Cold atmospheric plasma for the ablative treatment of neuroblastoma

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Abstract
Background: Recent breakthroughs have allowed for production of plasma at room temperature. Cold atmospheric plasma (CAP) may offer the capability of delivering reactive oxygen species directly into tissues, representing a novel modality for targeted cancer therapy. We studied helium-based CAP’s effect on neuroblastoma, both in-vitro and in an in-vivo murine model.

Methods: Mouse neuroblastoma cultures were treated with CAP for 0, 30, 60, and 120 s and assayed for apoptotic and metabolic activity immediately and at 24 and 48 h post-treatment. Five-millimeter tumors were ablated with a single transdermal CAP treatment, and tumor volume and mouse survival were measured.

Results: CAP decreased metabolic activity, induced apoptosis, and reduced viability of cancer cells in proportion to both duration of exposure and time post-treatment. In-vivo, a single treatment ablated tumors and eventual tumor growth was decelerated. Furthermore, survival nearly doubled, with median survival of 15 vs. 28 days (p < 0.001).

Conclusions: Our findings demonstrate the sensitivity of neuroblastoma to CAP treatment, both in-vitro and in an in-vivo mouse model of established tumor. While further investigation is necessary to establish the mechanism and optimize the treatment protocol, these initial observations establish cold atmospheric plasma as a potentially useful ablative therapy in neuroblastoma.

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Plasma, the “fourth state of matter,” is a partially ionized gas, containing a mixture of electrons, positive and negative ions, radicals, and various excited molecules [1]. Historical ly, plasmas could be produced only at high temperatures or in vacuums; however, recent breakthroughs in plasma physics have allowed development of plasma at room temperature and atmospheric pressure, so-called “non-thermal” or “cold” atmospheric plasma [2].

In simple terms, cold atmospheric plasma (CAP) is produced by applying a high voltage electric field to a plume of compressed gas; the intricacies of CAP production have been described elsewhere [1–3]. While any gas, or mixture of gases, can theoretically be used; researchers have chiefly

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Abbreviations: CAP, Cold Atmospheric Plasma; ROS, Reactive oxygen species.

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studied helium and argon. Noble gases have the advantage of being monatomic and chemically inert, characteristics that allow production of a stable and predictable plasma [2]. In comparison with argon, helium has the benefit of ionization at lower voltages, as well as production of more reactive species [2,4].

Recent investigation has focused on applying CAP to cancer treatment. Cancer cells are particularly sensitive to reactive oxygen species (ROS) [5,6]; a product of CAP. Conversely, non-tumor cells seem tolerant or even resistant to CAP [7–9]. This technology thus represents a potential strategy for selective, targeted, cancer therapy. Investigators have thus far demonstrated *in vitro* efficacy in a variety of cancers [4,7,8,10,11]. More importantly, reports of *in vivo* efficacy are beginning to emerge, specifically in mouse models of both melanoma [9] and glioma [12].

Given the broad range of potential applications, much investigation is directed at delineating the mechanism of CAP. Predictably, as ions and reactive species are present in plasma by definition, mounting evidence suggests that the majority of effects are mediated through production of reactive oxygen species [7,11,13–17]. This effect leads to apoptosis [7,13,18–20], loss of cell adhesion [1,3,7,13,21], decreased cell migration [3,20], and even cell membrane permeability and consequent destruction [22,23].

We explored the use of CAP in the treatment of neuroblastoma, the most common solid extracranial malignancy in children. This disease accounts for nearly 50% of malignant tumors diagnosed in the first year of life [24]. While survival for early and intermediate stage neuroblastoma is excellent [25], the majority of patients are unfortunately diagnosed with advanced disease [26]. In an effort to improve outcomes for these children, treatment strategies beyond traditional surgery and chemotherapy are investigated, including radiation [27] and immunotherapy [28]. Despite this, the prognosis for late-stage disease is very poor: only 34% survive 5 years [29]. Hence, the need for improved or additional therapeutic approaches is clear. Here, we present our initial experience using CAP in a murine neuroblastoma model [30]. We demonstrate both *in vitro* and *in vivo* efficacy for tumor ablation.

