DNA damage and repair in tumour and non-tumour tissues of mice induced by nicotinamide

AR Olsson¹, Y Sheng¹, RW Pero¹, DJ Chaplin³ and MR Horsman⁴

¹Department of Molecular Ecogenetics, University of Lund, 220 07 Lund, Sweden; ²Institute of Environmental Medicine, New York University Medical Center, New York, NY 10016, USA; ³CRC Gray Laboratory, PO Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, UK; ⁴Danish Cancer Society, Department of Experimental Clinical Oncology, Aarhus, Denmark.

Summary In vivo DNA damage and repair was induced by nicotinamide (NAM) in adenotype 12 virus-induced mouse sarcoma A12B3 and sarcoma F inoculated into CBA mice. DNA damage, NAM and NAD concentrations were measured after in vivo exposure to NAM, in tumours and spleens by alkaline elution and by HPLC analysis. Our results indicate that NAM between 100–1000 mg kg⁻¹ causes a high level of in vivo DNA strand breaks in tumours and normal tissues in mice bearing the immunogenic sarcoma A12B3 but not in the non-immunogenic sarcoma F. The repair process was also delayed by the NAM treatment probably due to inhibition of the DNA repair enzyme, poly(ADP-ribose)polymerase, as evidenced by accumulation of NAM and NAD. These data are consistent with NAM having a mechanism of action as a radiosensitiser at least in part due to DNA repair inhibition. In addition, it should also be considered that high doses of NAM might cause considerable complications to normal tissue in tumour-bearing individuals.

Keywords: nicotinamide; NAD; in vivo DNA damage; DNA repair; mouse sarcoma; poly(ADP-ribose)polymerase

Nicotinamide (NAM), the amide derivative of nicotinic acid (niacin, vitamin B₃) is the main precursor for the formation and support of the cellular pool of NAD (Berna Fsky, 1980; Olsson et al., 1993). NAM is considered relatively non-toxic at gram doses (Zackheim et al., 1981) and it has been used clinically to treat pellagra (Green, 1970), schizophrenia (Greenbaum, 1970) and to prevent type I diabetes mellitus (Elliot and Chase, 1991). NAM has also been shown to be a potent radiosensitiser in vitro and in vivo (Jonsson et al., 1984a; Kjellén et al., 1988; Horsman, 1995). This enhancement of radiation response by NAM is believed to be due to an increased tumour blood flow which in turn increases oxygenation of radiosensitive hypoxic cells (Chaplin et al., 1991; Kjellén et al., 1991; Horsman et al., 1989, 1995) and/or by inhibition of DNA repair via the nuclear enzyme poly(ADP-ribose)polymerase (PARP, EC 2.4.2.30) (Ben Hur et al., 1984; George et al., 1986). Furthermore, these radiosensitising effects only occur at relatively high NAM levels of 50–100 mg kg⁻¹ which is far above the threshold limit value of NAM dietary intake (i.e. 20 mg per individual).

NAD is essential for cellular ATP production, maintenance of the cell’s redox potential, and as a substrate for PARP and for the other ADP-ribose transfer reactions (for review see Althaus and Richter, 1987). The PARP enzyme is a DNA-binding enzyme with two zinc fingers in the DNA-binding domain (De Murcia et al., 1989; Molinet et al., 1993). The enzyme activity has been associated with DNA repair, apoptosis, cell proliferation, cell differentiation and genome surveillance (Althaus and Richter, 1987; Satoh et al., 1994; Kaufman et al., 1993; Dai et al., 1997). PARP is activated by DNA strand breaks, which results in consumption of NAD (Jonsson et al., 1984b; Rawling et al., 1993) by producing long-branched polymers of the ADP-ribose moieties and free NAM (De Murcia et al., 1988). NAM, benzamide and 3-aminobenzamide (3aBAM) are all potent inhibitors of PARP at doses above 10–100μM (Rankin et al., 1989) by preventing production of polymer and reduction of the NAD pool after induction of DNA damage (Lautier et al., 1990, 1994; Uchida et al., 1988).

