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Identification of Protein (S) in Ischemia Induced PC12 Cells after Induction with Conditioned Media of IMR-32 and U87mg Cells

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Abstract: Hypoxia to the tissue plays a significant role in ischemia. In spite of the development of various *in vitro* models of neuronal cultures to study the mechanism of ischemia-induced cell death, not much is known about the role of protein expression in ischemia induced pheochromocytoma (PC12) cells. Present study was done to study the expressions of proteins in hypoxia induced PC12 cells and after induction with conditioned media of human neuroblastoma (IMR32) and human glioblastoma (U87MG). We found down regulation of four protein markers with molecular weight of 97, 75, 40 and 14 kDa in hypoxic PC12 cells. Majority of these proteins reappeared after treatment with the conditioned media of IMR32 and U87MG cell lines. Identified proteins will be helpful to understand the mechanism of hypoxia/ischemia induced cell death and also the neuroprotective role of conditioned medium of IMR32 and U87MG cell at the proteomic level.

Key words: Hypoxia · pheochromocytoma cells · conditioned medium

INTRODUCTION

Hypoxia or lack of oxygen supply to the tissue plays a significant role in diseases like ischemia. Various *in vitro* models of neuronal cultures have been developed to understand the mechanism of ischemia-induced cell death at the cellular and molecular level [1, 2]. Pheochromocytoma (PC12) cell is well known and widely used in-vitro neuronal model to study hypoxia [3, 4]. In our previous work, we have demonstrated that PC12 cell when cultured with conditioned media of human neuroblastoma (IMR32) and human glioblastoma (U87MG) cell lines induce neurite outgrowth within 48 hr [5]. However, there are very few published reports which indicate the importance of specific protein expression in PC12 cells under ischemic condition [6-8]. Gonzalez *et al*. demonstrated that proteins of the immediate early genes c-Fos and c-Jun were found to be significantly increased in PC12 cell after *in vitro* ischemia [9]. Similarly Schmidt *et al.* showed high mRNA expression of neuroglobin in cell culture after severe prolonged hypoxia [10].

In the present study protein expression using one-dimensional polyacrylamide gel electrophoresis (PAGE) was studied in PC12 cell line with induction of hypoxia and after treatment with conditioned media of IMR32 and U87MG. Additionally hypoxia induced cell death was also studied at morphological and the biochemical levels by estimating lactate dehydrogenase activity (LDH).

MATERIALS AND METHODS

Pheochromocytoma (PC12), human Neuroblastoma (IMR32) and Human Glioblastoma (U87MG) cell line were purchased from National Center for Cell Science, Pune, India, Dulbecco's Modified Eagle Medium (DMEM), Sodium azide were purchased from Himedia, India, heat-inactivated horse serum and newborn calf serum were purchased from Sigma MO. USA and Albumin depletion kit from Bio-Rad, USA.

Cell culture: IMR32 and U87MG cell line were routinely maintained in DMEM medium supplemented with 5% newborn calf serum, 10 µl ml^{-1} penicillin, 25 µg ml^{-1} streptomycin and 25 µg ml^{-1} amphotericin B (complete medium), while PC12 cell line was routinely maintained in

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complete DMEM medium supplemented with 10% heat inactivated horse serum. Cells were routinely sub-cultured every 4-5 days.

Preparation of conditioned medium: $3x10^6$ cells of IMR32 and U87MG were seeded in tissue culture flask (Orange Scientific, USA) in 10 ml of complete DMEM medium and were allowed to grow for 3 to 4 days or till confluence was observed. When 90 % confluence was obtained the medium was removed and centrifuged at 2000 rpm for 20 minutes to remove the suspended cells. The supernatant (conditioned medium) collected was filtered through 0.22 um membrane syringe filter (Laxbro, India) and was stored at -20°C till further use.

Experimental protocol for *in vitro* chemical ischemia: PC12 cells were seeded in 24-well plates at a density of 2×10^5 cells ml^{-1} in complete DMEM medium containing 10% heat inactivated horse serum. Chemical ischemia was achieved in experimental sets by seeding PC12 cell in complete DMEM medium containing heat inactivated horse serum and 4 mM sodium azide in presence and absence of IMR32 and U87MG conditioned medium. Cells were then incubated for different time intervals, 20 min, 60 min, 90 min, at 37°C in a humidified 5% CO2 atmosphere. At the end of incubation period medium was removed and centrifuged at 2000 rpm for 10 min and supernatant was collected for analysis of alteration in morphological and biochemical parameters.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): Supernatant collected after chemical ischemia was subjected to electrophoresis after albumin depletion. For electrophoresis the sample was prepared by mixing albumin depleted supernatant containing 5 µg of protein with 10 µl of tracking dye. One dimensional Sodium dodecyl sulphate Polyacrylamide gel electrophoresis was performed with the vertical slab gel electrophoresis system (Broviga, India) using the standard Laemmali Method with 5% stacking and 10%, running gel. Electrophoresis was carried out at 150V. After completion of electrophoresis gel was developed using silver staining to observed protein profile.

