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Oxidative status in neuroblastoma: a source of stress?

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Abstract

Aim of Study—Reactive oxygen species have been shown to be initiators/promoters of tumorigenesis. Because evidence supports the role of increased oxidative stress in solid tumors, we sought to establish this relationship in neuroblastoma (NB). The aim of the study was to investigate the extent of oxidative DNA damage and antioxidative status in a progressive animal model of human NB.

Methods—Tumors were induced in the left kidneys of nude mice by the injection of cultured human NB cells (10^6). Blood was collected from tumor-bearing mice and controls at 2, 4, and 6 weeks. Peripheral blood leukocyte oxidative DNA damage was determined using single-cell gel electrophoresis (comet assay), and plasma antioxidant capacity was assessed by the Trolox equivalent antioxidant capacity method.

Main Results—Levels of oxidative DNA damage in peripheral blood leukocytes of NB-bearing mice were increased by 166%, 110%, and 87% as compared with healthy controls at 2, 4, and 6 weeks, respectively. Plasma total antioxidant values for tumor-bearing mice were not significantly different from control mice.

Conclusions—Our results indicate an increase of oxidative stress in an animal model of human NB, especially in the early stages of growth. Yet, we did not observe an appreciable response in plasma antioxidant activity. Because an altered redox status has been implicated in tumor maintenance and progression, these findings support the notion of a complex oxidant-antioxidant imbalance contributing to NB growth.

Index words

Neuroblastoma; Oxidative status; Oxidative stress; Comet assay; DNA damage; Antioxidants; Animal neuroblastoma model

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Neuroblastoma (NB) is a common pediatric solid tumor that often presents with advanced-stage disease. Because therapies for disseminated NB are frequently plagued with relapse and poor outcomes, innovative approaches to high-risk malignancy are warranted. To this effect, a large body of evidence implicates oxidative stress contributes to the pathogenesis of cancer and has been linked to more than 50 disease states [1]. An oxidative and antioxidant imbalance has been shown to exist in the circulations of children with liquid and solid cancers [2]. With regard to NB, we observed the secretion prooxidant polypeptides in NB cell lines grown in conditions simulating oxidative stress [3]. To continue to study whether perturbations of the host-tumor redox state are involved in NB progression, we sought to evaluate whether plasma measurements of oxidants/antioxidants are in an animal model of human NB correlates with tumor growth.

By describing the oxidation status in the circulation of a well-established NB mouse model, we hope to gain insight into the host oxidative response to tumor burden. Past work evaluating tumor growth has concentrated on studying how cancer cell growth adapts and modulates to hypoxic conditions [4–6]. Because oxygen deficiency elevates the expression of reactive oxygen species (ROS) production, antioxidants are generated within the cell to defend against excess ROS production. By analyzing how the host responds to tumor challenge, ramifications of this work include improved understanding of the oxidative microenvironment surrounding NB growth and potentially lead to the design of new therapeutic strategies based on a child's redox status.

1. Materials and methods

1.1. NB athymic mouse model

The human NB cell line, SK-N-AS, was grown as a monolayer in Dulbecco's modified Eagle's medium with 4 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum at 37°C in 5% CO₂. Cells were harvested, counted (10⁶), and prepared as previously described [7]. A total of 29 NCR female nude mice (4–6 weeks old) were used. After a 7-day acclimation period, mice were separated into 4 groups; group 1 (control, n = 6), group 2 (2-week tumor, n = 7), group 3 (4-week tumor, n = 7), and group 4 (6-week tumor, n = 7). Controls for each experimental group underwent a sham operation, and the left kidneys were injected (100 µL) with phosphate-buffered saline. Mice were anesthetized and implanted with tumor, and blood was obtained by cardiac puncture as described by Sandoval et al [8]. Mice were euthanized as per Indiana University Institutional Animal Care and Use Committee approved protocol. Whole blood was transferred to iced Eppendorf tubes containing 100 µL of EDTA.

1.2. Assessment of direct and oxidative DNA damage: comet assay

Whole blood and EDTA mixture was subsequently added to a 1% low melting point agarose at a ratio of 1:200 and applied to Trevigen CometSlides (Trevigen, Gaithersburg, Md). The comet assay was then performed as described by Pu et al [9].

1.3. Quantification of total antioxidant levels: Trolox equivalent antioxidant capacity assay

Antioxidant capacity was evaluated with a colorimetric assay, the Trolox equivalent antioxidant capacity (TEAC) assay. This assay is based on scavenging of relatively stable blue/green ABTS radical (ABTS⁺) and converting it into a colorless product. The antioxidant capacity is expressed as relative scavenging activity to the water-soluble vitamin E analogue, Trolox. The entire amount of plasma was diluted 1:20 in phosphate-buffered saline. Standards at varying concentrations of Trolox (Aldrich catalog 238813; Sigma-Aldrich, St Louis, Mo) were made, and a spectrophotometer provided a standard curve. The TEAC assay was

performed as described by Miller et al [10,11]. The TEAC assay measures absorbance at 724 nm.

