A study on per os drug administration

E. KJELLÉN, R. W. PERO, R. CAMERON and J. RANSTAM

Abstract

Nicotinamide is an inhibitor of adenosine diphosphate ribosyl transferase (ADPRT) which is involved in the mechanism of DNA repair after high doses of ionizing radiation. C3H mice with transplanted mammary adenocarcinomas were treated with low doses of nicotinamide, 10 mg/kg, 5 days a week, and in combination with ionizing radiation, 30 Gy, using different drug dose schedules. Mice given nicotinamide in combination with irradiation took a longer time to reach a tumor volume of 1 000 mm^3 and a higher complete response rate (i.e. defined as total disappearance of the tumor for at least 7 days) than those given radiation alone. This was true whether nicotinamide was given daily from one week before tumor transplantation until the animal was killed or from transplantation day until day of irradiation. In addition, nicotinamide given per os at a dose between the recommended maximum daily allowance for human subjects (20 mg/70 kg), and the therapeutic allowance (1 g–12 g daily) 5 days a week for 9 weeks, showed a radiosensitizing effect without any histologically detectable damage to the normal tissues of the mouse, including bone marrow, intestine and the liver.

Key words: Radiation biology; nicotinamide; radiosensitizing effect; mice.

Adenosine diphosphate ribosyl transferase (ADPRT) is a chromatin associated enzyme which attaches ADP-ribose moieties derived from nicotinamide adenine dinucleotide (NAD^+) to chromatin proteins (10, 17). The enzyme is activated by DNA damaging agents such as ionizing or UV radiation and by certain cytotoxic drugs (1, 2, 7, 19), indicating a role in the repair mechanism of DNA damage. Nicotinamide, the amide form of vitamin B_3, is a potent inhibitor of the enzyme (16, 18). We have previously reported on the radiosensitizing effect of nicotinamide on a C3H mouse adenocarcinoma (11, 12). In these studies we used a single intra-peritoneal injection of the drug, 0.2 g/kg, and the drug was given 30 minutes prior to the radiation. Since our ultimate objective is to evaluate the radiosensitizing effects of nicotinamide in man, we have in the present work studied the radiosensitizing effects of a per os administration of nicotinamide in mice at a dose between the human daily allowance, 20 mg/70 kg, and the therapeutic dose used for the treatment of various disorders in human subjects, 1 g to 12 g daily (9, 14), i.e. 700 mg/day or about 10 mg/kg. Here we report that nicotinamide given per os in this concentration radiosensitizes a C3H mouse mammary adenocarcinoma.

Material and Methods

Tumor transplantation. The tumor model used was a spontaneous mammary adenocarcinoma occurring in C3H mice and which has been propagated by serial transplantations in our department for three years. Animals were divided into groups, with ten in each group. Viable tumor pieces were cut with scissors and brought into suspension with small tumor fragments according to the method described by BORELL (5), and the cells were washed in Parker tissue medium (Gibco). The cell suspension was adjusted to about 2×10^6 cells/ml and a 0.15 ml aliquot was injected subcutaneously above the right gastrocnemius muscle in 6 to 8 weeks old mice of both sexes. The tumor take was close to 100 per cent. The tumors were approximately ellipsoids in shape and the tumor volume (V) was calculated as $V=a^2b/2$ where a is the shorter axis and b the longer perpendicular axis. Tumor volumes were meas-

Accepted for publication 26 September 1986.

Table 1

Median time for tumors to reach a volume of 1 000 mm³ in controls and after irradiation with a single dose of 30 Gy alone or combined with nicotinamide (NAM) given according to three different schedules (see Figure)

Group	No. of animals	Median time (days)	p-value
Control	26	18[a]	0.345
NAM schedule A	23	16[a]	
30 Gy+NAM schedule A	10	52[b]	0.032
30 Gy	18	35[b]	
30 Gy+NAM schedule B	19	>60[b]	<0.001
30 Gy	18	35[b]	
30 Gy+NAM schedule C	10	41[b]	0.532
30 Gy	18	35[b]	
30 Gy+NAM schedule A	10	52[b]	0.25
30 Gy+NAM schedule B	19	>60[b]	

[a] Day 1 is day of transplantation.
[b] Day 1 is day of irradiation.

