Examination of Oncogenic Effects of Environmental Pollutants

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Chemical byproducts produced by surrounding industrial complexes have polluted the local estuaries of Georgetown, South Carolina for several decades[1]. Previous studies completed by the EPA and SCDNR showed that the estuaries contained carcinogenic pollutants released from the local manufacturing plants[1]. One of the mentioned pollutants was dioxins which are produced during the paper bleaching process[2]. Dioxins are highly toxic compounds that have various carcinogenic effects on the organisms that inhabit the area. These previous studies were conducted over thirty years ago. Therefore, a current study to determine if chemical byproducts are contaminating local estuaries is crucial in determining if companies are abiding by the limits set by the EPA. To complete this experiment, water was collected from Sampit River and filtered through vacuum filtration. The collected water was added to media in a Boyden chamber assay containing neuroblasts in order to observe cell migration. qPCR was completed to analyze ID2, which was utilized as a biomarker for possible tumorigenic expression within the neuroblasts. Large populations of the neuroblasts in the Boyden chamber with the same starting seeding density in control vs experimental samples resulted in increased migration. In further spontaneous motility assays, tumor-like aggregated morphologies formed, showing that carcinogens are possibly present in experimental samples that are leading to oncogenic effects. qPCR analysis showed increased ID2 expression in the neuroblasts, which is a biomarker of tumorigenicity in the neuroblasts. These results indicated that chemical byproducts could be in the local watersheds of Georgetown, South Carolina.

Introduction

Neuroblastoma is a disorder of the nervous system that affects nerve cell precursors called neuroblasts. Neuroblasts are neuronal precursor cells that develop during embryonic neural crest development with the process of neurulation to later form neural cells[3]. This strongly invasive type of cancer, neuroblastoma, typically arises from the adrenal glands and metastasizes to other parts of the body[4]. To study if Georgetown water samples potentially had oncogenic effects, the SH-SY5Y cell line was utilized in conjunction with other biochemical methods. The SH-SY5Y cell line is an immortalized cell line derived from the SK-N-SH neuroblastoma cell line that was created in 1970 from metastasized neuroblastoma cells in a four-year-old female patient[5]. Previous studies several decades ago revealed that dioxins were being released from industrial effluents. However, today it is unknown if there is still a presence of dioxins in the local Georgetown, SC area or if there are additional chemicals in the waterways of Georgetown, SC. Dioxins are a group of organic chemical compounds that can be highly toxic and carcinogenic based on the location of their chlorinated group in the chemical structure of the molecule[2]. The three main types of toxic dioxins are polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs)[2]. Dioxins can cause carcinogenic effects due to the levels of dioxins in an area and in the ways that an organism tries to rid itself of the harmful toxins through the aryl hydrocarbon receptor (AhR). AhR is a transcription factor that is induced by dioxins when trying to excrete the harmful toxins[6].

Dioxins are produced as byproducts of many industrial processes and can contaminate a wide radius surrounding the industrial complexes[2]. Dioxins have long half-lives and take many years to break down in an environment[2]. Although, when areas become contaminated, the dioxins seep into all aspects of the environment, making them extremely difficult to mitigate, and they are able to contaminate the area for decades after its production. Dioxins can contaminate the local watersheds but are able to additionally contaminate the entire food chain (both aquatic and terrestrial) due to biological magnification as prey organisms are eaten by predator organisms. People that are the most at risk of the effects of dioxins are fetuses and infants[2]. They can also cause carcinogenic effects in not only the nervous system, but also the immune, reproductive, and endocrine systems. Dioxins can lead to chemotaxis. Chemotaxis is the process of cellular migration that can be initiated from exogenous chemical gradients[7]. Chemotactic cytokines (chemokines) are protein signaling molecules that are secreted in cells that interact with, for example, surface G protein heptahelical chemokine receptors[8]. These chemokine signals lead to cellular migration and are vital in immune system development and response (such as NK cell signaling). Genes responsible for chemokine signaling can be mutated to induce haptotaxis, chemokinesis, haptokinesis, and apoptosis[8]. The hypothesis was that the surrounding industries in the Georgetown area are potentially non-compliant with environmental regulations set by the Environmental Protection Agency (EPA), resulting in discharge of toxic chemicals into the local estuaries. Exposure to the chosen Georgetown sample experimental conditions was hypothesized to induce carcinogenic effects in the tested neuroblasts. In this study, ID2 was examined. The ID gene family are the Inhibitors of DNA binding and encode proteins that negatively regulate the cell cycle and cellular differentiation and are biomarkers in neuroblastoma[9]. ID2 is a tumor suppressing gene that encodes a helix-loop-helix protein that regulates E-proteins that are involved in the processes of cellular differentiation and cellular determination[10, 11].