Lactate dehydrogenase estimation: LDH activity in chemical ischemia induced PC12 supernatant was measured using commercial available LDH assay kit (Agappe Diagnostic pvt. India) by Erba Mannheim analyzer (Germany) at 340 nm.LDH activity was expressed as unit L^{-1}

RESULTS

Figure 1 shows the protein patterns in Sodium dodecyl sulphate polyacrylamide gel electrophoresis gels of supernatant samples obtained from PC12 cells with and without hypoxic condition, treated with IMR32 and U87MG conditioned medium. Expression of four proteins of PC12 cells under hypoxic condition with molecular weight of 97, 75, 40 and 14 kilo Dalton was down regulated as compared to normal PC12 cells (without sodium azide treatment). After treatment with conditioned medium of IMR32 the expression of three proteins 97, 40 and 14 kDa were up regulated. However in case of U87MG condition medium treated PC12 cells only two proteins i.e 40 and 14 kDa were upregulated.

Fig. 1: Shows10% SDS PAGE electrophoretogram of PC12 cell supernatants. Lane 1. Control (Normoxia), lane 2. Hypoxia with 4mM sodium azide, lane 3. Hypoxia in presence of IMR-32 Conditioned Medium, lane 4. Hypoxia in presence of U87MG conditioned medium

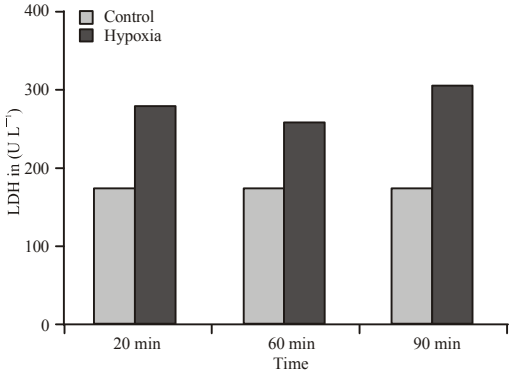

Fig. 2: Depict LDH activity in control and hypoxia induced PC-12 cell Supernatants at different time interval. In PC12 cell hypoxia was induced using 4 mM sodium azide

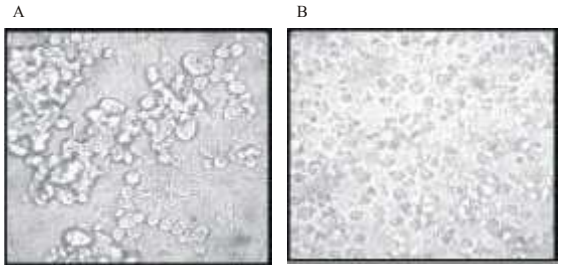

Fig. 3: Shows light microscopic photograph of the A) control and B) Sodium azide treated PC-12 cell

Figure 2 shows LDH estimation in PC12 cell to study the hypoxia induced cell death. LDH level is very high in PC12 cell under hypoxia as compare to normal PC12 at different time intervals of hypoxia. Figure 3 shows the morphological study of PC12 cells with and without sodium azide treatment. Hypoxia induced cell death was also noted in the PC12 cells which are treated with sodium azide. However, no changes were observed with respect LDH and morphological level after treatment with the IMR32 and U87MG conditioned medium.

DISCUSSION

PC12 cells treated with sodium azide is well established and widely used *in vitro* model for hypoxia studies. The present study focuses on three major issues; these are alterations in 1) protein expression 2) morphological appearance and 3) lactate dehydrogenase activity in PC12 cells (under hypoxia condition) and after treatment with the conditioned media of IMR32 and U87MG cell lines.

In our preliminary study, we have demonstrated relatively low expression of four protein markers with molecular weight of 97, 75, 40 and 14 kDa in PC12 cells under hypoxic condition as compared to normal PC12 cells. The marked visualization of these proteins reappeared except 75 kDa after treatment with the conditioned media of IMR 32 cell lines. However only 40 and 14 kDa proteins reappeared after treatment with conditioned media of U87MG cell line. These results reinforce our argument that these proteins are important markers to assess the neuroprotective response after hypoxia. These results suggest that cells treated with conditioned media of U87MG and IMR32 cell lines may protect the cells from hypoxia. This data also supports our earlier study where PC12 cells lines induced neurite outgrowth when treated with conditioned medium of IMR32 and U87MG cell lines [5]. Similarly the hypoxia induced cell death demonstrated an increase in LDH level and alteration in morphology of PC12 cells. However, we did not observe similar changes with respect to these two parameters (LDH level and morphological alteration) after treatment with the conditioned medium of IMR32 and U87MG cell line.

Previous publications have demonstrated either decrease or an increase in several proteins in PC12 cell lines under hypoxic conditions. Thuyan Tran *et al*. have shown the up regulation of early growth response protein 1(EGR 1) in PC-12 cells under hypoxic conditions [11]. The same authors have reported high expression of vascular endothelial growth factor (VEGF) in hypoxic PC12 cell line. These studies suggest the importance of identification of potential markers in PC12 cells with or without hypoxia to understand the mechanism of ischemia-induced cell death at the proteomic level.

The low expression of 97 and 75 kD proteins in PC12 cells under hypoxic condition is very interesting and suggests that these proteins play important role in maintenance of integrity of neuronal cells. A key feature of cerebral ischemia is the blocking of translation at the initiation step, as indicated by severe suppression of the incorporation of amino acids into proteins. However, the up regulation of suppressed proteins often indicates that these proteins are again resynthesised by the cells. It is believed that that ischemia-induced suppression of protein synthesis is reversible in resistant neuronal cells. The identification and characterization of such proteins in PC-12 cells will help in identification of cells responsible for production of these proteins.

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