### 1. Materials and methods

#### 1.1. Mice

Female A/J mice were purchased at 6 weeks of age from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in specific pathogen free conditions in the Animal Laboratory at Children’s National Medical Center. All mice were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC).

#### 1.2. Murine neuroblastoma cells

Neuro2a cells, a murine neuroblastoma line derived from AJ mice, were purchased from the American Type Culture Collection (Manassas, NJ). Cells were cultured in D10 media (DMEM media containing 10% fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine).

#### 1.3. Cold atmospheric plasma

The CAP device utilized for these experiments was previously described [3] and additionally modified for handheld application of the CAP. All treatments, both *in vivo* and *in vitro* were administered at 4 kV using industrial-grade helium at a flow rate of 4 l/min.

#### 1.4. MTT assay

Triplicate samples of $1 \times 10^4$ neuroblastoma cells were plated in 96 well plates in 100 μl of D10 media. MTT Reagent was prepared by mixing 5 mg of Thiazolyl Blue Tetrazolium Bromide (M5655) (Sigma-Aldrich; St. Louis, MO) in 1 ml of phosphate-buffered saline. The solution was filter sterilized and stored in the dark. After CAP treatment cells were either immediately analyzed or incubated for 24, 48 or 72 h then analyzed; analysis involved incubation with 10 μl per well of MTT reagent for 3–4 h. Plates were centrifuged and supernatants were removed. Cells were lysed with 100 μl per well of dimethyl sulfoxide (D8418) (Sigma-Aldrich; St. Louis, MO) and mixed for 5 min. Plates were read. Spectrophotometric analysis was performed with the EnSpire™ Multilabel Plate Reader (Perkin Elmer; Waltham, MA) at 540 nm.

#### 1.5. Annexin-V/7-AAD apoptosis assay

Triplicate samples of $1 \times 10^4$ neuroblastoma cells were plated into 96 well plates with 100 μl of D10 media per well. After treatment with CAP, cells were collected and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and 7-Aminoactinomycin D (7-AAD) obtained from BD Biosciences (San Jose, CA). Flow cytometry was performed using FACS Calibur (BD Bioscience, San Jose, CA); results were analyzed using FlowJo software (Ashland, OR).

#### 1.6. *In-vivo* assays

$5 \times 10^5$ Neuro2a cells were subcutaneously injected into the right hind legs of 14 female A/J mice after fur removal using a standard depilatory. Mice were monitored for tumor growth on a daily basis. Tumors were treated with CAP once they were approximately 5 mm in maximum diameter (Fig. 1A). 7 control mice received no therapy after inoculation. All 7 treated mice received 5 min of CAP after administration of intraperitoneal ketamine and...
xyllazine as well as inhaled isoflurane for anesthesia. Tumors were treated through the skin; no overlying incisions were made (Fig. 1B, C). Mice received one round of treatment only. Following treatment, tumors were measured on a daily basis. Tumor volumes were calculated using the formula $V=0.52(X^2Y)$. Control and CAP treated mice were sacrificed when tumors reached a maximum diameter of 20mm if tumor bleeding or ulceration occurred, or if the mice appeared moribund. Mice that were successfully treated (Fig. 1D) were sacrificed at the termination of the trial.

1.7. Statistical analysis

For **in-vitro** assays, results are representative of three independent experiments. For **in-vivo** experiments, seven mice were randomized to the untreated and CAP-treated groups after tumor growth was evident. Error bars represent standard error of the mean. Statistical analysis was performed using GraphPad Prism 5 Software (GraphPad Software, Inc., La Jolla, CA, USA). For **in-vitro** assays, one-way ANOVA with Bonferroni’s post-test was performed to determine the differences in viable cells, both between all groups and between treatment groups and controls. For **in-vivo** survival, Kaplan–Meier curves were developed and Log-rank (Mantel–Cox) testing was performed.