Methods

Water samples were collected from the Sampit River, in Georgetown South Carolina, across from the International Paper Company Industrial Complex. The water samples were filtered using vacuum filtration to rid the samples of sediments, while still allowing for the potential chemicals to pass through. During cell culture experiments, control groups contained 25% of non-chemically exposed water and 75% of the typical cell culture media to keep the cells alive while examining the effects of the water with potential pollutants. Experimental groups contained 25% of the Georgetown water samples with 75% of the typical cell culture media to keep the neural cells alive. The cell culture media for the neuroblast cells was DMEM medium + 10% FBS and 5% Pen-strep. While conducting experiments, the neural cells were examined for cell viability.

Examination of cellular migration was conducted by a Boyden chamber assay. During this assay, Corning 0.4 μm pore size tissue culture treated Transwell-24 plates were utilized. Cells were seeded at the same starting seeding density at 50,000 cells per well and their migration was monitored over a six-day period across non-chemically exposed and Georgetown water sample groups. Additionally, a spontaneous motility test was conducted across six days at the same seeding density as the Boyden chamber assay. Wells were examined before migration to ensure half of the plate of cells was empty and half had seeded cells at the same seeding density across control and experimental groups prior to examination at three and six days later. FIJI (ImageJ freeware) was utilized to quantify cellular migration studies and a t-test was performed for significance testing.

To further examine if there was a possibility of environmental pollutants in Georgetown water samples, genetic studies were conducted. cDNA samples were synthesized from the extracted cell lysate with RNA using SuperScript IV CellsDirect cDNA Synthesis kits for reverse transcription and were quantified by nanodrop. Gene expression levels were determined by qPCR using SYBR green with IDT-designed

primers and using a Bio-Rad CFX Connect System. Optical spectroscopy was also completed to attempt to determine the wavelengths of the pollutants in the Georgetown sample water. Georgetown water samples were tested against blank samples during optical spectroscopy in order to determine the specific wavelengths of the potential chemicals undergoing analysis. A Naphthalimide-based probe that selectively binds to tumor-like cells was also utilized to assist with imaging samples and was added to wells after culturing cells as described above and imaged.

Results

Quantification of the spontaneous motility migration assays across control water-media mix and experimental water-media mix showed an increased trend of migration in neuroblasts exposed to experimental water-media mix over six days **(Figure 1)**. This result shows that the Georgetown sample water potentially caused genetic and morphological changes to the neuroblasts to cause the increased migration, supporting the hypothesis that there are pollutants in the Georgetown estuaries. Neuroblasts exposed to the Georgetown sample water showed increased migration over six days in a Boyden Chamber assay as well compared to control samples **(Figure 1)**. Genetic expression study results, with qPCR, resulted in an over-expression of ID2 by a fold change of 2.745, showing the possibility of increased tumorigenesis in the cells treated with the Georgetown water samples **(Figure 2)**. However, the results from this experiment did not show statistical significance and contained variability within the control and experimental groups. Optical Spectroscopy of the Georgetown water samples showed a wavelength peak pattern near 300 nm that correspond with wavelength peaks similar to dioxins **(Figure 3)**. Morphological studies of the Georgetown water sample treated cells showed an increase in 3D tumor-like neuroblastoma aggregates and irregular morphologies **(Figure 4)**. The cells in the image from Figure 4 also had binding of the Naphthalimide-probe in experimental samples. This result shows the morphological changes in the neuroblasts exposed to the Georgetown sample water, supporting possibility of pollutants in the Georgetown estuaries having carcinogenic effects.

Discussion

Results from this experiment demonstrate that there may be contaminants in the local watersheds of Georgetown, South Carolina with harmful tumorigenic effects on human neuroblasts. The results from this experiment did not show any statistical significance, but the results could be influenced by several factors. For example, the discharge with potential pollutants may have been at different times than when the samples were collected, which could have created variability in the concentration of the potential pollutants within the samples that were collected (some samples having more contamination and others less). The positioning of the wavelength peaks from the optical spectroscopy could also be influenced by a bathochromic shift due to the different positioning of the chlorinated groups in the potential dioxin pollutants[12, 13]. There are other chemicals as well that could have contributed to the carcinogenic effects.

