Nicotinamide Can Lower Tumor Interstitial Fluid Pressure: Mechanistic and Therapeutic Implications

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Abstract
Several investigators have shown that nicotinamide (NA) may increase the tumor blood flow and/or alleviate temporal fluctuations in tumor blood flow and, consequently, increase pO2. However, the mechanisms of these changes in tumor blood flow are not understood, especially because NA lowers the mean arterial blood pressure in mice. Our hypothesis is that NA may decrease flow resistance in tumors, which would lower vascular pressure and tumor interstitial fluid pressure (TIFP). To test this hypothesis, we measured the physiological parameters: mean arterial blood pressure, TIFP, tumor water content, and hematocrit in C3H mice bearing FSall tumors, before and after treatment with 500 mg/kg of NA. In control animals, TIFP increased with tumor growth up to 400 mm3, reached a plateau, and then decreased when the tumor size was above 1000 mm3 (n = 135). Tumor water content correlated significantly with TIFP (for tumors <500 mm3) (n = 26). NA caused approximately a 15% decrease in mean arterial blood pressure (P < 0.05) and a 35% decrease in TIFP (P < 0.001) at 2 h postinjection, without any change in hematocrit. The change in TIFP was found to be tumor size dependent. Specifically, NA decreased the TIFP by 47% (P < 0.001) and 39% (P < 0.001) in medium (200 to 500 mm3) and large (500 to 800 mm3) tumors, respectively. The decrease in TIFP in small (<200 mm3) and very large (>800 mm3) tumors was not statistically significant (P > 0.1). Our results explain the size-dependent enhancement in pO2 and radiation response reported by I. Lee and C. W. Song (Radiat. Res., 130: 65-71, 1992) for this tumor line. If our results could be confirmed in human tumors in situ, they would have significant implications in noninvasive measurements of TIFP using NMR and in cancer treatment using radiation, chemotherapy, and immunotherapy.

Introduction
Since the original study of Jonsson et al. (1), several investigators have shown that NA, the amide form of vitamin B3, can radiosensitize murine tumors (2-4). This radiosensitization presumably results from the increased oxygenation by NA, as directly measured using microelectrodes by Lee and Song (4). The increased oxygen tension in tumors results from reduced oxygen utilization and/or increased oxygen availability through modifications in tumor blood flow (5-7). While there are no direct measurements of in vivo oxygen utilization following NA injection, several investigators have shown that NA increases TBF and/or decreases transient fluctuations in TBF (3, 4). However, what factors led to these changes in TBF is not known.

It is well known that TBF is proportional to P\_A - P\_V and inversely proportional to the FR (7). Stone et al. (8) and Horsman et al. (9) have shown that NA decreases the MABP of mice. Therefore, the only mechanism of the observed changes in TBF is a decrease in P\_V and/or FR. How is it possible to lower P\_V and FR with NA? FR has two components: geometric and viscous resistance; both are elevated in tumors (10, 11). The former results from the peculiar morphology of the tumor vasculature (10) and the latter from the altered rheology in tumor vessels (11). If NA decreases the rigidity or adhesion of RBC, WBC, or cancer cells in the tumor vasculature, it will lower the transient fluctuations in TBF as well as FR. The P\_V in tumors is also elevated, presumably due to elevated FR, and is the main source of interstitial hypertension (12). Therefore, if NA lowers FR, we should see a decrease in P\_V and, hence, in TIFP. The goal of this work was to test if NA decreases TIFP.

MABP, TIFP, tumor water content, and HCT were measured in control and NA (500 mg/kg)-treated C3H mice bearing FSall tumors. This dose of NA has led to increased pO2 and radiosensitivity in this tumor model (4). Furthermore, tumor oxygenation in response to NA was found to be tumor size dependent. Therefore, we carried out these studies for tumors between 100 and 1700 mm\(^3\). Since edema may contribute to interstitial pressure, we also sought possible correlation between TIFP and tumor water content. Finally, to exclude the possibility of a water shift from the vascular compartment to the peritoneal cavity due to an i.p. injection of NA, we measured the HCT before and after NA treatment.

Materials and Methods

Animals. Female C3Hf/Sed mice, 8 to 10 wk of age, were used in this study. Animals were kept under pathogen-free conditions in the animal facility maintained at 25 ± 3°C. The mice were allowed food and water ad libitum.

Tumors. Frozen FSall tumor cells (third generation) were thawed from the liquid N\(_2\), cultured, and grown in vitro in C3Hf/Sed mice. The FSall (fourth generation) tumors were harvested 7 to 10 days after the inoculation, and single cell suspensions were prepared using 0.25% trypsin solution. About 2 \(\times\) 10\(^6\) viable cells suspended in 0.05 ml of RPMI 1640 medium were injected s.c. into the right thighs of mice. Experiments were carried out when the tumor volume was between 100 and 1700 mm\(^3\). Tumor volumes were calculated using the formula \(V = 0.4 \times ab^2\), where \(a\) and \(b\) are the longer and shorter diameters of the tumors, respectively.