2. Results

2.1. CAP inhibits the metabolic rate of neuroblastoma cells

**MTT** (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a reagent utilized to determine cell viability. The MTT reagent is reduced to purple formazan crystals by dehydrogenase enzymes located in the mitochondria of viable, metabolically active cells. This method is particularly useful in terms of comparing the cytotoxicity of different therapeutic treatments. In order to determine the effect of CAP on the metabolic rate of neuroblastoma cells **in vitro**,
we performed MTT assays on treated cells. Cells were treated for 0, 30, 60, and 120 s with the CAP jet as described above. Metabolic rates were first determined immediately after treatment, then at 24, 48 and 72 h post-treatment. As demonstrated in Fig. 1, Neuro2a cells show a decrease in MTT activity that is directly proportional to the duration of treatment. Additionally, in the two groups treated for more than 30 s, this effect appears to propagate with time, as the cells’ activity continues to decline over the ensuing 72 h post-treatment.

### 2.2. CAP induces apoptosis in neuroblastoma cells

While the tumor cell decrease in metabolic activity is evidence for the efficacy of CAP, we sought to determine whether these findings translate into apoptosis and cell death, as a more direct measure of cell injury. To that end, neuroblastoma cells were treated for 0, 30, 60, and 120 s, and Annexin V and 7-AAD staining was performed for flow cytometry analysis. Cells were again measured immediately post-treatment, then at 24 h, 48 h and 7 days after treatment. Four-quadrant analysis of the flow-cytometry results characterizes the cells as viable (unstained), apoptotic (Annexin V positive), late-apoptotic (double positive), and dead (7-AAD positive). While percentages of stained cells in the treatment groups appear similar to the control immediately after treatment, over 24 and 48 h the CAP treatment demonstrates a clear pro-apoptotic effect (Fig. 2). At seven days out from treatment, the results demonstrate a lasting...
effect on cell viability. Likewise, numbers of viable cells decrease in direct proportion to both the duration of therapy and the time post-treatment.

2.3. Mice treated with CAP show decreased tumor growth rates and improved survival

We next sought to determine whether our findings could be applied to an in-vivo model of CAP neuroblastoma treatment. AJ mice were injected with Neuro2a cells and either treated with CAP when tumors were first noted or observed. As shown in Fig. 3, CAP initially ablated the tumors. While the tumors eventually recurred in some mice, their growth rate was decreased as is seen in the growth curves. Furthermore, median survival of the mice in the treatment group was nearly doubled, from 15 to 28 days, as demonstrated by the Kaplan–Meier survival curves (p<0.001) (Fig. 4).

3. Discussion

Our findings establish the efficacy of CAP in the treatment of neuroblastoma, both in-vitro and in an accepted in-vivo mouse model. CAP decreases metabolic activity, induces apoptosis, and dramatically reduces numbers of viable cancer cells in direct proportion to the duration of treatment. More importantly, CAP markedly decelerates tumor growth in-vivo, leading to dramatically improved survival (Fig. 5).

Current hypotheses regarding the mechanism of this therapy lie primarily in the effect of ROS. ROS are well known to be harmful to cells inducing apoptosis, senescence or cell cycle arrest [31]. This effect has already been harnessed therapeutically in radiation therapy [31] and pharmacology [32]. Sensenig et al. proposed that ROS are the mechanism through which CAP induces apoptosis. [33] We hope to add to this body of data supporting a mechanism for CAP therapy in the future.

CAP therapy provides a unique method of treatment. Unlike argon plasma, cryoablation, or electrocautery, this treatment is atraumatic to the tumor and surrounding tissue. A prior paper utilizing our device investigated surface temperatures during treatment and found a 2°C rise from room temperature [8]. There is no evidence of thermal damage to skin following treatment. Application of the beam to one’s skin is tolerable and pain-free.