1.4. Biostatistical analysis

Because of the exploratory nature of this study, power calculations were not estimated for effect size. Moreover, because of the small numbers of controls at each time-point ($n = 2$), we tested differences in values for the comet and TEAC assay at weeks 2, 4, and 6 among controls. Because there was no difference between controls by week (P values $>.999$ and $>.724$, respectively), all controls ($n = 6$) were combined for comparison with each of the 3 tumor-bearing groups ($n = 7$ per group; total, $n = 21$). Nonparametric analyses using ranked data were performed as the assumption of normality was violated. The Mann-Whitney U test was used to compare differences among the 3 time-points, whereas the Wilcoxon rank sum test was used for pairwise comparisons of controls. One-sided tests were conducted to compare controls vs tumor-bearing mice for comet and TEAC assays. P values of $\leq .05$ were considered significant. Analysis was performed in SAS version 9 (SAS Institute Inc, Cary, NC).

2. Results

2.1. Growth of human NB in athymic mice

NB was established in our animal model and allowed to grow for 2, 4, and 6 weeks. Fig. 1 shows a representative NB tumor at the 6-week time interval. Table 1 shows descriptive statistics for tumor weights by duration and group.

2.2. Oxidative damage of peripheral blood leukocytes during NB progression

The comet assay, also known as single-cell gel electrophoresis, is a microgel electrophoresis technique that detects DNA damage and repair in individual cells. Damage is represented by an increase of DNA fragments that have migrated out of the cell nucleus in the form of a characteristic streak similar to the tail of a comet. The DNA fragments are generated by DNA double-strand breaks, single-strand breaks, and/or strand breaks induced by alkali-labile sites in the alkaline version of the assay. The length and fragment content of the tail is directly proportional to the amount of DNA damage (Fig. 2). During NB progression, we observed significantly increased oxidative stress at 2, 4, and 6 weeks compared to controls; levels of oxidative damage were elevated 166%, 110%, and 87% vs controls, respective to time interval (Table 2). Interestingly, the extent of oxidative damage during tumor growth was inversely proportional. A significant peak of prooxidant stress was evident at 2 weeks (2.39 vs 0.90, $P = .03$). While still significant, we observed less oxidative damage at 4 and 6 weeks of NB growth compared with controls (1.89 vs 0.90, $P = .03$) and 1.68 vs 0.90 ($P = .02$), respectively. These results suggest oxidation stress is generated during NB progression, particularly in the early stages of growth.

2.3. Antioxidant capacity during NB growth

The TEAC assay considers the cumulative action of all the antioxidants present in plasma, thus, providing an integrated parameter rather than the simple sum of measurable antioxidants. The capacity of known and unknown antioxidants and their synergistic interaction is, therefore, assessed, thus, giving an insight into the in vivo balance between oxidants and antioxidants. As shown in Table 3, we observed an initial 9% increase ($P =$ not significant [NS]) in antioxidant response among the 2-week NB animals. This was followed by a decrease in antioxidant capacity for the subsequent time intervals (4 weeks, 5.57 vs 5.21, $P =$ NS; and 6 weeks, 5.65 vs 5.57, $P =$ NS). The pattern of antioxidant capacity during NB progression in our model suggests an initial burst that correlates with the greatest amount of oxidative stress then an interval decline as tumors enlarged within the mice. Overall, these findings are of

sufficient magnitude to support our conclusions that a significant association between oxidation stress and NB exists. At minimum, this pilot study serves as a demonstration of method and warrants further confirmation on a larger scale.

3. Discussion

Innovative therapeutic approaches are needed to extend survival for high-risk NB. For these reasons, targeting the interaction between host and tumor, or the cancer micro-environment, has become a promising strategy for the new generation of oncologic therapy. In fact, the relevance of the host-tumor interface with regard to angiogenesis has proven to be successful in preclinical models and reached clinical testing for NB [12]. Because a detailed understanding of dysregulated oxidation in NB may offer therapeutic options, we used an animal model of human NB to examine whether peripheral blood oxidation/antioxidant levels correlated with disease progression.