Table 2

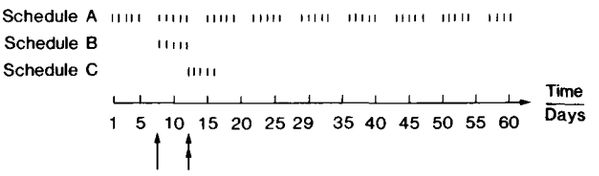
Number of complete responses and number of animals with complete tumor control at day 60 in relation to different treatments (for explanation see Figure and Table 1). Complete response was defined as total disappearance of the tumor for at least 7 days

Group	No. of animals	Complete response	No remaining tumor at day 60
Control	26	0	0
NAM schedule A	23	0	0
30 Gy	18	6	1
30 Gy+NAM schedule A	10	9	4
30 Gy+NAM schedule B	19	15	4
30 Gy+NAM schedule C	10	2	1

ured twice weekly, and when they reached an approximate volume of 200 mm³ the tumors were irradiated.

Nicotinamide treatment. Nicotinamide tablets (Amidex, a subsidiary of C. E. Jamieson & Co. (Dominion) Ltd., Ontario, Canada) containing 500 mg/tablet were used. The tablets were dissolved in tap water at a concentration of 0.6 mg nicotinamide/ml. The animals, about 25 g in weight, were given 0.5 ml of this solution of nicotinamide orally on Monday through Friday, following three different schedules (Figure). The nicotinamide was administered through a hard oral gastric tube to assure a correct drug dose. The dose of 0.3 mg/animal/day (i.e. 12 mg/kg bodyweight if the animals weighed 25 g) corresponds approximately to a human dose of nicotinamide of 750 mg/70 kg.

Schedule A. Nicotinamide treatment, 0.3 mg/day 5 days a week, was started one week before tumor transplantation. The animals were given nicotinamide until they were killed (Figure).



Schemes of nicotinamide treatment in combination with irradiation. Intention of schedule B was nicotinamide treatment for 5 days before irradiation but it could, due to tumor growth time, be extended to 14 days. Tumor transplantation (↑). Irradiation 30 Gy (↕). Nicotinamide 0.3 mg (ı).

Schedule B. The intention here was one week of nicotinamide pretreatment, i.e. 5 daily nicotinamide doses before irradiation, starting on the day of tumor transplantation. However, in some cases the tumor size had not reached the proper volume (200 mm³) for irradiation by this day. In these cases, nicotinamide treatment continued for an additional 1 to 9 days (Figure).

Schedule C. Nicotinamide, 0.3 mg/day, was given on 5 consecutive days, starting 2 h before irradiation (Figure).

Radiation treatment. The tumors were irradiated with a ^{60}Co gamma-ray source (Siemens, Gammatron S) with a dose rate of 0.7 Gy/min. The animals were placed unanesthetized in a plexiglas holder with the tumor-bearing leg stretched out in the radiation field. A 5 mm water bolus was placed around the tumor in order to ensure a homogeneous dose.

Histologic examination. The animals were killed in ether 60 days after irradiation, and dissected immediately for histologic examination. Tumor-bearing animals receiving nicotinamide and irradiation according to schedule A were used to assess long term response to nicotinamide. This experiment was performed in duplicate. Portions of tumor, liver, intestine and bone marrow were fixed in formalin. There were several cases where portions of pancreas were adherent to the bowel. Trimmed tissue blocks were routinely processed, microtomed at 4 to 5 μm, and stained with hematoxylin and eosin. Slides were studied with respect to tumor appearance and cellular viability, and damage in the tumor as well as in the intestine, bone marrow and liver.

Statistical methods. The time for the tumors to reach a volume of one cm³ was evaluated by the Kaplan-Meier method. Differences in times were tested by the generalized Wilcoxon test. The rates of complete response, as defined in Table 2, were analysed by the Fisher exact test.