Other experiments have been conducted in the past that have shown a possible trend of pollutants disrupting various cellular receptors and signaling pathways[14]. The exposure from the chemical pollutants may alter ligand formation properties that AhR receptors recognize differently than the normal ligand molecules, causing signaling disruption, tumor formation, or metastatic migration[14]. The trend of increased migration in the results supports the hypothesis that the Georgetown water sample may contain potential pollutants that have disrupted the signaling pathways involved in regulation of cellular migration along with other morphological changes observed as seen in the microscopy experiments.

In current times, mass spectroscopy can be completed to determine more about the exact pollutants in the Georgetown water samples similar to methods the EPA did in the past when determining the pollutants in the water and contact environmental consultants[1]. There are non-significant results, however, with the current possible environmental

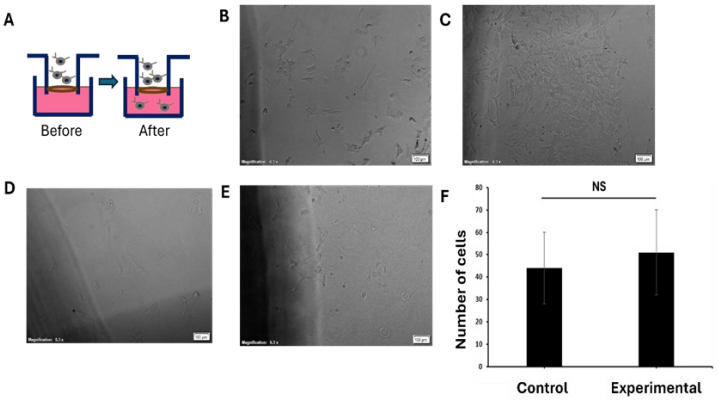

Figure 1. Migration Assays. (A) Diagram of the Boyden chamber migration assay (B) Control image after 6 days of migration in neuroblastoma cells (C) Experimental Georgetown water sample example image after 6 days of migration showing enhanced migration in experimental samples (D) Spontaneous motility (SM) assay image after 6 days of migration in control neuroblastoma cells (E) Image of experimental cells on day 6 in the SM assay (F) Average with standard error of the mean of the quantification of control vs experimental cell counts over 3 SM assay trials

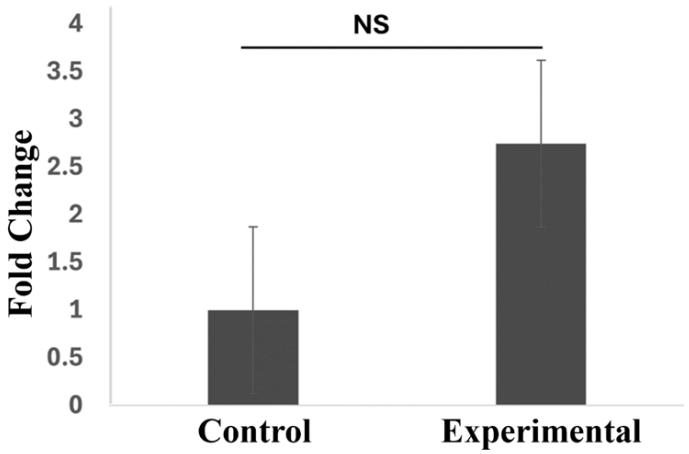

Figure 2. Expression of ID2, a known oncogenic factor in neuroblastoma. (A) qPCR quantification of the expression of ID2 in control vs experimental samples across 5 replicates

toxicity trends, future practices need to be on stand-by in Georgetown to make sure that the waterways are safe and that an increase in the severity of pollution leading to oncogenic effects does not occur. Editing of standard operating procedures and utilization of more environmentally safe chemicals for the same industrial procedures can also occur. A process by which dioxins are filtered out of the water and broken down much faster needs to be developed if pollution becomes severe to rid the Georgetown watershed of the dioxins at a much faster rate than they would by simply decomposing on their own[15]. Implementation of more biofilters can occur within the industrial practices in Georgetown to prevent chemicals from polluting the Georgetown watersheds[16]. Additionally, local manufacturers should contact environmental consultants to determine what the future plans are to fully eradicate the pollution that has already been done[2]. There are also more natural methods as well to assist in improvements in water quality such as incorporating mussel populations[17]. An on-site autonomous water monitoring sensor system with AI incorporation could also be implemented to monitor the level of pollutants in the watershed. However, an anti-fouling coating would have to be implemented on these sensors especially if used in conjunction with mussel populations.