While previous studies have established the efficacy of CAP for treatment of other cancers, to our knowledge this represents the first report of CAP in the treatment of neuroblastoma. Furthermore, in comparison with the two previously mentioned in-vivo studies, we additionally provide survival data, demonstrate a more dramatic treatment effect, and follow a larger group of animals for a longer duration. Vandamme’s treatment of an undisclosed number of U87 glioma-injected nude mice resulted in a reduction of tumor bioluminescence of between 54% and 83%, which correlated to a reduction in tumor volume of approximately 30% over five days of follow-up [12]. Kim’s in-vivo experiment demonstrated no initial reduction in melanoma size, but did show CAP’s ability to inhibit tumor growth in four mice over ten days of post-treatment follow-up [9]. We show a 100% resolution of tumor burden after treatment. While tumors eventually recur, growth is markedly reduced over 21 days, resulting in a 100% survival difference between the two groups over the same period. Interestingly, while the other two groups required multiple treatments for CAP to have an effect [9,12], our results were obtained with a single treatment. Two important variables in our study potentially explain these discrepancies: the device utilized and the cancer treated.

Plasma therapy, as a nascent technology, is not generated by a standardized device. While the CAP device utilized for our experiments was similar to those of other labs, each device is designed and fabricated in-house; there is no standard. Unfortunately, while broad conclusions can be drawn from multiple reports of CAP, comparisons between devices is made difficult due a number of variables including voltages, currents, gas composition and gas flow rate. Until a standardized or superior device emerges, result comparison will be difficult.

Nonetheless, our experience treating both melanoma [8] and neuroblastoma using identical settings has
demonstrated increased sensitivity in the neuroblastoma model. Admittedly, while myriad other variables may contribute to this difference, the possibility that neuroblastoma may be especially sensitive to CAP is not only intriguing, but also supported by accepted tumor microbiology. Amplification of the MYCN oncogene has long been the primary adverse prognostic factor for this cancer [34]. Recent evidence has demonstrated that MYCN functions by regulating the expression of focal adhesion kinase (FAK). This non-receptor tyrosine kinase controls a number of cell signaling pathways including proliferation, viability, and survival [35]. Notably, CAP has been shown to inhibit cell-surface FAK expression [21], perhaps offering a strategy for specifically targeting the most aggressive tumors.

We have several proposals regarding clinical applications of this technology. Given the small size of the plasma aperture and the presently indeterminate depth of tissue penetration, in its current form the device seems most readily translated for use in “painting” tumor beds following primary resection. Presumably, this would be beneficial in eliminating microscopic rests of tumor, thereby limiting recurrent disease. Given the technology’s ability to preferentially induce apoptosis in tumor cells [8], the effect on adjacent bystander cells should be minimal. Laparoscopic or thoracoscopic administration is theoretically possible. As the device applies specifically to the treatment of neuroblastoma, however, metastatic disease is of greater concern. We are hopeful that intravascular, transcatheter delivery of CAP will be feasible, permitting treatment of disease via an endovascular approach. Obviously, significant modifications and improvements to the technology will be necessary to realize this ambition.

4. Conclusions

CAP represents a novel treatment modality, potentially providing targeted and specific anti-tumor therapy. Our device delivers this therapy practically, with a clear pathway for translation from the laboratory to the operating room or clinic. While the specific mechanism or mechanisms behind CAP’s anti-neoplastic effects remain to be conclusively established, preliminary results indicate that the therapy may deliver ROS directly into tumor cells. Moreover, the modality may precisely inhibit known neuroblastoma signaling pathways. Accordingly, this study establishes striking efficacy of CAP in the treatment of neuroblastoma. We have clearly demonstrated both dose-dependent declines in metabolic activity and corresponding increases in cellular apoptosis, with notable propagation of effects over time. Most importantly, we have established the in-vivo efficacy of CAP in a mouse model of neuroblastoma. These initial results beg further investigation into what we feel may be a promising emerging therapy.

References

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