Reduction of Tumor Hypoxia and Inhibition of DNA Repair by Nicotinamide after Irradiation of SCCVII Murine Tumors and Normal Tissues

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ABSTRACT

The alkaline comet assay was used to measure both tumor hypoxic fraction and DNA strand break rejoining kinetics in individual cells from tumors and tissues of C3H/HeN mice exposed to ionizing radiation and nicotinamide. The percentage of hypoxic cells in SCCVII murine squamous cell carcinoma decreased from 18.4 to 4.4% in mice injected with a clinically relevant dose of 200 mg/kg nicotinamide 30 min before irradiation. At higher doses (500 and 800 mg/kg), nicotinamide also increased the half-time of strand break rejoining in tumor, thymus, spleen, bone marrow, and testis from 10–20 min to 40–80 min. Cells from the brain rejoined radiation-induced breaks 3–5 times more slowly than did cells from other tissues and showed no additional delay after nicotinamide. Cells with extensive numbers of strand breaks appeared 24 h after treatment with nicotinamide and radiation and 48 h after treatment with radiation alone. For most tissues, damage was more consistent with necrosis than with apoptosis. The percentage of heavily damaged cells was dependent on tissue type, time after irradiation, radiation dose, nicotinamide dose, and sequence of administration. In SCCVII tumors of air-breathing mice, nicotinamide enhanced radiation-induced cell killing primarily in cells close to the vasculature, but in tumors clamped before irradiation, 500 mg/kg nicotinamide did not increase cell kill. It appears that in addition to promoting tumor reoxygenation, nicotinamide inhibits DNA strand break rejoining in tumors and most normal tissues and promotes the earlier appearance of radiation-damaged cells, perhaps through inhibition of poly(ADP-ribose) polymerase.

INTRODUCTION

In 1986, nicotinamide, the amide derivative of vitamin B3, was reported to significantly improve the response of solid murine tumors to ionizing radiation (1). This drug is now in Phase II clinical trials in combination with carbogen breathing and accelerated radiotherapy with the expectation that this combination will be particularly effective in sensitizing rapidly proliferating tumors containing radiation-resistant hypoxic cells (2, 3). The rationale for combining these agents with radiation is that carbogen will increase the oxygenation of diffusion-limited hypoxic cells distant from blood vessels, whereas nicotinamide will increase the oxygenation of tumor cells that undergo perfusion-related (transient) hypoxia (4, 5). However, initially it was thought that radiosensitization by nicotinamide was due to inhibition of DNA repair by interference with the function of the nuclear enzyme PARP. This enzyme is activated by the presence of DNA strand breaks to bind sites of DNA damage and influence accessibility to repair enzymes (6). Moreover, poly(ADP-ribosyl)ation results in modification of important nuclear proteins, including histones, topoisomerases, DNA polymerase α and β, and DNA ligase 2 (7). Although growth of cells in nicotinamide-deficient media can inhibit DNA strand break rejoining (8), addition of nicotinamide was also shown as early as 1970 to enhance cell killing by ionizing radiation (9), and later experiments revealed that it inhibited DNA strand break rejoining (10, 11). These observations are consistent with its role as an inhibitor of PARP (12). However, the predominant mechanism of solid tumor radiosensitization by nicotinamide is now believed to be reoxygenation via an increase in tumor perfusion at the microregional level (5). Although nicotinamide does not appear to inhibit oxygen consumption rate (4), it can increase microregional tumor blood flow even in the face of a decrease in blood pressure (13–15). Those tumor cells subjected to transient hypoxia through occasional stoppages in tumor blood flow are believed to be most responsive to reoxygenation by nicotinamide (16). Nicotinamide has been shown to decrease interstitial fluid pressure (17, 13), which is believed to contribute to this form of tumor hypoxia (18).

The combination of nicotinamide and carbogen breathing appears so effective in sensitizing tumors to radiation that the suggestion has been made that perhaps there is an additional mechanism of interaction with radiation that goes beyond reoxygenation (3, 19). The fact that there is a small radiosensitization of normal tissues would also support this theory. Ono et al. (20) observed an enhancement ratio of 1.07 for jejunum and 1.15 for bone marrow for relatively low doses of nicotinamide, and Kjellen et al. (21) found an enhancement ratio of 1.07 for kidney in air-breathing animals. The combination of nicotinamide, carbogen, and accelerated radiotherapy is not without toxic side effects (2). The idea that DNA repair might be compromised by nicotinamide was therefore reexamined using a method that can be applied to quantify both tumor oxygenation and DNA single-strand break rejoining in vivo.