We found increased levels of oxidative stress in tumor-bearing mice than nonstressed controls because elevated DNA damage in peripheral blood leukocytes of NB mice was detected compared with healthy controls (Table 1). This supports the notion of a prooxidant tumor environment arising during tumor formation [13,14]. Yet, contrary to our hypothesis, which sought to evaluate whether a greater oxidative response correlated with a progressively enlarging tumor, we interestingly observed a greater extent of oxidation stress during the early time intervals than the 6-week time-point. Given the evidence that ROS support tumor growth and progression [15,16], we surmise that at 2 weeks, where oxidative damage is highest, the ROS generated were as a result of a highly proliferative NB cell mass. At 4 and 6 weeks, as the tumor enlarges and develops a necrotic core, subsequent hypoxia ensues, which triggers an elevation of intratumor ROS. Because a threshold effect limits the level of ROS at which they induce cytotoxicity [17], we rationalize that the tumors reduce ROS produced by decreasing oxidative damage. To further support this theory, data suggest that low levels of intracellular ROS promote cellular proliferation and cell cycle progression [18–20]. It should be recognized that an alternative theory would involve the host generating an overwhelming oxidative response by activated macrophages as a result of tumor implantation. This trend may be a reflection of intratumor regulation of ROS to promote progression or simply a function of host-mediated response to tumor implantation. Further work is needed to clarify these observations.

To mitigate the detrimental effect of oxidation stress, cells maintain redox homeostasis by activating complex antioxidant defenses [21]. We assessed total antioxidant capacity during NB progression at 2, 4, and 6 weeks, and hypothesized that antioxidant load would increase as tumor bulk amassed. Our results reveal a relatively small increase (21%) in antioxidant levels as compared with 2-week controls. As NB progressed, we observed a decrease in antioxidant capacity at 4 and 6 weeks. We reason that as the TEAC assay measures global capacity to neutralize ROS, at the early time-point, the overabundant ROS formation corresponds to the upregulation in oxidative stress and concomitant activation of antioxidant defenses. Mice in the latter groups generated less oxidant stress and, as a result, lower than anticipated antioxidant responses. Moreover, we did not study intratumoral antioxidant levels, and effective antioxidant mechanisms may be restricted at the cancer cell level. Analysis of cancer cell lines antioxidant profiles reveals a variation in glutathione peroxidase, catalase, and superoxide dismutase activities such that some malignant cells have a diminished capacity to detoxify ROS [22,23]. These findings underscore the complex redox biochemistry regulating host-tumor interactions.

In this study, we evaluated blood levels of oxidative stress and antioxidant activity in a progression animal model of human NB. Our findings show that oxidation stress occurs in NB;

yet, there is lack of oxidative burden and antioxidant imbalance with tumor growth. Strategies aimed at targeting the redox microenvironment of NB are not new. Hyperbaric oxygen therapy is based on the theory that increasing intratumor oxygen tensions in solid tumors such become sensitized to radiation therapy. For instance, *meta*-iodobenzylguanidine, a radio-iodinated form of norepinephrine, has been used in combination with hyperbaric oxygen in high-risk NB with varying results [24,25]. As we gain better comprehension of the redox status of children with NB, therapies targeting oxidative stress pathways may improve outcomes for advanced-stage malignancy.

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Fig. 1. NCR nude mouse implanted with SK-N-AS human neuroblastoma (NB) cell line at 6 weeks. The figure shows a large intraabdominal tumor at necropsy. On removal of tumor, a large mass is appreciated (white arrows), displacing abdominal contents (black arrow). Weight of tumor was 7.1 g.

Fig 2. Comet assay image exhibiting DNA damage after electrophoresis. Area 1 is the “head” that corresponds to the single cell being analyzed. The amount of damage can be calculated by determining the percentage length of the “tail” shown in area 2 here. See Materials and methods for details.

Table 1

Descriptive statistics for tumor weight by duration

Analysis variable: tumor weight						
Duration (wk)	n	Mean	SD	SE	Median	Minimum Maximum
2	7	0.10	0.11	0.04	0.07	0.00 0.27
4	7	3.43	2.20	0.83	4.10	0.70 6.83
6	7	6.04	4.18	1.58	7.06	0.78 11.24

Table 2

Comet assay results indicating oxidative DNA damage for experimental and control groups at 2, 4, and 6 weeks

	Mean	Percent difference	SD
Controls	0.90		0.32
2 wk			
Experimental	2.39	+166	0.90
<i>P</i>	.03		
4 wk			
Experimental	1.89	+110	1.19
<i>P</i>	.03		
6 wk			
Experimental	1.68	+87	0.27
<i>P</i>	.02		

Table 3

Trolox equivalent antioxidant capacity assay values from plasma of NB implanted and control mice at 2, 4, 6 weeks

	Mean	Percent difference	SD
Controls	5.57		0.84
2 wk			
Experimental	6.06	+9	0.48
<i>P</i>	>.05		
4 wk			
Experimental	5.17	-8	0.91
<i>P</i>	>.05		
6 wk			
Experimental	5.65	+1	1.34
<i>P</i>	>.05		