Looking towards the future, an epidemiological study quantifying the normal cancer-per-capita rate in other areas without surrounding industrial complexes and comparing these numbers with the amount of cancer cases-per-capita in Georgetown may provide insight into if

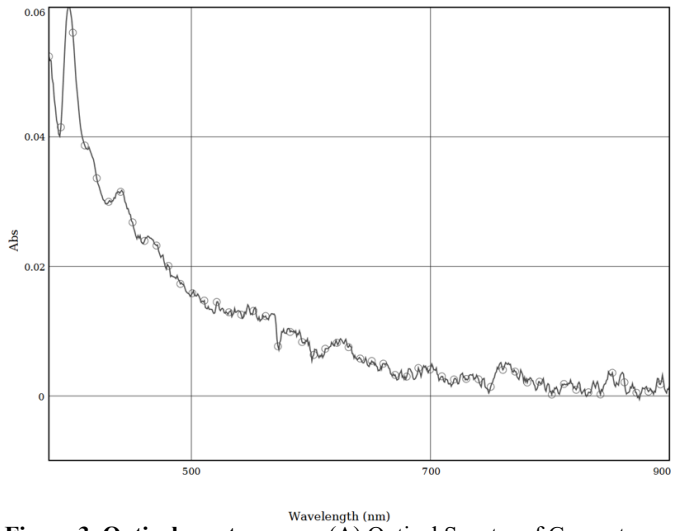

Figure 3. Optical spectroscopy. (A) Optical Spectra of Georgetown experimental sample water

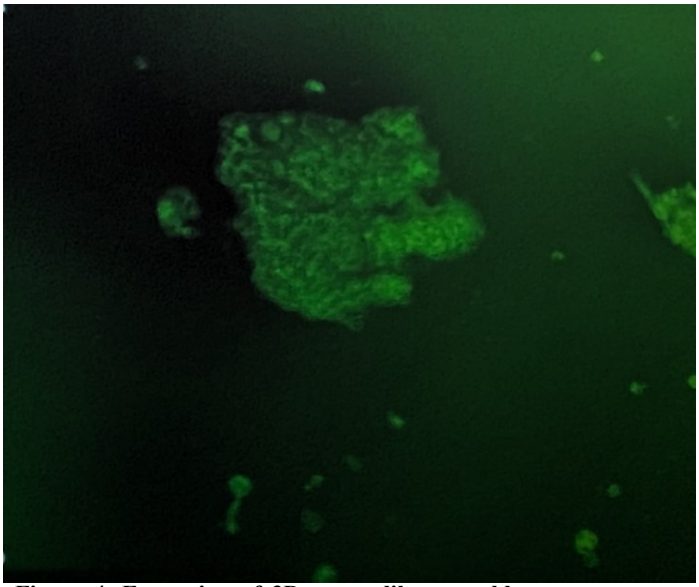

Figure 4. Formation of 3D tumor-like neuroblastoma aggregates after exposure to experimental Georgetown, SC water samples. (A) Image with the Naphthalimide-based probe that has selective binding to tumor-like cells.

pollution from industrial complexes could correlate with the cancer-per-capita statistics. These results are also of importance to bring awareness to because of the impact on the Gullah-Geechee cultural heritage corridor. In the future, biotherapeutics can continue to be developed to counterbalance the effects of these carcinogenic chemicals that are causing differential expression of known oncogenes. Potentially, these biotherapeutics can be derived from Gullah Geechee anecdotal evidence and later scientifically examined evidence. Current broad spectrum cancer therapies can also leave other normal cells in harm's way. With the continuation of research, these newer therapies that are specifically targeted to malignant neuroblastomas can be further developed as complementary medicine to help alleviate the growth, spread, and inflammation within the tumor's microenvironment from external chemical influences.

Acknowledgements

This work was funded by the SC-INBRE and BREP program at Francis Marion University. We would like to thank Andrew Ackerman for running most of the experiments, writing the original manuscript within the Vacharasin Lab with writing suggestions with other authors, and collecting samples. We would like to acknowledge Teil Fuller, Cyrus Ingram, Matthew Dixon, and Elaina Ponder of the Vacharasin Lab for their support of feedback. The Vacharasin Lab would also like to thank the members of the Department of Biology and the Department of Chemistry at Francis Marion University and Tanzil Ur Rehman at the National Center of Excellence in Physical Chemistry at the University of Peshawar for the insightful support.

Notes and References

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