The comet assay, originally developed to measure DNA damage in individual cells (22), has been adapted for the detection of hypoxic cells in solid tumors and normal tissues (23–25). Ionizing radiation produces 3 times more DNA single-strand breaks in aerobic cells than in hypoxic cells (26, 27), forming the basis for the measurement of hypoxic fraction using the comet assay. Some studies have shown that nicotinamide increases tumor oxygenation measured using oxygen electrodes (28, 29), whereas other studies using electrodes or cryospectrophotometry have shown little or no increase in oxygenation after treatment with nicotinamide (5, 15, 30–32). The reason for these inconsistencies is not clear; however, analysis of hypoxia at the level of the individual tumor cell may be important to demonstrate the effect of an agent the primary action of which is to modify tumor perfusion at the microregional level. Therefore, our first goal was to measure cellular hypoxic fraction in murine tumors exposed to nicotinamide before radiation. In SCCVII murine tumors used for this study, nicotinamide appears to decrease both perfusion and diffusion-limited hypoxia (16, 33). The comet method has also been used to measure rejoining rates of DNA strand breaks in vivo (34, 35), and from the few studies examining break rejoining in normal tissues, it is clear that induction and repair of strand breaks can be influenced by differentiation state and hypoxia (36). Cell separation or novel labeling methods have been used in the past to examine the DNA rejoining capacity of different cell subpopulations in normal tissues (37–39). However, analysis of DNA repair at the level of the individual cell using the comet assay can provide information on heterogeneity in response within a complex normal tissue. We therefore applied the comet assay to examine radiation-induced DNA damage...
and repair in cells obtained from tumors and a variety of murine tissues and then to determine whether nicotinamide would reduce single-strand break rejoining rates after irradiation.

MATERIALS AND METHODS

SCC VII Tumor Cells. SCC VII squamous cell carcinoma cells were transplanted s.c. over the sacral region of inbred male C3H/HeN mice weighing approximately 30 g. Tumors were used for experimentation approximately 2 weeks later when they had reached a weight of 400–600 mg. Nicotinamide was injected i.p. from a fresh stock solution (48 mg/ml) in sterile PBS. Mice received whole-body irradiation from a 250-kV X-ray unit at a dose rate of 3.3 Gy/min. After irradiation, mice were sacrificed by cervical dislocation; tumors were rapidly excised and placed in ice-cold PBS. A single-cell suspension was prepared from the entire tumor by mincing the tissue and filtering the suspension through 30 μm nylon mesh. The suspension was then centrifuged and resuspended in ice-cold complete medium to provide a cell yield of approximately 10⁷ cells/g.

Preparation of Tissues for Comet Analysis. Organs were removed to a large volume of ice-cold PBS within 30 s after irradiation. Bone marrow was obtained by flushing a core of tissue from the tibia. Entire testis, thymus, tumor, and spleen were examined. Tissues were chopped with crossed scalpels in ice-cold PBS containing 2 mm EDTA, and the 3-kW mesh was filtered through 30 μm nylon mesh. Cells were centrifuged, and pellets were resuspended in PBS for dilution for the comet assay. All tissues were treated in an identical fashion, and no changes were required in the standard comet preparation or analysis method. The one exception was the analysis of DNA damage and rejoining in some cells from testis. Elongated spermatids were routinely omitted from our analysis because cells failed to lyse under standard conditions. Lysis could be achieved by the addition of β-mercaptoethanol to the lysis solution, but this was avoided because it produced unacceptably high background levels of damage in the alkaline comet assay as reported previously (40). Images of elongated spermatids were omitted from analysis based on DNA content (haploid), lack of DNA damage, and characteristic ovoid shape of the propidium iodide-stained comet.

Hoechst 33342 Tumor Cell Sorting. To examine the response of tumor cells to radiation as a function of their distance from the functional vasculature, mice were injected i.v. with 0.1 ml of the fluorescent perfusion stain Hoechst 33342 (8 mg/ml in PBS) approximately 20 min before asphyxiation or irradiation. After irradiation, mice were sacrificed and tumors were removed and disaggregated enzymatically as described previously (41). Tumor cells were exposed for 5 min on ice to a 1:100 dilution of FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) to stain macrophages, which are the most abundant normal cell constituent of this tumor (41). IgG-negative cells were sorted on the basis of Hoechst 33342 concentration into the 10% most dimly fluorescent and 10% most brightly fluorescent populations.

