Study of Cytochrome P450s in Differentiating Neuronal Cells Derived from Human Hematopoietic Stem Cells

Synopsis

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DOCTOR OF PHILOSOPHY
In
BIOTECHNOLOGY

By
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M.Sc.- Biotechnology (JRF-CSIR)

Under the Joint supervisions of

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The developing brain is particularly vulnerable to toxic agents, even at exposure levels that have no lasting effects in the adult nervous system. In part, this may be due to no or low metabolic functionality in differentiating cells. The expression, inducibility and regional specificity of several members of cytochrome P450s (CYPs) gene family, involved in metabolism, toxicity and detoxification have been documented in mature brain following experimental exposure of environmental chemicals and drugs. However, the expression and inducibility of CYPs during neurogenesis is poorly understood. The use of pure population of cultured cells of neuronal origin has been suggested as a powerful tool to study and elucidate such mechanism based studies. But in vitro studies with developmental stages have been hampered since most of the studies were conducted with mature adult cells only.

Stem cells isolated from human umbilical cord blood will be used as the primary source material, since stem cells are known to have comparable properties to primary human cells, i.e., unlimited proliferation ability, plasticity to generate other cell types and a more readily available source of human cells. It is also important to note that till date most of the mechanism understanding studies have been made using non-stem, proliferating normal, immortalized, or tumorgenic cells. Thus, we propose to exploit the proliferation and plasticity potential of human stem cells to convert them into neuronal subtypes. The stem cells isolated from human umbilical cord blood (which is non-neuronal origin and generally discarded material after the birth of child) will be subjected to differentiate into neuronal subtypes under the influence of specific growth conditions. Then the expression and inducibility of brain CYPs will be studied all through the differentiation following the exposure of known inducers of CYPs like 3-methyl cholanthrane, ethanol etc. Further, the system will be validated using monocrotophos (MCP), a widely used neurotoxic organophosphate pesticide known to induce brain CYPs in rat brain following experimental exposure, at various points of differentiation/maturity of cells. MCP is selected in this study since; it is most widely used organophosphate pesticide and known to have teratogenic effects in rat fetus and lactating pups. This will allow us to prove the concept that CYPs expression profile could be used as biomarkers of exposure and effects in developing brain for environmental chemicals and drugs.

Thus, in other word it is proposed to develop new and more sensitive stem cell based in vitro model to study the possible developmental neurotoxicity potential of environmental
chemicals, which are supposed to come in contact with human beings and developing fetus and growing children by invading our ecosystem. The chemical induced differentiating stem cells derived from human umbilical cord blood will be used to study the basal expression of marker genes involved in neurogenesis, neurodegeneration, neuroprotection and xenobiotic metabolism and their responsiveness to MCP.

**Objectives:**

- To convert the human umbilical cord blood stem cells into neuronal subtypes
- To study the expression and inducibility of xenobiotic metabolizing cytochrome P450s all through the differentiation following the exposure of known inducers of P450s
- To study the responsiveness of differentiating stem cells against known developmental neurotoxicant pesticide- monocrotophos

**Current Status:**

Human umbilical cord blood is non-controversial, easily available and richer source for hematopoietic stem (HSCs) and progenitor cells (Ha et al., 2001; Buzanska et al., 2002; Zigova et al., 2002) that are pluripotent having the capability of self-renewal and differentiation, under the influence of specific growth conditions, into various cell types like hepatocytes (Newsome et al., 2003), osteoblast (Rosada et al., 2003) and neuronal cells (Sanchez-Ramos et al., 2001; Bicknese et al., 2002; Buzanska et al., 2003; Zigova et al., 2002). These properties of stem cells have been utilized in toxicology (Davila et. al., 2004) to develop *in vitro* model system to test the toxicity of environmental chemicals and drugs in specific cell type differentiated from these cells. To anticipate the toxicity of various xenobiotics, the role of cytochrome P450 (CYP) enzymes are undertaken which are complex multigene superfamily of heme proteins and metabolize a wide variety of xenobiotic compounds. The CYPs are abundant in the liver which is the primary organ to mediate CYPs associated metabolism. The expression, inducibility and regional specificity of several members of CYP gene family involved in metabolism, toxicity and detoxification have been well documented in mature brain following experimental exposure of environmental chemicals and drugs (Ravindranath et al., 1989; Meyer et al., 2007). However, the information about the expression and inducibility of CYPs in HSCs and progenitor cells are still limited.

To date, the expression of mRNA for various CYPs like CYP1A1, 2D6, 2E1, and 3A4 are demonstrated in human bone marrow (Bernaure et al., 2006), macrophages (Hutson et al. 1999), as well as in mononuclear cells (Ali Asghar et al., 2002). Various metabolites of
benzene induce the expression of CYPs mRNA in human leukemia hematopoietic stem cell lines KG-1, U937 as well as in bone marrow culture (Henschler et al., 1995). For the first time the expression and specific enzymatic activity of CYP2E1 in HSCs was reported by Kousalova et al. (2004). Later on Pavel et al. (2005) reported the constitutive expression of CYP1B1 and CYP2E1 mRNA and proteins in purified population of HSCs and progenitor cells. The expression of CYPs at RNA level is also reported in fetal liver hematopoietic stem cells (Jing Shao et al., 2007).

