Elucidation of Neuroprotective Properties of Astrocytoma (Astrocytes-like) Cells in Neural Cell Culture Models *In Vitro*: Applications in Tissue Engineering and Nanotoxicology

Y.Y.W. Wong[*], A.R. Jaiswal[**], V.V. Dukhande[***], A. Bhushan[****], S.W. Leung[*****] and J.C.K. Lai[******]

[*]Presenting author: Department of Biomedical & Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 469-463-5139, email: wongyin@pharmacy.isu.edu

[**] Department of Biomedical & Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-705-2447, email: jaisashv@pharmacy.isu.edu

[***]Department of Molecular and Cellular Biochemistry, College of Medicine, University of Kentucky, Lexington, KY 40536-0509, USA Fax: 859-257-2283; Tel: 208-315-3370, email: vikas.dukande@gmail.com

[****]Department of Biomedical & Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-282-4408, email: abhushan@pharmacy.isu.edu

[*****]Department of Civil and Environmental Engineering, College of Engineering, and Biomedical Research Institute, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4538; Tel: 208-282-2524; email: leunsolo@isu.edu

[******]Corresponding author: Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-282-2275; email: lai@pharmacy.isu.edu

ABSTRACT

Evidence is accumulating that cell culture models *in vitro* facilitate high throughput and mechanistic studies in tissue engineering. Astrocytes are known to protect neurons against pathophysiological assaults *in vivo* and *in vitro* without fully elucidated underlying mechanism. Previously, we investigate several of the putative neuroprotective properties of astrocytes in cell culture model. Those studies led us to hypothesize that different stress factors can elicit and/or enhance the neuroprotective effects of astrocytes through the activation of astrocytic signaling and alteration of astroglial function. The results of this study suggest that U87 (astrocytes-like) astrocytoma cells exert some protective effects on SK-N-SH (neurons-like) neuroblastoma cells against pathophysiolo-gical assaults (e.g., oxidative stress) under several sets of culture conditions not previously studied. Thus, our results may have pathophysiological implications and applications in neuroprotection, tissue engineering, and nanotoxicological research.

Key Words: Cell culture models; human neural cells; U87 astrocytes-like astrocytoma cells; neuroprotection; tissue engineering; nanotoxicology

Topic Area: Neurotoxicity of Nanomaterials (Under Environment, Health & Safety of Medical and Bio Health)

1. INTRODUCTION

There is increasing evidence that cell model systems *in vitro* play vital roles in recent tissue engineering research. The additional structural and functional perspectives of co-culture cell systems *in vitro* start to gain prominence over single cell type cell systems because unlike the latter, the former more closely resemble cell types in a tissue/organ *in vivo*.

Astrocytes (non-nerve, glial cells) in the brain play important roles in protecting neurons (nerve cells) against various assaults; yet, the cellular and molecular mechanisms underlying this protection is far more being understood [1]. Among the research on further elucidating such mechanisms, more efforts appear to center on commonly known neurodegenerative diseases such as Alzheimer's disease (AD), followed by Parkinson's disease (PD) [2]. AD is a progressive and irreversible neurodegenerative disorder in human brain leading to key symptoms of memory loss [3]. No known cure is available for AD as current therapy primarily treats AD symptoms and presents them from worsening. Thus, there is an urgent need to better understand how astrocytes exert their protective effects on neurons in a co-culture cell system *in vitro*. Once this protection is elucidated, then one could design treatment strategies and/or tissue engineering application to prevent the occurrence of accelerated neuronal loss in neurodegenerative diseases such as AD and PD.

Our project seeks to examine the underlying neuroprotective properties offered by astrocytes to protect neurons, employing a co-culture cell model system *in vitro.* We have been developing several model systems to investigate putative cytotoxic and inflammatory mechanisms associated with induction of neuronal cell damage and cell death [4-6]. Our previous data have shown that U87 astrocytoma and SK-N-SH neuroblastoma cells constitute good co-culture model systems for astrocytes and neurons, respectively, *in vitro* because of their close functional resemblance to normal astrocytes and neurons and that U87 cells protect SK-N-SH cells from several pathological assaults [4-5]. We therefore hypothesized that different stress factors can elicit and/or enhance the neuroprotective effects of astrocytes through the activation of astrocytic signaling and alteration of astroglial function. In this study, we have investigated this hypothesis further.

2. MATERIALS AND METHODS

2.1 Cell culture of U87 and SK-N-SH cells

The human astrocytoma (U87) and neuroblastoma (SK-N-SH) cell lines were obtained from (ATCC; Manassas, VA, USA) and cultured in MEM supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA), 1% (w/v) sodium pyruvate (Sigma Aldrich; St Louis, MO), 0.292 g/L L-glutamine (Sigma), 1.5g/L sodium bicarbonate (Sigma) and 1% (v/v) antimycotic (Atlanta Biologicals; Lawrenceville, GA). Cells were maintained at 37°C and 5% (v/v) CO_2 as described previously [4-5].