DNA Damage Measured Using the Comet Assay. For the alkaline comet assay, single cells from spheroids and SCC VII tumors were suspended in ice-cold PBS at a concentration of 2 × 10⁴ to 4 × 10⁵ cells/ml. Then 0.5 ml cell suspension (10⁴ cells) was placed in a 5-ml disposable tube and 1.5 ml 1% low gelling temperature agarose (Sigma type VII prepared in distilled water and held at 40°C) were added to the tube. Then 1.5 ml were quickly pipetted onto a half-frosted microscope slide and allowed to gel for about 1 min on a cold surface. Slides were carefully submersed in an alkaline lysis solution containing 1 M NaCl, 0.03 M NaOH, and 0.1% sarkosyl for 1 h followed by a 1-h wash in 0.03 M NaOH plus 2 mM EDTA before electrophoresis in a fresh solution of 0.03 M NaOH plus 2 mM EDTA at 0.6 V/cm for 25 min. Slides were rinsed and stained for 10 min in 2.5 μg/ml propidium iodide.

Individual cells or comets were viewed using a Zeiss epifluorescence microscope attached to an intensified solid-state charge-coupled device camera and image analysis system (34). As the number of DNA strand breaks increased, the amount of DNA able to migrate away from the comet head increased proportionate to dose. The "tail moment," defined as the product of the fraction of DNA in the comet tail multiplied by the distance between the means of the head and tail distributions, and "DNA content," defined as the total fluorescence associated with an image, were calculated (34). Tail moment histograms generated from 200 or more comets from the same treated population.

RESULTS

Nicotinamide injected i.p. 30–90 min before irradiation significantly reduced the fraction of hypoxic cells in SCC VII tumors (Fig. 1). The percentage of hypoxic cells decreased from 18.4 ± 6.6% in control tumors to 2.9 ± 2.8% for tumors of mice injected with 800 mg/kg nicotinamide 60–90 min before irradiation and to 4.4 ± 7.6% for mice injected with 200 mg/kg nicotinamide 30 min before irradiation (Table 1). A shorter interval between nicotinamide injection and irradiation was chosen for lower doses of nicotinamide due to the earlier peak drug concentration at lower doses (42).

Irradiation of cells from tumors and normal tissues on ice in vitro resulted in comet dose-response curves with identical slopes (Fig. 2, B). For cells from normal tissues irradiated in vivo and subsequently extracted for comet analysis, there was a decrease in the slope of the dose-response curves because rapid repair occurred in these tissues during and after irradiation and continued until repair was inhibited by immersion of tissue in ice-cold PBS. A larger difference observed between the in vitro and in vivo dose-response curves for a tissue can therefore be an indication of a more rapid DNA repair rate. The displacement between the two curves was smallest for cells from cerebellum. For SCC VII tumors and testis, the large displacement between the curves is a result, in part, of the presence of radiosensitive hypoxic cells in vivo.

To examine the kinetics of DNA strand break rejoining in vivo, tumors and normal tissues were excised at various times after 15 Gy, and single-cell suspensions were analyzed for DNA damage using the comet assay. Results shown in Fig. 3 indicate a range of DNA repair rates for these different tissues. The estimated time to rejoin half of the breaks (uncorrected for repair occurring during irradiation or for residual unrepaired damage) is indicated in Table 2. Note that this is not the rejoining half-time of the "fast" component, as is often reported, but is simply the time required to rejoin half of the breaks.

Fig. 1. Effect of nicotinamide on hypoxic fraction in SCC VII tumors measured using the comet assay. Mice were exposed to 15 Gy only (a–c), to 200 mg/kg nicotinamide 30 min before 15 Gy (d–f), or to 800 mg/kg nicotinamide 60–90 min before 15 Gy (g–i). Immediately after irradiation, tumors were excised, and single cells were analyzed for DNA damage using the alkaline comet assay. Tail moment histograms were fit to two normal distributions representing the hypoxic and aerobic populations in the tumor (23).

2802
Nicotinamide (800 mg/kg) administered 60–90 min before irradiation inhibited the rate of DNA strand break rejoining in all normal tissues with the exception of brain (Fig. 3). Injection of 500 mg/kg 60 min before irradiation was less effective in inhibiting single-strand break rejoining, and no significant inhibition was observed if only 200 mg/kg were administered 30 min before irradiation. In tumors and testes, there was also an apparent increase in initial DNA damage in the nicotinamide-treated animals, presumably as a result of nicotinamide-induced reoxygenation. By 4 h after treatment, there was no significant difference in residual damage in normal tissues of irradiated animals treated with or without nicotinamide. However, at 24 h after treatment, a large increase in DNA damage was observed in tumors and most normal tissues exposed to both nicotinamide and radiation. Intercellular heterogeneity in DNA damage or rejoining kinetics in response to radiation alone was relatively small (Fig. 4), although there was some indication of heterogeneity in the 15-min repair results. At 48 h after treatment, extensive DNA degradation in some cells of the population was apparent in all tissues.