The potential of NAM as a radiosensitiser is currently being evaluated in several clinical trials (Kaanders et al., 1995). However, there have been a number of disturbing toxic side-effects reported in the cancer patients receiving therapeutic doses of NAM (ESTRO, 1994; van der Maazen et al., 1995). In order to understand better the contribution of the possible modes of action of NAM at increasing blood flow or inhibiting DNA repair, we have studied DNA damage and the NAD pool as indicators of toxic end points in CBA mice with or without transplantable mouse sarcoma tumour lines (i.e. sarcoma A12B3 and sarcoma F). The levels of DNA damage and DNA repair were evaluated in vivo to emphasise the potential importance of these parameters, in understanding the mechanism of radiosensitisation and normal tissue toxicity induced by NAM.

Materials and methods

Chemicals

NAD was from Boehringer Mannheim (Germany), 3-aminobenzamide (3aBAM), thymidine, proteinase K and dimethyl sulphoxide (DMSO) were from Sigma Chemical Co. (St Louis, MO, USA), Triton-X 100 was from Eastman (Kodak, Rochester, NY, USA) and RPMI-1640 medium was supplied from Gibco (USA). NAM was purchased from E Merck (Darmstadt, Germany) and was administered i.p. after dilution in 0.9% sodium chloride solution (0.01–0.02 ml g⁻¹ body weight) at a dose of 10–1000 mg kg⁻¹. 3aBAM was dissolved in 50% DMSO and was injected i.p. (5 μl g⁻¹ body weight) at doses of 100, 600 and 1000 mg kg⁻¹.

Cell and tissue sampling

In this study 10–15-week-old CBA mice were inoculated with an immunogenic mouse sarcoma line (adenotype 12 virus, A12B3) (Olsson et al., 1995) or the sarcoma F line (Alison et al., 1991). The tumours were inoculated (one tumour per animal, subcutaneous, rough suspension) in the right flank (A12B3) or in the back (sarcoma F) 12–14 days before the experiments were performed. The final tumour weights were in the range of 200–500 mg. The animals were treated according to the Swedish and the UKCCR guidelines for humane treatment of laboratory animals and the experiments were approved by the ethical committee at the University Hospital in Lund, Sweden.

Correspondence: A Olsson and RW Pero, Department of Molecular Ecogenetics, The Wallenberg Laboratory, University of Lund, Box 7031, S-220 07 Lund, Sweden
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Experimental design and statistics

Any effect of variation in the initial tumour weights (i.e. range, 200 – 500 mm³) was controlled by always including 1 – 3 non-treated tumour-bearing animals in each separate experiment involving evaluation of NAM treatment of tumour-bearing animals (i.e. 6 – 10 animals). The animals were randomly assigned into different dose groups including controls, and the results were calculated as the percentage of the controls in each specific experiment. The separate experiments were then pooled as controls (non-treated) and NAM-treated groups for statistical analyses of an overall effect.

All data were converted to percentage of controls for analysis and presentation. Calculations were carried out by SPSS statistical software. For comparison between NAM doses at any time point, analysis of variance (Anova) was used, and for comparison between two groups the Duncan test was used.

Results

In vivo DNA damage in tumour tissue induced by NAM

The exposure of CBA mice bearing mouse sarcoma A12B3 to a single dose of NAM at 100 or 1000 mg kg⁻¹ (i.p.), in the absence of any other DNA-damaging agent, resulted in a statistically significant induction of DNA strand breaks in tumour tissue after 1.5 h after injection (10 – 20% of control DNA retained on filter), when monitored and quantified with alkaline elution (Figure 1a). However, at a NAM dose of 10 mg kg⁻¹, there was no statistically significant induction of DNA strand breaks in the A12B3 sarcoma line (Figure 1a). CBA mice bearing mouse sarcoma F were much less affected by NAM with significantly different DNA damage levels being only around 85 – 90% of the control values at 100 and 1000 mg kg⁻¹ (Figure 1b). In addition the DNA repair for the sarcoma A12B3 was delayed with less than 25% of the induced damage repaired 24 h after NAM injection of 1000 mg kg⁻¹ and about 50% of the induced damage remained after 48 h (Figure 1a). A similar pattern was observed from the 100 mg kg⁻¹ dose of NAM where it was not possible to measure the repair until 8 h after injection (Figure 1a) and the DNA damage was still 50% of control level after 24 h.