2.2 Treatment of U87 cells

The U87 cells were cultured in T-75 tissue culture flasks to ~50% confluent in 20 ml of 10% FBS-containing MEM medium. Then the medium was discarded and changed to 20 ml of 5% FBS-containing medium and the cells incubated for 24 hours. After that, the medium was replaced with 20 ml of medium containing 0% FBS and the cells were incubated for an additional 24 hours. Cells were treated with L-sulfoximine buthionine (BSO), pioglitazone (PZ) or a combination of both. At the end of another 24 hours of incubation, the media were removed and frozen with liquid nitrogen immediately. The cell pellet was collected and both cell pellet and media collected were kept at -80°C until used for experiments.

2.3 Cell Survival (MTT) assay

The neuroprotective properties of U87 cells on SK-N-SH cells were determined in different combinations of regular medium (RM) and conditioned medium (CM) collected from U87 cells cultured under various conditions (see above). RC/CM ratio of 100/0, 70/30, 50/50, 40/60, 30/70, and 0/100 were used. SK-N-SH cell survival after the treatments was assessed employing the MTT assay [5].

2.4 Western Blot Analysis

Western blot analysis was employed to assess protein expression in cell lysates to determine the effects of media, BSO and PZ. SDS-PAGE electrophoresis was performed as previously described [7]. The separated proteins were visualized using chemiluminesence detection kit while β-actin was the loading control.

3. RESULTS AND DISCUSSION

Our culture model consists of U87 (astrocytes-like) cells and medium collected from U87 cells cultured under specified conditions to grow SK-N-SH (neurons-like) cells. This is a new modified co-culture strategy to allow the characterization of putative "protective factors" secreted by U87 cells to protect SK-N-SH cells in a non-contact cell culture setup. In addition, we can characterize the putative structural and functional properties of U87 cells under the different specified conditions.

A selective inhibitor of glutathione (naturally-occurring antioxidant) synthesis, L-buthionine sulfoximine (BSO), is used to induce oxidative stress in cells. A peroxisome proliferator activated receptor gamma (PPARγ) agonist, pioglitazone (PZ), is used to block the inflammation which is usually unregulated in AD [8-9]. We have designed studies whereby we could utilize BSO and PZ in addition to serum depletion to further elucidate the putative protective mechanism(s) of U87 cells on neurons-like SK-N-SH cells. Serum depletion and drug treatment for U87 cell cultures were prepared as shown in the flow chart below (Fig. 1). U87 cells cultured in medium containing 10% FBS were

used as control (not shown in Fig. 1). Medium containing 0% FBS were defined as serum free (SF) medium.

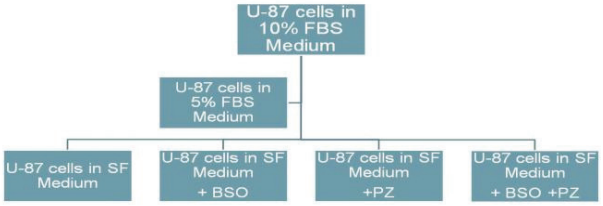

Figure 1. Experimental design of U87 cells treated with BSO, PZ or in combination. The U87 cells were grown to ~50% confluent in 10%-FBS containing medium and subsequently discarded and changed to 5% FBS-containing medium and incubated for 24 hours. After that, the medium was replaced with medium containing 0% FBS and incubated for an additional 24 hours. Cells were treated BSO or PZ or a combination of both for another 24 hours of incubation.

Morphological and functional changes/adaption in astrocytes *in vivo* and *in vitro* is known to associate with oxidative and other environmental stresses. We employed light microscopy to examine the effects of BSO treatment and FBS depletion to induce oxidative and environmental stress, respectively, on U87 cells and assess the effects of PZ treatment thereon.

Treatment with SF medium induced U87 cells to put out multipolar processes and the other treatments accentuated this effect (Fig. 2). Clearly, these observations raise interesting mechanistic questions that need to be addressed further (see below).

Glial fibrillary acid protein (GFAP) is a well-known and classical marker for astrocytes: its altered expression is associated with changes in astroglial morphology. Thus, we employed western blot to determine if the morphological changes in U87 cells exposed to SF medium with and without BSO/PZ treatment were seen also associated with changes in GFAP expression. An increased GFAP expression was observed when U87 cells were cultured in SF medium compared to those cultured in 10% FBS medium (data not shown), suggesting a correlation between changes in cellular morphology and GFAP expression.

Growth factors and neuronal survival enhancers are putatively released into the medium by astrocytes to protect their surrounding neurons. To investigate this possibility, we collected media in which we grew U87 cells under the conditions specified in Figure 1 above and assessed their ability to influence survival of SK-N-SH cells. Our results suggested that exposure of SK-N-SH cells to various combinations of SF media allowed them to survive better than when cultured in 10% FBS medium (control condition) alone (Fig. 3). Moreover, exposure to PZ enhanced this protective effect, even under conditions of oxidative stress.

