Ozone Treatment Inhibits Proliferation in Human Neuroblastoma SK-N-SH Cells

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SUMMARY - Tissue hypoxia results from an inadequate supply of oxygen (O2) that compromises biologic functions. Recent experimental and clinical studies suggest that intratumoral oxygen levels may influence a series of biologic parameters affecting the tumor’s malignant potential. Indeed, sustained hypoxia in a growing tumour may cause cellular changes that can result in a more clinically aggressive phenotype. During the last two decades it has been shown that ozonated autohemotherapy is therapeutically useful in arteriopathic patients because it increases oxygen delivery to hypoxic tissues, leading to normoxia. Although several oxygenation approaches have been tested, none is able to restore normoxia permanently in cancer. Since a prolonged cycle of ozonated autohemotherapy has been postulated to correct tumor hypoxia leading to a less aggressive behavior, we studied whether ozone treatment could affect cell growth and cell cycle perturbations on the human neuroblastoma SK-N-SH cell line. These data indicate that ozone acts as a real chemical drug capable of inhibiting cell growth suggesting its possible antineoplastic role in neuroblastoma cancer.

Introduction

Tissue hypoxia results from an inadequate supply of oxygen (O2) that compromises biologic functions.

Traditionally, tumour hypoxia has been considered a potential therapeutic problem because it renders solid tumours more resistant to ionizing radiation. More recent experimental and clinical studies suggest that intratumoral oxygen levels may influence a series of biologic parameters affecting the tumor’s malignant potential. Indeed, sustained hypoxia in a growing tumour may cause cellular changes that can result in a more clinically aggressive phenotype.

During the process of hypoxia-driven malignant progression, tumour may develop an increased potential for local invasive growth, perifocal tumour spreading and regional and distant tumour spreading.

Experimental evidence indicates that hypoxia not only induces proteome changes influencing tumour propagation but also drives malignant progression through transient and persistent genomic changes in neoplastic cells. Hypoxia promotes genomic instability (through point mutations, gene amplification, and chromosomal rearrangements) and may unveil pre-existing cryptic genetic variations, thus increasing the number of genetic variants.

Tumour hypoxia, when assessed by polarographic probes, is an independent prognostic factor for response to treatment and/or survival of patients with head and neck, uterine cervical tumour and sarcoma. As demonstrated by Overgaard’s meta-analysis, improving tumor oxygenation can lead to better local control and increased overall survival rates following chemo-radiotherapy. Consequently, several strategies have been proposed to enhance tumor oxygenation inducing a constant restoration of normoxia. Ozone therapy is a technique that has been used in the treatment of ischemic syndromes.

Bernardino Clavo described a relationship between oxygenation in head and neck cancer and in anterior tibialis muscles, and an ozone therapy-induced improvement in the oxygenation of the ‘most-hypoxic’ anterior tibialis muscles, together with improvement in the most hypoxic tumors. These data suggested that ozone therapy could have some positive effect during the treatment of head and neck cancer.

Nevertheless, the potential usefulness of ozone therapy alone or as an adjuvant in neoplastic
patients receiving chemo–radiotherapy warrants further investigation.

The aim of the present study was to evaluate whether O₃ treatment could affect cell growth and cell cycle in the SK-N-SH human neuroblastoma cell line.

**Materials and Methods**

**Cell Culture**

Human SK-N-SH cell line was maintained as a monolayer culture in EMEM supplemented with 10% heat-inactivated FCS (Life Technologies, Scotland, United Kingdom), antibiotics and L-glutamine (2 mM) at 37°C in a 5% CO₂/95% air atmosphere in a humidified incubator.

**Ozone Generation**

Ozone therapy was administrated using clinical grade O₂, the O₃/O₂ gas mixture was prepared with an ozonosan α plus photonic device 1014/10 (Dr. Hansler, Germany) and sterilized by passage through a sterile 0.20 µm filter.

**Treatment and Growth Inhibition Assay**

Cells were seeded at a density of 1.0x10⁶ cells per T25 flask and after 24 hours of cell culture were treated at room temperature using an ozonosan α plus photonic device 1014/10 (Dr. Hansler, Germany) and sterilized by passage through a sterile 0.20 µm filter.

**Propidium Iodide Cytotoxicity Assay**

The capacity of 6, 12, 25, 50 and 80 µg/ml O₃ used in a single or repeated-regimen, to produce cell death was determined by PI staining exclusion test and flow cytometry. Cytotoxicity was defined as the cellular damage identified by PI staining, which shows the loss of structural integrity of the plasma membrane, a typical event of necrotic cell death. SK-N-SH cell line was stained with 100 µl of the supravital PI (4 mmol/L) for three minutes. After incubation, cells were washed with 5% bovine serum albumin in PBS and kept on ice until flow cytometry analysis.