Elevated NAM levels in A12B3 sarcoma

The delay in DNA repair in sarcoma A12B3 at a dose of 100 and 1000 mg kg⁻¹ NAM (Figure 1a) may be a result of inhibition of the DNA repair enzyme PARP. Our results have clearly shown that the NAM concentration measured by HPLC (see Material and methods) was above the reported IC₅₀ value for PARP in vivo (IC₅₀= 100 µM, Rankin et al., 1989), at both 100 and 1000 mg kg⁻¹ (Figure 2). When NAM levels in tumour tissue (sarcoma A12B3) declined to a level close to 100 µM the DNA repair was initiated (Figure 1a). At 10 mg kg⁻¹ there was also a significant elevation of NAM tumour levels compared with control tumours, but the tissue concentration neither reached PARP-inhibiting levels nor was enough to induce a significant amount of DNA damage.

NAD pools in tumour tissue

To investigate whether the PARP enzyme was inhibited in tumour tissue after NAM exposure, the NAD pools, as an indirect measurement of the PARP activity, were determined at 1.5, 4, 24 and 48 h after injection of NAM (Figure 3). If the PARP enzyme was inhibited by NAM levels in vivo, then NAD pools would be expected to increase because PARP consumes NAD as a co-substrate during its participation in DNA repair. In such a way the NAD pools can serve as an indirect measure of PARP activity because DNA damage stimulates this enzyme activity to consume NAD.
The NAD pools were significantly higher at 1.5 h and 4 h after injection of 1000 mg kg\(^{-1}\) in both tumour lines compared with unexposed tumours (NAD levels between 131% and 170% of control tumours, Figure 3). The NAD pool in A12B3 tumours exposed to 1000 mg kg\(^{-1}\) NAM, was significantly higher at 1.5 h but they returned to control levels 4 h after injection, and there was even a slight decrease compared with control levels after 24 h. This reduction may reflect PARP activity and consumption of NAD during DNA repair (Figure 1a). At the 1000 mg kg\(^{-1}\) dose, the pools were back to control levels in both tumour lines after 24 h which corresponded well to the repair of strand breaks (Figure 1a and b).

Any significant difference from control levels in tumours exposed to 10 mg kg\(^{-1}\) was not detected at any time point. This was expected since there was no induction of DNA strand breaks that could activate PARP (Figure 1), and the tumour level of NAM was not high enough to inhibit the PARP enzyme. Moreover, there was only minor induction of DNA damage assessed by alkaline elution or alteration in NAD pools determined in tumours exposed to 3aBAM, a well defined inhibitor of PARP (Table 1), which may exclude the possibility that DNA damage was induced as a result of inhibition of PARP (IC\(_{50}\)=5.4 \(\mu\)M in vivo, Rankin et al., 1989) and DNA repair.

In vivo, DNA damage in normal spleen tissue

To investigate whether the induction of DNA damage was a result of the presence or absence of tumours, normal spleens originating from CBA mice without tumours, and spleens from mice carrying sarcoma A12B3 or sarcoma F tumours, were analysed by alkaline elution after exposure to single doses of NAM at 10, 100 and 1000 mg kg\(^{-1}\). The A12B3 sarcoma-bearing animals had similar levels of DNA damage in their spleens compared with tumour tissues from the same mouse strain at 1.5 h, but the repair was quicker (when compared with tumour tissues (Figure 4), i.e. the half repair time was <4 h). This fact was interpreted to indicate that the induction of DNA damage by NAM is an immediate event even though the tissues are exposed to NAM for a relatively long period of time (Figure 2; Horsman et al., 1993).

Nevertheless, spleens from mice carrying the sarcoma F had only a minor but statistically significant increase in DNA damage at 1.5 h compared with tumour tissue (50–65% for spleen vs 85–100% for tumour, Figure 1b and 5). Spleen tissue from non-tumour-bearing animals did not show any significant induction of DNA damage at any dose (Figure 5), which supports the conclusion that tumour burden is essential for induction of DNA damage by NAM in spleen tissue.

**Discussion**

The data reported in this study contribute two important observations to our understanding of how NAM could
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Figure 3 NAD pools in tumour tissue (a) A12B3 mouse sarcoma and (b) mouse sarcoma F as a function of time after injection (i.p.) of NAM at (□) 100 and (△) 1000 mg NAM kg\(^{-1}\). NAD pools are expressed as a percentage of control±s.e. of 3–7 exposed animals at each data point (unexposed tumours: A12B3 tumours mean: 225 ± 44 μM, \(n = 18\) and F tumours mean: 210 ± 27 μM, \(n = 13\). Indicated areas show 95% confidence interval of mean). NAD content was analysed by HPLC (see Material and methods).

