UV-B INDUCED STIMULATION OF PHOSPHATIDYLINOSITOL 3-KINASE IN HUMAN FETAL HEPATOCYTES

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ABSTRACT

We investigated the effect of UV-B irradiation on PI 3-kinase activity in human fetal hepatocytes. When cells were exposed to monochromatic (304 nm) UV-B light, a significant increase in intracellular PI 3-kinase activity was observed in a dose dependent manner with maximal activity upon 1500 Jm⁻² irradiation. At 1500 Jm⁻² dose PI 3-kinase activity increased by 80% in membrane fraction of fetal hepatocytes of 25 weeks gestation. PI 3-kinase inhibitors wortmannin and LY294002 specifically inhibited the UV-B induced lipid kinase activity and blocked significantly the UV-B induced cell viability. The data suggests a correlation between cell survival and elevated levels of PI 3-kinase and suggest that UV-B irradiation at a dose of 1500 Jm⁻² is ideal for fetal hepatocyte transplantation. Also, PI 3-kinase levels could be a representative marker for viable UV-B irradiated fetal hepatocytes for transplantation.

KEY WORDS

Signal transduction, Lipid kinase, Hepatocytes transplantation, Xenotransplantation

INTRODUCTION

Cells exposed to various stress factors such as UV, X-rays, reactive oxygen species (ROS) and photodynamic action induce the activation of stress-induced protein kinase and a number of biochemical processes (1-3). As ultraviolet (UV) irradiation causes alterations in cell surface proteins, it emerged as one of the most potent modes of immunosuppression. Xenotransplantation of UV-B (290 – 320 nm) irradiated hepatocytes are a viable alternative to facilitate clinical transplantation of irradiated hepatocytes without rejection (1). Human fetal hepatocytes exposed to UV-B retained their viability, metabolic and detoxifying capacities and are suitable for transplantation (1). UV is known to interact quite differently with cells according to the wavelength range. Recent studies suggest that insulin growth factor1 (IGF-1) can protect fibroblasts from UV-B light induced apoptosis, but on the other hand, it was also found that this protection against UV-induced apoptosis was blocked by a fungal metabolite, wortmannin (potent inhibitor of PI 3-kinase), which implies a role for PI 3-kinase (4). Since UV-B exposed human fetal hepatocytes are commonly used for transplantation,

in the present study an attempt was made to find out optimal UV-B dose for viable fetal hepatocytes for transplantation.

MATERIALS AND METHODS

Fetal Calf Serum (FCS), DMEM/F12, Hank's buffer, phosphoinositides, Benzamidine, Tris, and Kodak X-Omat AR films were obtained from Sigma Chemicals Co. St.Louis, USA.

(γ -³²P)-ATP was obtained from BRIT (Board of Radiation and Isotope Technology) India.

PI 3-kinase antibodies (specific to p85 α subunit) were purchased from Upstate Biotech Inc., USA. Silica gel 60 plates were obtained from E-Merck, USA. All other chemicals used were of analytical grade.

Human fetus collection

Human fetuses were collected within 1-2 hr of abortion. The samples obtained were mainly after spontaneous abortions, and occasionally, from pregnancies terminated under medical supervision after the consent from parents/guardians. Mothers with a history of alcohol abuse, diabetes, jaundice or any other metabolic disorder and also stillborn deaths with growth retardation (e.g. Anencephalic cases etc.) were excluded from the study. The age of the fetuses was calculated by measuring the crown rump length using Hamilton chart and also from the history of mother's last menstrual period. The ethical committee of the

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Indian Council of Medical Research (ICMR), New Delhi, permitted the use of liver tissue from these fetuses for the present research work.

Hepatocyte Isolation

Liver cells from human fetuses of 20-28 weeks of gestation were prepared by a two-step perfusion method (5). In brief, the human fetal liver was perfused *in situ* through the umbilical cord with Ca^{2+} ion - free Hank's buffer (pH 7.3), followed by 0.025% collagenase digestion for 15 min. The liver was then excised, decapsulated, minced and filtered through a 35 μm porosity nylon mesh by slow agitation. The hepatocytes were then finally separated by elutriation centrifugation in Hank's medium (pH 7.3).

Viability of Hepatocytes

Fetal hepatocytes (25 weeks gestation period) were maintained in Hank's medium for 12 h after UV-B irradiation and viable cells counted by trypan blue dye exclusion technique (6). To assess the effect of PI 3-kinase inhibitors, hepatocytes were pretreated with wortmannin (10^{-7} M) and LY294002 (10^{-5} M).