**Results**

The oxonation experiments were conducted to investigate whether O₃ could be considered an antineoplastic drug. First we determined the optimal schedule of ozone treatment. We exposed cells to 6, 12, 25, 50 and 80 µg/ml O₃ doses and examined the effects produced by treatment on cell growth and the cell cycle at different times after ozone exposure (4, 24, and 48 hours). At each time, cells were harvested and counted using the trypan blue dye exclusion test. After addition of O₃ given in a single dose 6, 12, 25, 50 and 80 µg/ml, a dose-dependent inhibition of cell proliferation was observed (figure 1A). Four hours after exposure of cells to 80µg/ml, a cell growth inhibition of 58% was observed. The inhibitory effect become 99% after 48 hours, whereas growth inhibitions of 33% and 29% were obtained after 4 hours of exposure to 50 and 25 µg/ml O₃ and persisting during the 48 hours following treatment. When cells were exposed to the lowest O₃ doses of 12 and 6 µg/ml the growth inhibitions were about 24% and 17%, respectively. These latter effects were partially lost during the following days, with cell growth inhibition values evaluated 48 hours after O₃ treatment of about 17% and 10%, respectively.

We also measured the effect of repeated doses of 6, 12, 25, 50 and 80 µg/ml O₃. Figure 1B shows that the different doses of 6, 12, 25, 50 and 80 µg/ml given 24 hours after seeding and repeated three hours after the first treatment (repeated dose-regimen) enhanced cell growth reduction. The
maximum antiproliferative effect was observed at 80 µg/ml dose with similar values to those obtained with the previous regimen of O₃ treatment.

Four hours after the start of 50 and 25 µg/ml exposure we already observed cell growth inhibition of about 61% and 50% respectively (versus 33% and 29% obtained with the single-dose regimen) and it was maintained up to the last time point of the cell growth experiment (days 3 = 48 hours after start of O₃ treatment). The percentage of inhibition was about 37% and 20% in the 12 and 6 µg/ml O₃-treated cells in comparison with the single-dose regimen (24% and 17%, respectively) and it was partially recovered during the following 48 hours.

To evaluate whether O₃-induced cell growth inhibition could be related to cell cycle perturbations, PI staining and fluorescence-activated cell sorting analysis were done on SK-N-SH cells exposed to 6, 12, 25 and 50 µg/ml O₃ in single or repeated-dose regimen (table 1A and B). This analysis, performed at different times after 6, 12, 25 and 50 µg/ml O₃ exposure, given in a single dose regimen A) and a repeated dose regimen B). The analysis was performed 4, 24 and 48 hours after treatment. At each time point the cells were harvested and counted by trypan blue exclusion test. Cell counts are reported as percentages of control. Data are representative of three different experiments. Values are means of triplicate samples.
confirmed that 80 µg/ml O₃ is a lethal dose. These findings indicate that ozone acts as a real chemical drug capable of inhibiting neuroblastoma cell growth suggesting its possible antineoplastic role in neuroblastoma cancer.

Discussion

In solid tumours, oxygen delivery to the respiring neoplastic and stromal cells is frequently reduced or even abolished by a deteriorating diffusion geometry, severe structural abnormalities of tumour microvessels, and impaired microcirculation \(^{39}\). In an increasing number of reports on tumour oxygenation, the addition, anaemia and formation of methemoglobin or carboxyhemoglobin reduce the blood’s capacity to transport O₂. As a result, areas with very low (down to zero) oxygen partial pressure exist in solid tumours, occurring either acutely or chronically. These microregions of very low or zero O₂ partial pressures are heterogeneously distributed within the tumour mass and may be located adjacent to regions with normal O₂ partial pressure. In contrast to normal tissue, neoplastic tissue can no longer fulfil physiologic functions. Thus, tumour hypoxia cannot be defined by functional deficits, although areas of necrosis, which are often found in tumour tissue on microscopic examination, indicate the loss of vital not evaluable cellular functions. Actually hypoxia (defined as the fraction of measured O₂ partial pressures of <5 mmHg) is a statistically significant adverse prognostic factor of disease-free survival. A Kaplan–Meier analysis showed statistically significantly shorter survival and recurrence-free survival for patients with hypoxic tumors. The results were consistent with the hypothesis that radio-biologically hypoxic tumors (i.e., tumors with a reduced radiosensitivity at critically low O₂ levels) are less curable \(^{40}\), even though the role of hypoxia in conventional antineoplastic drug resistance could not be excluded.

These data were supported by Cox regression analysis which revealed tumor oxygenation as the strongest independent prognostic factor, followed by FIGO stage \(^{12}\). Of special interest was the fact that the disadvantage in outcome for patients with hypoxic tumor was independent of primary treatment (radiation therapy or radical surgery). Sundfor et Al \(^{41}\) reported a poor outcome associated with low oxygen tension in 40 patients with advanced squamous cell carcinoma of the uterine cervix. These findings are in agreement with