Figure 4 DNA damage and repair measured by alkaline elution in spleen tissue from tumour-bearing animals (sarcoma A12B3). Tumour-bearing CBA mice were treated with NAM at doses of (○) no exposure, (□) 10, (□) 100 and (△) 1000 mg kg\(^{-1}\), and sacrificed at indicated time points after the drug was administrated. Results are presented as means±s.e. of 3–6 animals. *Statistically significant difference (\(P<0.05\), Duncan test) compared with the control group.

Figure 5 DNA damage in spleens from CBA mice (□) without tumour, (□□□) with subcutaneously transplanted sarcoma F tumours, and (□□□□) with subcutaneously transplanted A12B3 sarcoma tumours. The data represents DNA retained on filters as percentage of control and staples show the mean±s.e. of 3–5 animals. *Statistically significant difference (\(P<0.05\)) compared with non-tumour-bearing animals and **statistically significant difference (\(P<0.05\), Duncan test) compared with animals with sarcoma F tumours.

Radiosensitise or induce toxic side-effects; namely (1) NAM can by itself, without co-administration of radiation, induce DNA damage and inhibit DNA repair at doses that are known to radiosensitise, and therefore, increasing tumour blood flow and overcoming tumour hypoxia is not the only radiosensitising mechanism that NAM can modulate; and (2) NAM can induce DNA damage in normal tissue of tumour-bearing animals which may in turn contribute to its toxic side-effect profile.

Our results could have been influenced by differences in tumour biology such as hypoxia or necrosis. However, we have considered this variable by always including non-treated tumour-bearing animals as a control over variations in tumour physiology for each separate experiment. As a consequence, our data has indicated that within the tumour weight range of 200–500 mg there was no measurable influence of the effects of tumour size on the NAM treatment.

The predominant radiosensitising mechanism of NAM is believed to be through increased tumour blood flow and oxygenation leading to an increase in radiation-induced DNA damage (Horsman et al., 1989; Lee et al., 1993; Hirst et al., 1993). In this study the induction of DNA strand breaks at NAM doses of 100 mg kg\(^{-1}\) and 1000 mg kg\(^{-1}\) (Figures 1 and 2) could have been explained by enhanced tumour oxygenation of hypoxic cells resulting in increased production of oxygen radicals. However, this explanation cannot explain the induction of DNA damage in spleens from tumour-bearing animals where the tissue is expected to be fully oxygenated (Figure 5). Moreover, DNA damage induced by NAM in spleens from tumour-bearing animals had a repair half-time less than 4 h (Figure 4) compared with >24 h in tumour tissue (Figure 1a). This point could be explained if NAM clears much faster from the spleen than from tumours, and hence there would be less of an opportunity for NAM to accumulate and inhibit DNA repair.
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Figure 6 DNA damage in spleen cell suspensions 1.5 h after injection of NAM at a dose of 1000 mg kg⁻¹ plotted against the average spleen weight of CBA mice; (○) without tumours; (x) with subcutaneously transplanted sarcoma F tumours; (*) with subcutaneously transplanted sarcoma A12B3 tumours. Data points represent means ± s.e., n ≥ 3 animals. Linear regression analysis of all the individual data points included gave a correlation coefficient of 0.885; P < 0.001, n = 12.

Another important consideration is that NAM had a direct dose-dependent effect on DNA damage in spleens and tumours in tumour-bearing animals (Figures 1 and 5). Whether NAM produced DNA damage by direct interaction with DNA, or by NAM triggering some other cellular metabolic event leading to DNA damage, the consequence was a fast and huge induction of DNA damage (Figures 1, 4 and 5) similar to the dose-dependent induction of DNA damage by ionising radiation alone or in combination with metollopamide exposure shown earlier in the same experimental model (Olsson et al., 1995). One plausible mechanism that could explain these results is that certain normal tissues susceptible to infiltration by phagocytes may accumulate DNA damage via activation of the respiratory burst. Endogenous oxidative stress induced by NAM in phagocytes would then be the mode of action (Shacter et al., 1988; Cochrane, 1991). Support for this hypothesis has been obtained when the size of the spleens from tumour- and non-tumour-bearing animals were plotted vs the level of induced DNA damage in the spleens (Figure 6). The increase in weight of the spleen tissue could have been the result of an increased number of infiltrating phagocytes and leucocytes as a consequence of immunogenetic tumour burden. The extent of the immunogenetic response and the level of infiltration in the sarcoma A12B3 and sarcoma F tumours are currently under investigation in our laboratory. These data could be very important from a clinical perspective because they indicate that in the presence of tumour burden, NAM could cause unexpected side-effects like DNA damage to normal tissues.