The increase in damage 24 h after nicotinamide and radiation was also characterized by the presence of a fraction of heavily damaged cells. Fig. 5 shows results for testis exposed to radiation alone (a–c), drug alone (d–f), or the combination of the two agents (g–i). No heavily damaged cells were observed when tissues were excised 24 h after treatment in animals exposed to 15 Gy alone or 800 mg/kg nicotinamide alone (Fig. 5, d–f). With the exception of the cerebellum, all normal tissues exposed to both nicotinamide and radiation showed an increase in the presence of heavily damaged cells, which was not observed 24 h after 15 Gy alone (data not shown). Interestingly, injection of nicotinamide immediately after irradiation (Fig. 5k) did not produce the same population of damaged cells at this time.

A time course of the effects of radiation and nicotinamide is shown in Fig. 6. In mice treated with both nicotinamide and radiation, the presence of heavily damaged cells in testis appeared to be maximum around 24 h and returned to background levels of damage by 48 h. However, heavily damaged cells were observed in testis treated with 15 Gy alone when analyzed 48 h after treatment. Irradiation followed by 800 mg/kg nicotinamide did not promote the earlier appearance of damage (Fig. 6b, □). Analysis of the effects of radiation dose and nicotinamide dose on this delayed damage are shown in Fig. 7 for testis and SCCVII tumor cells examined 24 h after treatment. Reduction of the dose to 2 Gy considerably reduced but did not eliminate damage by 800 mg/kg nicotinamide. Similarly, 200 mg/kg nicotinamide was still capable of producing some heavily damaged cells in both tissues (Fig. 7). Histological examination of tissue sections 24 h after treatment with radiation alone or radiation after treatment with nicotinamide confirmed the earlier appearance of apoptotic cells.
Table 2  Inhibition of DNA strand break rejoining by nicotinamide

Mice were irradiated with 15 Gy, and cell suspensions were prepared and analyzed for DNA damage using the comet assay.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>t_{1/2}^a</th>
<th>t_{1/2} + 800 mg/kg</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>16</td>
<td>60</td>
<td>3.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>22</td>
<td>86</td>
<td>2.9</td>
</tr>
<tr>
<td>Thymus</td>
<td>12</td>
<td>41</td>
<td>3.4</td>
</tr>
<tr>
<td>Testis</td>
<td>10</td>
<td>35</td>
<td>3.5</td>
</tr>
<tr>
<td>Tumor</td>
<td>9</td>
<td>55</td>
<td>6.1</td>
</tr>
<tr>
<td>Brain</td>
<td>48</td>
<td>48</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a t_{1/2}, time in min required to rejoin one-half of the initial amount of strand breaks, determined by interpolation of results shown in Fig. 3 after correction for average background damage.

(shrunken cells, densely-staining condensed chromatin, and apoptotic bodies) in spleens of mice exposed to nicotinamide before irradiation. However, similar apoptotic changes were not observed in sections from tumor or testis at this time. Gel electrophoresis indicated the presence of nucleosomal ladders in DNA samples from irradiated spleen and thymus 4–8 h but not 24 h after irradiation and nicotinamide (data not shown).

Treatment of mice with 500 mg/kg nicotinamide 60 min before irradiation increased tumor cell killing when the tumor was excised and cells analyzed for clonogenicity immediately after irradiation (Fig. 8a). Interestingly, nicotinamide increased radiation cell killing to a much greater extent in the population of tumor cells closest to the functional vasculature (sort fraction 1). This population should be largely aerobic but includes cells that are affected by transient fluctuations in perfusion (43). Qualitatively similar results were obtained using 800 mg/kg nicotinamide 90 min before irradiation. Nicotinamide alone at 500 or 800 mg/kg did not affect plating efficiency. Tumors of mice clamped 90 min after i.p. injection of 500 mg/kg nicotinamide, then irradiated and excised immediately after irradiation, showed no additional cell killing relative to asphyxiation and irradiation only (Fig. 8b).