Irradiation of Hepatocytes

Isolated human fetal hepatocytes (25 weeks) were serum starved for 24 h and irradiated according to Lahari *et al.*, (2). Briefly, the cells were taken in a quartz petridish at a concentration of $3 \times 10^6/\text{ml}$ in Hank's medium then it is transferred into DMEM media and irradiated at 500, 1000, 1500, 2000, 2500 and 3000 Jm^{-2} (cells were irradiated at a distance of 10cm from lamp, $\lambda=302$ nm, for different time intervals) with a UV-lamp (UV M-57 ultraviolet products, USA) and radiometer assembly (UVX-31). Cells not exposed to UV-B were used as controls. To study the effect of PI 3-kinase inhibitors hepatocytes were preincubated with wortmannin and LY294002 for 15 min. After irradiation cells were maintained for 12 h in Hank's medium and analyzed for PI 3-kinase levels and cell viability.

Immunoblotting of PI 3-Kinase

Fetal hepatocytes (1×10^6 cells) of different gestation periods were lysed by suspending in RIPA buffer (20mM Tris-HCl, pH 7.4, 50mM NaCl, 50mM NaF, 1mM PMSF, 1mM sodium orthovanadate) and lysed with hand homogenizer. Cytosolic and membrane fractions were prepared by centrifugation at 30,000 x g, and were resolved on 10% SDS-PAGE (7). Following SDS-PAGE, the proteins were transferred on to nitrocellulose membrane and incubated for 90 min in blocking solution (TTBS: 20mM Tris-HCl, 0.05% Tween-20, 15mM NaCl, pH 7.4) supplemented with 3% gelatin. Nitrocellulose membrane was washed (15ml x 3) for 5 min with TTBS before incubating for 90 min with the monoclonal anti PI 3-kinase antibodies specific to p85 α

subunit (1:5000) in TTBS at 4°C with gentle rocking. The membrane was washed in TTBS (10 ml x 4), and incubated with secondary antibody solution (alkaline phosphatase-conjugated, diluted in TTBS; 1:3000) for 1 h at 4°C. Finally, the blot was developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) as the substrate.

PI 3-Kinase assay

PI 3-kinase activity was measured by the method of Carpenter *et al.*, (8). Membrane and cytosolic fractions were analyzed for the PI 3-kinase activity in fetal hepatocytes (25 weeks gestation period), which were previously exposed to different doses of UV-B radiation. The reaction mixture containing 100mM ($\lambda\text{-}^{32}\text{P}$)-ATP (0.2mCi/mmol), 5mM MgCl_2 , 100 mM phosphatidylinositol 4,5-bisphosphate in a final volume of 25 ml was incubated at 37°C for 10 min. The reaction was terminated by the addition of 105ml of 1N HCl, followed by the addition of 160 ml of chloroform: methanol (1:1 v/v). The samples were vortexed vigorously and centrifuged to separate the aqueous and organic phases. The organic phase (30ml) was resolved by thin layer chromatography (TLC) using oxalated silica gel 60 plate (9). The plate was developed with 1M acetic acid and 2-propanol (35:65), air dried plate was subjected to autoradiography by exposing to Kodak X-Omat film. Alternatively, the radioactivity incorporated was measured after drying the organic phase and counting using Beckman liquid scintillation counter (LS 1801 Beckman Scintillation Counter).

RESULTS AND DISCUSSION

To assess the ideal conditions for hepatocyte transplantation, fetal hepatocytes of different gestation periods were screened for PI 3-kinase levels by probing the western blot with anti - p85 α (regulatory subunit of PI 3-kinase) antibodies. PI 3-kinase in hepatocyte. Hepatocytes obtained after 23-28 weeks gestation, expressed significant levels of PI 3-kinase, however, fractions of 20 weeks gestation period did not shown any cross reacting bands corresponding to regulatory subunit of PI 3-kinase (Fig. 1). Since highest levels of PI 3-kinase were detected in 25 & 28 weeks gestation, 25 weeks hepatocytes were used in further studies. UV-B exposure resulted in activation of PI 3-kinase activity and the increase is dose dependent up to 1500 Jm^{-2} (Fig. 2). Two fold increase in total hepatocytes PI 3-kinase activity was observed when hepatocytes were irradiated with 1500 Jm^{-2} . Exposure at higher doses above 2000 Jm^{-2} resulted in total loss of lipid kinase activity. Since receptor activated PI 3-kinase is always membrane associated we measured the membrane and cytosolic PI 3-kinase levels upon UV-B irradiation. The results indicate a UV-B dose dependent increase in PI 3-kinase activity in membrane fractions and a corresponding decrease in cytosolic fractions (Fig. 3)