Nicotinamide Treatment. Nicotinamide (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile saline solution (0.9% NaCl) before each experiment. The tumor-bearing mice were given i.p. injections at a dose of 500 mg/kg in a volume of 0.01 ml/g of body weight.

Measurement of MABP. Mice were anesthetized with an i.p. injection of sodium pentobarbital (60 mg/kg) and placed on a heating pad to keep the body temperature around 37.5°C. The right carotid artery was exposed and cannulated with a PE-10 polyethylene catheter (Clay Adams, Parsippany, NJ), using a binocular microscope. During the
insertion of PE-10 tubing into the artery, the artery was occluded with a small clamp. The tubing, previously filled with heparin (70 units/ml), was connected with a three-way stopcock to a pressure transducer (Gould, Inc., Valley View, OH). The clamp on the artery was then released, and the PE-10 tubing was checked for blood and tied with fine silk thread. The stopcock was then turned on to allow communication between the tubing and the pressure transducer. MABP was only measured in animals with small tumors. In animals with larger tumors, cannulation of the carotid artery and pentobarbital anesthesia induced the death of the animals. However, we were able to measure MABP in animals with larger tumors, anesthetized with ketamine (90 mg/kg)/xylazine (9 mg/kg).

Measurement of TIFP. TIFP was measured with the WIN technique using a 23 gauge needle with a side hole 5 mm from the tip (13). The experimental setup used for the measurements of TIFP was similar to that described in detail previously (14). Briefly, five nylon surgical sutures (6-0 ethilon; Ethicon, Inc., Somerville, NJ) were placed within the needle. To take the pressure measurements, the needle was connected to a pressure transducer by PE-50 polyethylene tubing filled with sterile heparinized (70 units/ml) saline. Before pressure measurements in each animal, the calibration of the pressure transducer setup was verified by applying pressures of 0, 5, 15, and 30 cm of saline (i.e., 1 mm of Hg = 1.36 cm of saline in height). Possible leaks in the system were tested by maintaining the pressure of 30 cm of saline for at least 5 min. For this protocol, the animals were anesthetized with 60 mg/kg, i.p., of sodium pentobarbital, and the skin was carefully removed above the tumors. The animals were placed on a hot water heating pad in order to keep the body temperature at 37.5°C. Two needles were inserted separately into the central region of the tumor, since TIFP as been shown to be relatively uniform throughout solid tumors growing as a single nodule (12). In animals not treated with NA, two measurements were made by introducing two needles in the central regions of the tumors. The animals given injections of NA were divided in two groups. In one group TIFP was monitored continuously with one needle before and after 2 h following the injection of NA. In the second group of animals, one measurement was obtained 2 h following injection of NA using a separate needle. Data of the second group were compared with those of untreated tumors of similar size. The dynamic changes in the TIFP signal were continuously monitored and recorded by a chart recorder. After measurements of TIFP, the WIN system was recalibrated. TIFP values were discarded when the values drifted by more than 1 mm of Hg.

Measurement of Tumor Water Content (Twc). Water content in a limited number of tumors was calculated using the formula

\[ T_{wc} = \frac{T_w - T_d}{T_w} \times 100\% \]

Tumors were weighed immediately after the excision for the wet weight (T_w) and after a drying period of 2 days at 50°C followed by 5 days at room temperature for the dry weight (T_d). The dry weight was not significantly different at 2 and 5 days at room temperature for tumors < 500 mm^3.

Measurement of HCT. The HCT was measured before and after the i.p. administration of nicotinamide (500 mg/kg) in a 70-μl sample of blood (approximately 3.0 to 3.5% of total blood volume) collected from the orbital sinus of each mouse in a 75-μl capillary tube. The capillary tubes were centrifuged for 10 min at 12,500 rpm, and separate bands of HCT were read.

Data Analysis. All values are shown as the mean ± SE of each group and time points for the parametric statistic. The changes by percentage were determined individually for each mouse, based on pretreatment values, and then averaged. The significance of the differences within a group before and after the nicotinamide treatment was evaluated with a paired t-test. Significant differences between treatment groups were checked with an unpaired t-test.

Results

Fig. 1.4 shows TIFP as a function of tumor volume (100 to 1700 mm^3) in untreated tumors. Note that TIFP increased with tumor volume from 100 to 400 mm^3 and then leveled off. Over 1000 mm^3, the TIFP decreased significantly. TIFP differed considerably among individual tumors of similar size. Fig. 1B shows TIFP and pO2 as a function of the tumor volume in FSAll tumors (<500 mm^3). TIFP increased when the tumor grew; however, pO2 decreased showing an inverse relationship
after treatment with NA (500 mg/kg), reaching 85% of the control value by 30 min \( (P < 0.05) \) and then fluctuated slightly for 2 h posttreatment. Fig. 2B shows the percentage of changes in TIFP after treatment with NA. TIFP also rapidly decreased within 10 min of treatment with nicotinamide, reaching a minimum of 60% of the control value by 2 h. In a few animals TIFP was measured for 4 h, and it did not return to the pretreated control value. For subsequent analysis, we chose values of MABP and TIFP at 2 h after an i.p. injection of nicotinamide, i.e., approximately a 35% decrease in TIFP \( (P < 0.001) \) and a 15% decrease in MABP \( (P < 0.05) \). In the saline-treated control group, no changes in MABP or TIFP were observed (data not shown).