DISCUSSION

Our results are consistent with many studies in the literature indicating that the major effect of nicotinamide is to reduce the fraction of radiobiologically hypoxic cells in solid tumors and thereby increase cell sensitivity to killing by ionizing radiation. The comet data clearly indicate that hypoxic fraction is reduced after treatment with nicotinamide. Reoxygenation as estimated using the comet assay was relatively independent of dose of nicotinamide over the range of 200–800 mg/kg, in agreement with
results by Chaplin et al. (44), who showed that 250 mg/kg nicotinamide was as effective as 1000 mg/kg in reoxygenating the SCCVII tumor. In our experiments, testis also responded to nicotinamide treatment by showing, like tumors, a significant increase in "initial" DNA damage (Fig. 2d), most likely the result of reoxygenation before irradiation. This finding is consistent with previous results by Schlappack et al. (45) in which the authors concluded that the PARP inhibitor 3-aminobenzamide increased the oxygenation status of the mouse testis before irradiation.

Cell survival data shown in Fig. 8a support results of Horsman et al. (4, 5) indicating that nicotinamide reduces perfusion-limited hypoxia. In these cell sorting experiments, the increase in cell killing occurred primarily in cells that stained most intensely with Hoechst 33342 (i.e., those cells closest to the functional blood supply and therefore more likely to be well oxygenated). However, the protocol used to stain these cells (i.v. injection 10 min before irradiation) does not ensure that they will be well oxygenated at the time of irradiation, and in fact the relatively poor radiation toxicity gradient through the tumor cord has been explained on the basis of changes in the pattern of perfusion occurring between the time of Hoechst 33342 injection and the time of tumor irradiation (43). The increase in the gradient of killing through the tumor cord for nicotinamide-treated animals is consistent with a decrease in these fluctuations in perfusion. Exposure of tumors to 15 Gy alone produced the same average cell killing as exposure to 10 Gy plus 500 mg/kg nicotinamide, indicating an enhancement ratio of 1.5.

Radiation-induced DNA strand break induction and rejoining were found to be tissue type dependent, with more rapid rates of rejoining in tissues maintaining some proliferative potential and a slower rate in the terminally differentiated, nondividing brain cells. The slopes of the radiation dose-response curves generated with tumor and normal cell types in vitro were essentially identical, indicating that there are no intrinsic differences in radiosensitivity as detected by single-strand break induction. As with earlier studies (36, 39, 46, 47), differences in amount of "initial" radiation damage in vivo can be explained by the presence of hypoxic cells (tumor, testis), or differentiated cells that

![Fig. 5. Heterogeneity in DNA damage in cells from testes after treatment with radiation and/or nicotinamide. DNA damage in cells from testes was analyzed at various times after 15 Gy only (a-c), 800 mg/kg nicotinamide only (d-f), and 800 mg/kg nicotinamide followed 60 min later by exposure to 15 Gy (g-i). Results are also shown for 500 mg/kg nicotinamide only (j), 500 mg/kg given i.p. immediately after 10 Gy (k), or 500 mg/kg nicotinamide given 60 min before 10 Gy (l). The time of analysis after irradiation or drug treatment is indicated for representative histograms.]

![Fig. 6. Time course for DNA degradation in the testis after nicotinamide and/or irradiation. a, exposure to 15 Gy alone or 500 mg/kg nicotinamide alone. b, exposure to combinations of nicotinamide and radiation. Data points, means for three independent experiments (100–200 comets each); bars, SE.]

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800 mg/kg inhibited DNA strand break rejoining, less effect was observed after 500 mg/kg, and no significant inhibition was evident after 200 mg/kg.

Extensive DNA fragmentation was observed in some cells of most normal tissues 48 h after whole-body irradiation. Although some studies indicate that PARP inhibitors can affect apoptosis (51–53), preliminary histological and gel electrophoresis results support necrosis rather than apoptosis as the primary mode of cell death at this time. Nucleosomal ladders were present in DNA samples from spleen 4–8 h after irradiation, but not 24 h later. The identification of a population of heavily damaged cells 24 h after treatment with radiation and nicotinamide argues for involvement of nicotinamide in DNA repair after radiation damage. Again, the only tissue that did not show heavily damaged cells was the brain, suggesting a possible link between inhibition of radiation-induced strand break rejoining and the appearance of damaged cells. Interestingly, nicotinamide given after rather than before irradiation did not promote the earlier appearance of DNA damage. Nicotinamide, by enhancing metabolic activity through a substantial increase in levels of NAD$^+$ (32), may have promoted the toxic effects of radiation so that they were evident at an earlier time. However, this mechanism should presumably operate whether nicotinamide is given before or after radiation, and clearly, the earlier appearance of damaged cells requires that nicotinamide be given before radiation.