| Table 1. Effect of O₃ on cell cycle distribution of SK-N-SH human neuroblastoma cells |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                 | Single dose-regime | Repeated dose-regime |
|                                 | (%)              | (%)              |                  | (%)              |
| **O₃**                          |                  |                  |                  |                  |
| **4 hours**                     |                  |                  |                  |                  |
| Control                         | G1   47  | S    34  | G2   19  | Toxicity 2  |
| 6µg/ml                          | 48   41  | 14   4  |                  |                  |
| 12µg/ml                         | 44   34  | 14   4  |                  |                  |
| 25µg/ml                         | 40   6   | 14   3  |                  |                  |
| 50µg/ml                         | 35   5   | 11   5  |                  |                  |
| 80µg/ml                         | nv   nv  | nv   nv |                  |                  |
| **24 hours**                    |                  |                  |                  |                  |
| Control                         | G1   44  | S    34  | G2   22  | Toxicity 2  |
| 6µg/ml                          | 45   37  | 18   3  |                  |                  |
| 12µg/ml                         | 41   37  | 18   3  |                  |                  |
| 25µg/ml                         | 39   20  | 11   5  |                  |                  |
| 50µg/ml                         | 40   13  | 19   8  |                  |                  |
| 80µg/ml                         | nv   nv  | nv   78  |                  |                  |
| **48 hours**                    |                  |                  |                  |                  |
| Control                         | G1   49  | S    39  | G2   12  | Toxicity 2  |
| 6µg/ml                          | 46   43  | 13   5  |                  |                  |
| 12µg/ml                         | 43   43  | 16   8  |                  |                  |
| 25µg/ml                         | 42   43  | 16   9  |                  |                  |
| 50µg/ml                         | 43   43  | 15   9  |                  |                  |
| 80µg/ml                         | nv   nv  | nv   80  |                  |                  |
| **4 hours**                     |                  |                  |                  |                  |
| Control                         | G1   43  | S    34  | G2   22  | Toxicity 2  |
| 6µg/ml                          | 41   37  | 18   3  |                  |                  |
| 12µg/ml                         | 44   37  | 18   3  |                  |                  |
| 25µg/ml                         | 20   40  | 19   5  |                  |                  |
| 50µg/ml                         | 40   13  | 19   8  |                  |                  |
| 80µg/ml                         | nv   nv  | nv   78  |                  |                  |
| **24 hours**                    |                  |                  |                  |                  |
| Control                         | G1   49  | S    39  | G2   12  | Toxicity 2  |
| 6µg/ml                          | 49   34  | 17   5  |                  |                  |
| 12µg/ml                         | 46   31  | 23   15 |                  |                  |
| 25µg/ml                         | 20   40  | 19   5  |                  |                  |
| 50µg/ml                         | 40   13  | 19   8  |                  |                  |
| 80µg/ml                         | nv   nv  | nv   88  |                  |                  |
| **48 hours**                    |                  |                  |                  |                  |

FCM cell cycle analysis of untreated and O₃-treated SK-H-SH cells at the 4, 24 and 48 after the treatment. The cell cycle phase percentages were estimated by applying the MODFIT software to the PI/DNA histograms. Data were means of three separate experiments with similar results.
Our findings demonstrated that O₃ affects cell neoplastic lesions warrants further investigation. Nevertheless, the potential usefulness of ozone, which has a higher oxidizing capacity than oxygen, with three atoms and two unpaired electrons, may slowly bring about a concerted activation of the immune system usually suppressed by tumor growth. In contrast to the claim of a possible O₃ toxic effect on blood cells, it has been reported that O₃-induced toxicity is overcome using the appropriate dose. Indeed, the range of the therapeutic window has been determined between 20 and 80 µg/ml per ml of blood cells (0.42-1.68 mM) which did not produce any toxic effect. Indeed, patients undergoing ozone therapy do not have adverse effects and most patients reported a feeling of wellness and euphoria. Normally a cycle of 14-15 treatments (twice weekly) significantly improves visual acuity in about 70% of patients with the atrophic form of age-related macular degeneration: (ARMD) and in most patients with chronic limb ischemia (stage II). Our data are in partial agreement with results obtained by Bocci et Al on O₃ cytotoxicity. We did not find toxicity at doses of 6, 12, 25 and 50 µg/ml when O₃ was administered in a single
dose-regime while toxicity was obtained when O₃ was given in a repeated dose-regime. The highest dose of 80 µg/ml produced an elevated cytotoxicity in both single and repeated-dose regime and was considered a lethal dose. We observed that the O₃ induced a dose dependent cell growth inhibition and that the highest dose was able to activate necrotic cell death by producing irreversible cellular damage inhibiting the progression of cells through the cell cycle. In agreement with Bocci et Al., we also believe that ozone acts as a real chemical drug.

**Conclusion**

Ozone directly inhibited neoplastic cell growth following injection into the neoplastic nodule or via reinfusion of ozonated blood in patients bearing neuroblastoma cells. In accordance with Overgaard, we think that improving tumor oxygenation can lead to better local neoplastic control, increased patient survival rates and a better quality of life. Indeed ozone therapy could be a new therapeutic approach targeted to specifically bypass the resistance implemented by hypoxic selection reducing the risk of hypoxia-mediated treatment failures and improving survival and disease-free recurrence in neuroblastoma cancer patients. Besides the normalization of hypoxia, ozone therapy displays other interesting biological effects that may enhance the therapeutic outcome.

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Bibliography


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