To delineate the effectiveness of nicotinamide in TIFP reduction in the same animals, we divided the tumors into four groups: small (100 to 200 mm\(^3\)); medium (200 to 500 mm\(^3\)); large (500 to 800 mm\(^3\)); and very large (800 to 1700 mm\(^3\)). A significant drop in TIFP was found for tumor volumes ranging from 200 to 800 mm\(^3\); the pressure decreased by 47 ± 9% \( (P < 0.001) \) and 39 ± 6% \( (P < 0.001) \) in medium and large tumors, respectively. A 13% reduction in TIFP was found in small tumors \( (P > 0.1) \). Nicotinamide did not influence the TIFP of very large tumors (Fig. 3).

Fig. 4 compares TIFP as a function of tumor volume for animals treated and untreated with NA. TIFP in untreated animals increased as a function of tumor size between 100 and 400 mm\(^3\). In animals given injections of NA, the increase in TIFP associated with tumor growth disappeared. The mean TIFP decreased to values between 2.8 and 3.8 mm of Hg as compared with untreated animals with TIFP between 4.5 and 9.0 mm of Hg. Also the major drop in TIFP of 5 mm of Hg was observed in tumors with a volume between 350 and 450 mm\(^3\).

The HCT of the control mice was 49 ± 0.7%, and that of mice bearing about 150-mm\(^3\) tumors was similar to the HCT of the control mice. However, as the tumors grew to about 700 mm\(^3\) and 1400 mm\(^3\), the HCT declined to 45 ± 0.9% \( (P < 0.05) \) and 42 ± 0.4% \( (P < 0.05) \), respectively. However, a single
injection of 500 mg/kg of nicotinamide did not alter the hematocrit for 2 h posttreatment ($P > 0.1$).

Discussion

The first goal of this study was to measure TIFP in FSAl tumors as a function of tumor size. Similar to prior studies with experimental and human tumors (14, 15), TIFP increased with tumor size up to 400 mm$^3$. However, unlike previous studies, TIFP decreased somewhat in tumors $> 1000$ mm$^3$. The only other study where TIFP was found to decrease beyond a critical size of the tumor was done using a chronic implant of micropore chambers pioneered by Gullino (5). Possible explanations of this decrease in TIFP are (a) decrease in microvascular pressure due to a large tumor burden and (b) ulceration of tumors resulting in increased tissue hydraulic conductivity (16). However, in this tumor model, MABP did not decrease when tumors grew up to 1700 mm$^3$.

The second goal of this study was to check if there is a correlation between TIFP and water content as postulated by Steen (17). Water content increased from $2$ mm of Hg to $14$ mm of Hg, in support of Steen's hypothesis. On the other hand, water content was nearly constant (82%) for larger tumors ($>500$ mm$^3$) and did not correlate with pressure. If this hypothesis is true for other experimental and human tumors, then the tumor water content as measured by $^1$H-NMR could be used to estimate TIFP, noninvasively.

The third goal of this study was to test the hypothesis that NA can lower TIFP. Indeed, the increase in TIFP in tumor with a volume between 100 and 400 mm$^3$ disappeared after the treatment with NA. Recently, Lee and Song (4) have shown in FSAl tumors that the decrease in pO$_2$ associated with tumor growth was abolished by NA. Interestingly, an inverse relationship between TIFP and pO$_2$ has also been seen in human cervical carcinomas by Roh et al. (18).

Since tumors have high vascular permeability and they lack functioning lymphatics (12, 14, 16), the TIFP is primarily governed by microvascular pressure. Microvascular pressure in tumor vessels in sandwich tumor preparation has been found to vary between 5.8 and 6.7 mm of Hg (19), and in tissue-isolated tumors it is very close to TIFP. Based on the current results we expect microvascular pressure to go down following NA injection in a size-dependent manner. Simultaneous measurements of microvascular pressure and interstitial pressure after treatment with NA are needed to confirm this hypothesis.

In addition to providing insight into the NA-induced TBF modification, our results suggest that NA may be useful for improving the delivery of novel therapeutic agents to tumors. Both hyperthermia (20) and photodynamic therapy can lower the TIFP but at the cost of lowering the tumor blood flow rate. On the other hand, NA can improve the blood supply and also lower TIFP, which is a major obstacle to the delivery of antibodies and other macromolecules (21). Since NA is relatively nontoxic in humans (22), its effects on the pathophysiology of human tumors in situ should be examined to check if the data from murine tumors can be extrapolated to humans.

In conclusion, NA can lower the elevated TIFP, which is a major obstacle to the delivery of macromolecules (e.g., monoclonal antibodies). Also, TIFP and pO$_2$ are inversely related. TIFP measurements are considerably easier to perform than pO$_2$ distribution; therefore, they may be useful in individualizing therapy and predicting the outcome of radiation therapy (18). Furthermore, a positive relationship between TIFP and water content in tumors was determined in this study. If this correlation is found in other experimental and human tumors, then water content as measured by $^1$H-NMR could be used to estimate TIFP noninvasively.

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