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rejoin breaks more slowly (cerebellum). An advantage of the comet assay over other methods used to measure DNA damage and repair is the ability to examine heterogeneity in response at the level of the individual cell. However, although each normal tissue is composed of a variety of cell types, heterogeneity in radiation-induced DNA damage and rejoining was comparatively small, so that the mean tail moment was fairly representative of the entire population (Fig. 4).

Nicotinamide inhibited rejoining half-time by a factor of 3–6 in all tissues except the brain. The lack of effect on brain tissue may relate to the much slower repair capacity of cells from this organ. Access of brain cells to the drug could also be an important consideration because positron emission tomography studies indicate that brain uptake of $^{11}$C-nicotinamide is low relative to uptake by liver, kidney, and lymph nodes (48). The degree of inhibition of strand break rejoining is in rough agreement with earlier results using various PARP inhibitors on cultured cell lines or thymocytes (11, 49, 50). SCCVII tumor cells appear to show a greater inhibition of rejoining by nicotinamide than normal tissues (Table 2), and tumor is the only tissue to show significantly more damage remaining at 4 h after nicotinamide and radiation than radiation alone, suggesting a favorable therapeutic ratio. However, although

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**Fig. 7.** Degradation of DNA in testis (a) and SCCVII tumors (b) 24 h after exposure to nicotinamide and radiation. Mice were injected with nicotinamide 60 min before irradiation. Twenty-four h later, tissues were removed and single cells were analyzed for DNA damage (average tail moment using the alkaline comet assay. Results of 30 independent experiments (100–200 comets each) are shown.

**Fig. 8.** Effect of nicotinamide on the response of SCCVII murine tumor cells to ionizing radiation. a, mice were injected i.p. with 500 mg/kg nicotinamide, and 70 min later, animals received an i.v. injection of Hoechst 33342. Twenty min later, mice were irradiated with 0, 10, or 15 Gy. Tumors were removed immediately and a single-cell suspension was prepared. Cells were sorted according to the Hoechst 33342 fluorescence gradient, in which fraction 1 represents the most fluorescent cells in the tumor (and therefore those cells closest to the functional vasculature), and fraction 10 represents those most distant from the functional blood supply. Data points, means for 3–4 experiments are shown; bars, SE. b, mice were injected i.p with 500 mg/kg nicotinamide and i.v. with Hoechst 33342; 70 min later; however, 20 min later tumors were clamped immediately before irradiation with 15 or 20 Gy. Open symbols, response without nicotinamide pretreatment; closed symbols, response with nicotinamide pretreatment before irradiation. Data points, means of 3–5 experiments are shown. Symbols near the ordinate show the response of unsorted samples.
Although the reported small enhancement of radiation toxicity by nicotinamide in normal tissues may be explained by high NAD+ levels or inhibition of PARP, in tumors it is less certain that nicotinamide acts by a mechanism other than reoxygenation to sensitize cells to killing by ionizing radiation. To address this question, the influence of nicotinamide on tumor reoxygenation must be eliminated. This is most easily accomplished by clamping the tumor or asphyxiating the mouse before irradiation. Horseman et al. (4) found that 1000 mg/kg nicotinamide given 90 min before 20 Gy was ineffective in increasing EMT6 tumor cell kill when tumors were clamped during irradiation. This result suggested that the only effect of nicotinamide was to reoxygenate the tumor. Our results agree with this conclusion because no additional cell killing could be detected in SCCVII tumors of mice treated with 500 mg/ml nicotinamide before asphyxiation and irradiation (Fig. 8b).

It is still possible, however, that a DNA repair-related radiosensi-

tection effect of nicotinamide is confined to well-oxygenated
tumors. This is most easily accomplished by clamping
the hypoxic fraction appeared to be marginally different in these
immediate after irradiation may prevent us from seeing this
effect of nicotinamide. In cell sorting experiments, radiosensitiza-
tion after 200 mg/kg nicotinamide was always less than radiosensi-

tization after 500 mg/kg (data not shown), in spite of the fact that
the hypoxic fraction appeared to be marginally different in these
two situations (Table 1).

Our results support the suggestion by Rojas et al. (19) that the
impressive gain shown by nicotinamide and carbogen combined
with radiation may not be solely due to an increase in tumor
reoxygenation but may involve inhibition of DNA repair by nicotin-
amide.

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