4. CONCLUSIONS

When taken together, our results suggest astrocytes-like U87 cells changed their cell shape and morphology when cultured under serum-free media with and without drug treatment. Those morphological changes correlated with upregulation of GFAP in such cells.

Moreover, exposure of SK-N-SH cells to various combinations of SF media allowed them to survive better than when cultured in 10% FBS medium (control condition) alone and exposure to PZ enhanced this protective effect, even under conditions of oxidative stress. Thus, our results provide additional evidence that our cell model can be productively employed to further elucidate the putative neuroprotective mechanisms of astrocytes. As such they assume pathophysiological importance in neuronal survival/death mechanisms in AD and PD. Clearly, this is an area that merits further study, especially in tissue engineering and nanotoxicological research and applications.

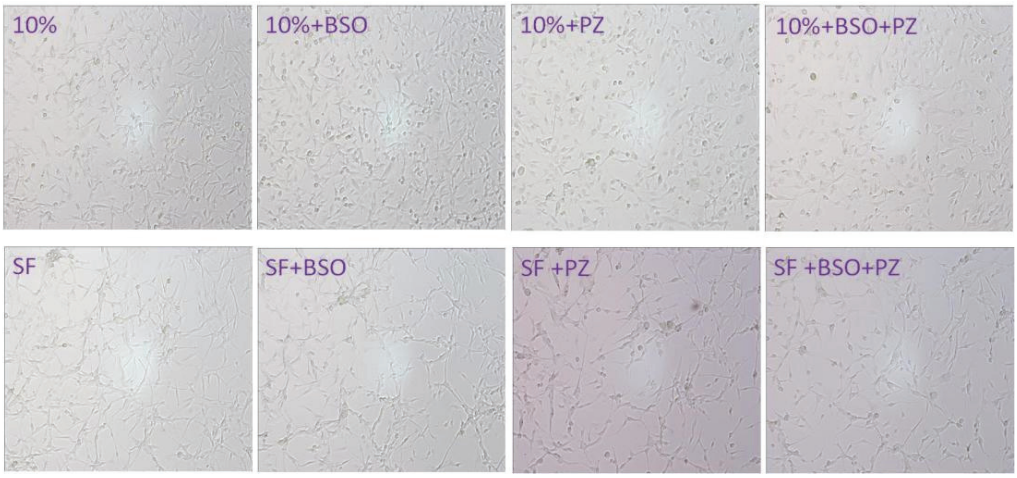

Figure. 2 Treatment-induced cellular morphology changes in U87 cells (100X magnification). Top panel of photomicrographs represents U87 cells cultured in 10% FBS medium treated with or without drug or drug combination while those of the bottom panel represents U87 cells cultured in serum-deprived (SF) medium treated with or without drug or drug combination.

5. ACKNOWLEDGMENTS

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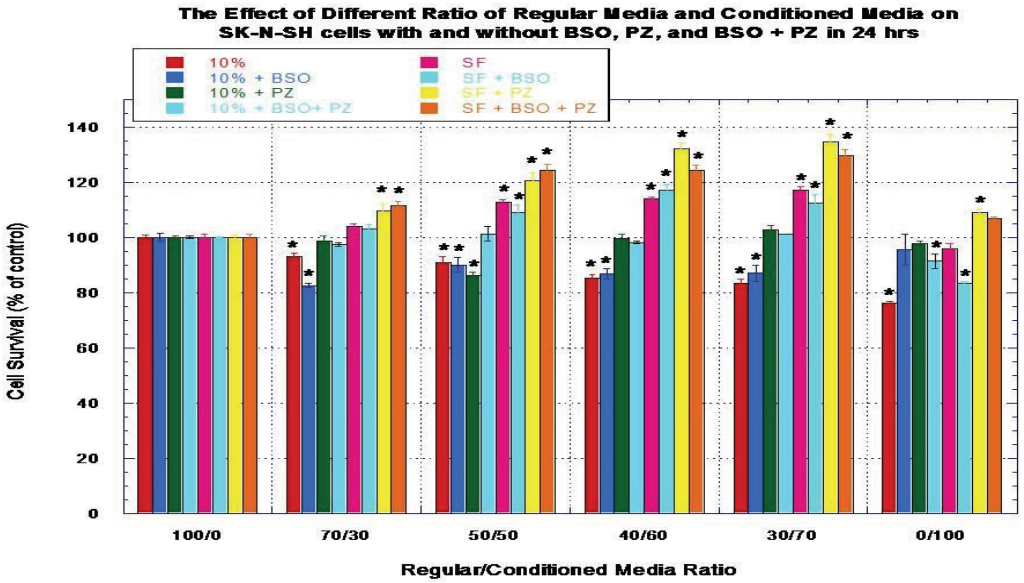

Figure 3. Treatment effects on SK-N-SH cell survival with different regular/conditioned media combination (see Fig. 1). SK-N-SH cells cultured in 10% FBS served as the control. Values are expressed as % of the control and are given as mean ± SEM of three separate determinations. *p<0.05 versus control.