Results

Tumor growth measurements. Table 1 shows the median time required for the tumors to reach a volume of 1 000 mm³ in unirradiated controls (day 1=transplantation

day) and for tumors given a single radiation dose, 30 Gy, with or without nicotinamide at different dose levels. Tumors in mice given nicotinamide alone (schedule A) did not differ in growth rate from the untreated controls ($p=0.345$). Tumors in mice given nicotinamide, 0.3 mg daily, from Monday through Friday (schedule A) plus 30 Gy showed a significantly longer time to reach a tumor volume of 1000 mm^3 compared with 30 Gy irradiation alone ($p=0.032$). A significantly longer delay in tumor growth was also found in animals treated with nicotinamide (schedule B) plus 30 Gy than in animals only irradiated ($p<0.001$). The part of the experiment using schedule B was performed in duplicate, with the same pattern of results recorded for each replicate experiment. The data in Table 1 are the pooled results. However, no significant differences in tumor growth delay were found when comparing nicotinamide using schedule C (Figure, Table 1) plus irradiation, with irradiation alone ($p=0.532$). There was also no difference in tumor growth between the groups given nicotinamide according to schedule A or B plus irradiation ($p=0.25$).

Tumor response. The complete response rate (CR) and number of animals without tumor at day 60 in the different groups appear in Table 2. In this study, a significantly better complete response rate was found in the animals given nicotinamide according to schedules A and B plus irradiation than in the groups treated by irradiation alone ($p=0.006$ and $p=0.008$, respectively). However, there was no significant difference between animals given nicotinamide according to schedule C plus irradiation and animals only irradiated ($p=0.67$).

Histologic findings. In *tumor tissue,* no significant differences with respect to cellular appearances or extent of necrosis could be seen between animals only irradiated and animals that had received both nicotinamide and irradiation. The number of mitoses per high power field was also similar in the two groups: 8.7 in nicotinamide and irradiated tumors and 9.0 in tumors only irradiated.

The *intestine and bone marrow* were chosen for study of the nicotinamide effects on normal tissue as they are partly composed of rapidly proliferating cells and are thus particularly sensitive to substances which disturb DNA synthesis. Sections of proximal small bowel showed villi which were of normal height and width and which were lined by regular tall columnar cells. At the base of the villi, cell proliferation was entirely normal. The fragments of pancreas, lymph node and fat seen in some sections of bowel showed no changes. Bone marrow was often somewhat hypercellular with some predominance of myeloid precursors. No degenerating hematopoietic cell foci or necrosis were seen.

The *liver* was examined as nicotinamide in very high doses has been shown to have a toxic effect on hepatocytes (20). In 5 of 13 animals treated with nicotinamide, small foci of liver cell necrosis were seen. In these, the hepatocytes had pyknotic nuclei and dark eosinophilic cytoplasm. The foci were usually mid-zonal but occasionally centri-lobular and almost always bland. A rare focus showed some inflammatory cell infiltrates of both lymphocytes and granulocytes. In 2 of 10 animals not treated with nicotinamide, similar small necrotic foci were seen. The liver morphology was otherwise entirely normal in all cases.

Discussion

The data now reported indicate a radiosensitizing effect of nicotinamide given in a daily dose of 10 mg/kg/day 5 days a week from transplantation day until irradiation. Nicotinamide given daily, 5 times a week, until the animals were killed did not, in this experiment, give a better tumor control rate than nicotinamide given until the day of irradiation. Nicotinamide alone had no significant effect on tumor growth. This low dose of nicotinamide is about 20 times lower than the single dose (200 mg/kg) we used with obvious radiosensitizing effect in previous papers (11, 12). In addition. BERGER et coll. (4) showed that mice bearing L 1210 cells and treated with N-methyl-N-nitrosurea in combination with nicotinamide at about the same dose of 200 mg/kg (i.e. 5 mg/mouse) had increased survival compared with animals receiving N-methyl-N-nitrosurea alone; a dose of nicotinamide below 200 mg/kg did not enhance the effect of the cytostatic drug.