The effective dose of NAM on blood flow is reported to be at concentrations from 100 mg kg⁻¹ and above (Chaplin et al., 1990). Horsman et al. (1993) showed that there is no significant difference between the radiosensitising effect of NAM at doses of 100 mg kg⁻¹ and 1000 mg kg⁻¹ in a C3H mammary carcinoma, even though the difference in plasma and tumour concentrations of NAM was 5- to 10-fold. These data have suggested an alternative explanation for the radiosensitising properties of NAM; namely that NAM at tumour concentrations of 100 μM or higher inhibits the PARP enzyme (IC₅₀ = 30–100 μM) (Molinet et al., 1993), which has been shown to be involved in DNA repair (Satho and Lindahl, 1994). The induced DNA damage at NAM doses of 100 and 1000 mg kg⁻¹ could be a result of PARP inhibition because of high tumour tissue levels of NAM (Figure 2), which in turn resulted in elevated NAD levels (Figure 5) and accumulation of DNA damage (Figure 1). Later, when NAM was cleared from tumour tissue and returned to its original, steady-state level, the DNA repair was initiated (Figures 1 and 2), and a drop in the NAD pool owing to PARP activity below control levels was expected and observed (Figure 3). Furthermore, the NAD pools in tumour tissue 1–2 h after administration of 100 mg kg⁻¹ or 1000 mg kg⁻¹ NAM was increased over controls presumably owing to PARP inhibition, and thereby, they decreased towards control levels between 24–36 h (Figure 3) when PARP was not inhibited and NAD was consumed.

In another effort to implicate PARP inhibition in the mode of action of NAM, we have used the more powerful PARP inhibitor, 3aBAM. It has been demonstrated previously that when PARP is inhibited by 3aBAM (IC₅₀ = 5.4 μM, Rankin et al., 1989) instead of NAM, the NAD pools are elevated both in vivo and in vitro in normal tissues (Uchida et al., 1988; Smit and Stark 1994). However, we could not show any elevation of the NAD pools in tumour tissues after exposure of tumour-bearing mice to 3aBAM at doses between 100 mg kg⁻¹ and 1000 mg kg⁻¹ (Table 1). Hence these data suggest that the observed DNA-damaging effects of NAM treatment as opposed to 3aBAM treatment are more influenced by NAD metabolism supplementing NAD pools than by PARP activity which is 3–8 times higher in tumour tissue compared with normal tissues (Hirai et al., 1983; Pero et al., 1985; Singh, 1991). For example both 3aBAM and NAM do not 100% inhibit PARP, and so there would always be a demand for NAD. NAM treatment would support NAD synthesis whereas 3aBAM treatment could result in NAD pool depletion especially in the presence of amplified PARP activity. Lautier et al. (1990, 1994) showed in C3H101/2 cells, that PARP-inhibiting doses of 3aBAM (100 μM) did not inhibit NAD catabolism completely after exposure to active oxygen species even if poly(ADP-ribose) polymer production was stopped. In addition, there was no effect on the level of DNA damage or changes in the NAD pool after exposure to NAM at 10 mg kg⁻¹ (Figures 1 to 3, a dose not sufficient to effect PARP inhibition or blood flow). Taken together these data are consistent with the primary effects of NAM treatment being on NAD metabolism as well as on PARP inhibition when considering the possible radiosensitising mechanism of induction of DNA damage.

Abbreviations
3aBAM, 3-aminobenzamide; DMSO, dimethyl sulphoxide; HPLC, high performance liquid chromatography; i.p., intraperitoneally; NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; PARP, poly(ADP-ribose)polymerase; PCA, perchorlic acid; SSB, single strand breaks; cTidH, thimidine.

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