The attempt will be made to anticipate the toxicity, metabolic capability and effects of various environmental chemicals and drugs in developing brain during gestational periods. To predict this, proliferation and plasticity potential of human umbilical cord derived stem cells will be exploited to differentiate them into neuronal subtypes in vitro under the influence of specific growth conditions to mimic the in vivo system of brain development and the level of CYPs expression will be measured at various maturity of neurogenesis following the exposure of Monocrotophos and known CYP inducer like 3-Methyl Cholanthrane.

Methodology:

Isolation of stem cells from umbilical cord blood: Stem cells will be isolated, characterized and cultivated from umbilical cord blood obtained from CSM Medical University, Lucknow, India as well as also being procured from commercial sources. Stem cells will be isolated by ficoll density separation method using RosetteSep human stem cell enrichment antibody cocktail followed by Robosep (magnet assisted robotic automated) separation method using EasySep CD34+ hematopoietic stem cells isolation kit.

Characterization of stem cells: The purity of freshly isolated and cultured cells at various passages will be analyzed by two-color flow cytometry on a FACS Canto II flowcytometer (Becton Dickinson, San Jose, CA, USA) using fluorescently tagged antibodies against specific antigenic markers of stem cells like CD34, CD133, CD38 and CD45. The purity of cells will also be assessed with the help of Real Time PCR and some specific dye test like Hoechst 33342 and Rhodamine 123.

Bulk production of stem cells: Purified population of characterized stem cells will be cultivated in Myelocult expansion medium supplemented with 20% FBS, 20% HS, Hydrocortisone (10^{-6}M), Penicillin 100 U/ml, Streptomycin 0.1mg/ml and glutamine (2mM) 10ng/ml IL-3, 10ng/ml IL-6, 25ng/ml rhTPO, 25ng/ml rhSCF, 50ng/ml rhFGF-
basic and 10ng/ml rhFLT-3L. Medium will be changed at an interval of 3 days till the confluence reached.

**Chemical induced differentiation of stem cells:** Confluent growing cells will be sub-cultured as per the experimental demand and allowed to differentiate in neuronal subtype in Serum free Neurobasal Medium supplemented with N5, B27 supplement, rhNGF (50ng/ml), rhFGF-basic (50ng/ml), rhTPO (25ng/ml), retinoic acid (10^{-5}M), for a period of 30 days (Kogler, et.al., 2004). At various time points of maturity, cells will be studied for the basal levels of expression profile of marker genes involved in neurogenesis.

**Identification of non-cytotoxic doses of known inducers of CYPs and monocrotrophos:** Prior to start the expression studies, non-cytotoxic doses of MCP and CYP inducers such as 3-methyl cholanthrane, ethanol and phenobarbital will be ascertained in chemical induced differentiating cultured cells using standard endpoints of cytotoxicity mainly including, morphometric analysis, MTT, NRU, trypan blue dye exclusion assays. The non-cytotoxic selected doses will be used in all further experiments.

**Expression studies for neuronal markers:** Constitutive expression (mRNA and Protein) and inducibility of marker genes involved in neuronal development, injury and repair will be studied using Real Time PCR with SYBR green and TaqMan Chemistry (Low Density Array Plate) following the standard protocols at various points of differentiation of neuronal cells. Immunocytochemical localization of antigens for marker genes will also be done using specific monoclonal antibodies to quantify and localization of physiological functional proteins in chemically induced differentiating cells at various time points of maturity.

**Expression studies for CYP450s:** The expression of various cytochrome P450s (CYP1A, 1B, 2B, 2E1, 3A, etc.) involved in toxicity and detoxification of various environmental chemicals/drugs will be studied following the exposure of known inducer of CYPs and known neurotoxicant MCP. The unexposed control sets will also be run parallel under identical experimental condition. The expression studies will be done with the help of Real Time PCR using SYBR Green Chemistry and Taqman chemistry and immunocytochemical localization following the standard protocols.

**Expected outcome:** For the first time, it seems possible to establish an innovative screening method for human specific xenobiotic metabolism and toxicity/detoxification. It will also be useful to identify biomarkers without animal based *in vitro* and *in vivo* systems by exploiting the outstanding feature of human stem cells from placenta, a waste material after the birth of a child. The expected *in vitro* models will be having a quality to
rationalize the specific response of each individual towards the specific chemical. Moreover, it will be genetically homogenous model of future research for more precise and mechanism specific assessment of vulnerability of human brain cells at different stages of development against drugs and environmental pollutant. These *in vitro* models will also be applicable to tell the role of specific marker genes and pathways involved in the neurogenesis, metabolism and chemical induced neurotoxicity and neuroprotection in the human beings. Further, the use of human stem cells will be ideal for extrapolation of ambiguous conventional test results to human and for prioritising conventional testing. Output of the study will also be helpful to regulatory agencies to incorporate a battery of new objective endpoint for the pre-screening of chemicals for developmental neurotoxicity.
Bibliography:


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