In mice with sarcoma S 180, CALCUTT et coll. (6) also found a better tumor control rate if the animals were treated with nicotinamide at 400 mg/kg and irradiation than if they were treated with radiation alone. They also reported on weight loss and changes in LD 50 for whole body irradiated animals in combination with or without nicotinamide, and found a decrease in the LD 50 radiation dose and an increased weight loss in animals treated with radiation and nicotinamide, compared with irradiation alone. In their experiment the authors used increasing doses of nicotinamide, from 200 mg/kg to 800 mg/kg. They found, however, no correlation between increasing doses of nicotinamide and changes in the LD 50 or weight loss. Hence, previous assessments of the sensitizing effects of nicotinamide are in agreement with the present results, but they were mainly performed with relatively high doses of nicotinamide.

Nicotinamide is an effective inhibitor of ADPRT (3, 4) and inhibitors of this enzyme are known to be effective radiosensitizers in the dose range of 200 mg/kg (6, 11, 12). However, nicotinamide is not only an inhibitor of ADPRT but it is also the natural precursor for NAD^+ synthesis in the cell (8). Exogenously supplied nicotinamide expands NAD^+ both in normal (13) and in malignant cells (6) and the level of NAD in the cell is thought to be regulated by two enzymes, ADPRT and NAD glycohydrolase (15). Nicotinamide can thus have metabolic effects on tumor and normal tissue responses apart from ADPRT inhibition and this could in turn affect the response to DNA damag-

ing agents. For example, tumor tissue has been shown to have hypoxic cell fractions, and under conditions of hypoxia nicotinamide and NAD metabolism can be changed, compared with oxic situations. Under hypoxia, tissue levels of reduced nucleotides, and thus total levels, have been reported to increase (15), as well as the activity of poly (ADP-ribose) synthetase. Hypoxia can thus change the metabolism of NAD and of nicotinamide, and since hypoxia is known to exist in neoplastic tissue the low dose of nicotinamide used in a daily schedule could, via changed metabolism, be sufficient to inhibit ADPRT and thus show a radiosensitizing effect, possibly due to inhibition of DNA repair. Moreover, it is also possible that other metabolites of nicotinamide could be increased in tumor tissue and exert a radiosensitizing effect. Furthermore, data exist indicating a better uptake of nicotinamide in log-phase cells than in plateau-phase cells, and this could also contribute to a preferential uptake of nicotinamide in the fast growing tumor cells (2).

As in a previous study (12), no microscopic differences could be detected between tumors of animals treated with nicotinamide and those not so treated. In this study, there was no significant difference in the mitotic frequency between the two groups, possibly due to the length of time between irradiation and killing.

The finding that nicotinamide, in these doses, does not result in microscopically detectable damage to intestinal mucosa or bone marrow is heartening. Some damage might be expected, considering the potent inhibitor effect of nicotinamide on ADPRT. The cause of the foci of liver necrosis is obscure. It is quite possible that nicotinamide may have some liver toxic effect. As the necroses were not seen in all the nicotinamide treated animals, and as they were observed also in a couple of animals not treated with nicotinamide, it is possible, however, that some other factors play a role.

ACKNOWLEDGEMENTS

The authors acknowledge the assistance of Mrs Margareta Olsson and Miss Maria Johansson. The tumor cell line used was kindly donated by the Department of Tumor Biology at Karolinska Institutet, Stockholm. The investigation was supported by grants from John and Augusta Persson's Fund for Medical Scientific Research and from the Swedish Cancer Society.

Request for reprints: Dr Elisabeth Kjellén, Department of Molecular Ecogenetics, Wallenberg Laboratory, University of Lund, P.O. Box 7031, S-220 07 Lund, Sweden.

REFERENCES

1. BEN-HUR E.: Involvement of poly(ADP-ribose) in the radiation response of mammalian cells. Int. J. Radiat. Biol. 46 (1984), 659.
2. — UTSUMI H. and ELKIND M. M.: Inhibitors of poly(ADP-ribose) synthesis enhance radiation response by differentially affecting repair of potentially lethal versus sublethal damage. Brit. J. Cancer 49 (1984) Suppl. No. 6, p. 39.
3. BERGER N. A. and SIKORSKI G. W.: Nicotinamide stimulates repair of DNA damage in human lymphocytes. Biochem. Biophys. Res. Commun. 95 (1980), 67.
4. — CATINO D. M. and VIETTI T. J.: Synergistic antileukemic effect of 6-amino-nicotinamide and 1,3-Bis(2-chloroethyl)-1-nitrosurea on L1210 cells in vitro and in vivo. Cancer Res. 42 (1982), 4382.
5. BORELL U.: Influence of ovarian hormones on the metabolism of nucleic acid and phospholipids in rabbit uterus. Acta Endocr. (Kbh) 9 (1952), 141.
6. CALCUTT G., TING S. M. and PREECE A. W.: Tissue NAD levels and the response to irradiation or cytotoxic drugs. Brit. J. Cancer 24 (1970), 380.
7. COHEN J. J. and BERGER N. A.: Activation of poly (adenosine diphosphate ribose) polymerase with UV irradiation and UV endonuclease treated SV 40 minichromosome. Biochem. Biophys. Res. Commun. 98 (1981), 268.
8. FOSTER W. and MOAT A. G.: Nicotinamide adenine dinucleotide biosynthesis and pyridine nucleotide cycle metabolism in microbial systems. Microbiol. Rev. 44 (1980), 83.
9. HAWKINS D. R.: Treatment of schizophrenia based on the medical model. J. Schizophr. 2 (1968), 3.
10. HAYAISHI O. and UEDA K.: Poly(ADP-ribose) and ADP-ribosylation of proteins. Ann. Rev. Biochem. 46 (1977), 95.
11. JONSSON G. G., KJELLÉN E. and PERO R. W.: Nicotinamide as a radiosensitizer of a C3H mouse mammary adenocarcinoma. Radiother. Oncol. 1 (1984), 349.
12. — — — and CAMERON R.: Radiosensitization effects of nicotinamide on malignant and normal mouse tissue. Cancer Res. 45 (1985), 3609.
13. KJELLÉN E., JONSSON G. G., PERO R. W. and CHRISTENSSON P. I.: Effects of hyperthermia and nicotinamide on DNA repair synthesis, ADP-ribosyl transferase activity. NAD^+ and ATP pools and cytotoxicity in γ-irradiated human mononuclear leukocytes. Int. J. Radiat. Biol. 49 (1986), 151.
14. MA A. and MEDENICA M.: Response of generalized Granuloma Annulare to high-dose Niacinamide. Arch. Dermatol. 119 (1983), 836.
15. MCCREANOR G. M. and BENDER D. A.: The role of catabolism in controlling tissue concentrations of nicotinamide nucleotide coenzymes. Biochim. Biophys. Acta 759 (1983), 222.
16. PREISS J., SCHLAEGER R. and HILZ H.: Specific inhibition of poly ADP ribose polymerase by thymidine and nicotinamide in HeLa cells. FEBS Lett. 19 (1971), 244.
17. PURNELL M. R., STONE P. R. and WHISH W. J. D.: ADP-ribosylation of nuclear proteins. Biochem. Soc. Trans. 8 (1980), 215.
18. SIMS J. L., SIKORSKI G. W., CATINO D. M., BERGER S. J. and BERGER N. A.: Poly (adenosine diphosphoribose) polymerase inhibitors stimulate unscheduled deoxyribonucleic acid synthesis in normal human lymphocytes. Biochemistry 21 (1982), 1813.
19. SKIDMORE C. J., DAVIES M. I., GOODWIN P. M. et coll.: The involvement of poly (ADP-ribose) polymerase in the degradation of NAD caused by gamma-radiation and N-methyl-N-nitrosurea. Europ. J. Biochem. 101 (1979), 135.
20. WINTER S. L. and BOYER J. L.: Hepatic toxicity from large doses of vitamine B_3 (Nicotinamide). New Engl. J. Med. 289 (1973), 1180.