Figure 1. Expression levels of PI 3-kinase in human fetal hepatocytes of different gestation periods. Fetal hepatocyte (2×10^6 cells) were boiled in SDS sample preparation buffer and subjected to SDS-PAGE using 10% gels. After electrophoresis proteins were transferred onto nitrocellulose membrane and probed with anti-p85 α monoclonal antibodies and detected using alkaline phosphatase coupled secondary antibodies. Lane 1 to 4 represents fetal hepatocytes of 28, 25, 23, and 20 weeks gestation periods respectively.

Figure 2. Effect of UV-B irradiation of PI 3-Kinase activity. Fetal hepatocytes of 25 weeks gestation were irradiated with different doses of UV-B (304nm) and assayed for PI 3-Kinase activity as described in methods. The reaction products were extracted into chloroform, dried and counted for incorporated radioactivity. Data are mean $\pm$ SEM (bars) values ($n = 3$).

However, at higher dose (2500 Jm^{-2}) upto 80% inhibition of PI 3-kinase activity was noted in both membrane and cytosolic fractions. The results thus indicate that increased PI 3-kinase activity in hepatocytes (fig. 2) is due to membrane translocation of cytosolic PI 3-kinase than regulation at gene expression. The lipid kinase activity is inhibited by Wortmannin (10^{-7} M) and LY 294002 (10^{-5}) (fig. 4). Effect of PI 3-kinase inhibitors on cell survival upon UV-B irradiation indicated that the viability of cell is due to activation of PI 3-kinase as shown in the presence of inhibitors wherein cell viability decreased fig. 5.

Figure 3. Effect of UV-B on membrane translocation of PI 3-kinase. Fetal hepatocytes irradiated with UV-B were lysed in RIPA buffer and membrane (·) and cytosolic (?) fractions were obtained by centrifugation and assayed for PI 3-Kinase activity as described in the methods using PI 4,5 P₂ as substrate.

Figure 4. Dose-dependent changes in intracellular PIP₃ levels in human fetal hepatocytes after UV-B irradiation. Hepatocytes on culture dishes were irradiated directly with overhead (10cm) UV-B lamp (1500 Jm^{-2}). The effect of PI 3-Kinase was evaluated using cells pretreated with Wortmannin (10^{-7} M) or LY294002 (10^{-5} M). After irradiation cells were kept in Hanks medium for 12 hr and assayed for PI 3-kinase activity. PIP₃ formed was separated by TLC and quantitated by autoradiography. Lane 1, cells not irradiated, Lane 2, Wortmannin, Lane 3, LY294002 and lane 4 irradiated hepatocytes.

The studies from Yao and Cooper (10) revealed that the inhibition of PI 3-kinase induced apoptosis. Further, there is a strong correlation between activation of PI 3-kinase and protection from cell death (11). Thus the present results highlight the effective use of UV-B irradiated hepatocytes (1500 Jm^{-2}) during liver transplantation, wherein apart from abolishing the immune response the cells possess activated PI 3-kinase ensuring better survival of the transplanted cells. Previous transplantation studies from our laboratory (1) indicated the safe use of UV-B treated (1000 Jm^{-2}) fetal hepatocytes in liver transplantation. However, the present data indicate that UV-B exposure up to 1500

Figure 5. Effect of PI 3-Kinase inhibitors on UV-B induced cell death. Hepatocytes pretreated for 15 min with PI3-kinase inhibitors, 10^{-7} M wortmannin (?) and 10^{-5} M Ly294002 (?), DMSO (●) and none (○) were irradiated with different UV-B doses. After 24 h cell viability was quantitated by trypan blue dye exclusion method.

Jm⁻² could be safe and might be a viable alternative to facilitate clinical transplantation of irradiated hepatocytes without rejection, thus overcoming the shortages of donor cells and / or the use of immunosuppressive agents.

The results presented here further corroborated with the fact that AT (Ataxia Telangiectasia, a human autosomal recessive disorder, characterized by cerebellar degeneration, chromosomal instability, cancer predisposition and hypersensitivity to irradiation) cells show a high degree of sensitivity to ionizing radiation. The ATM (mutated) gene shows striking similarity to 110-kDa subunit of PI 3-kinase and is also shown to possess lipid kinase activity (12). Since PI 3-kinase was detected in 23-25 weeks gestation period and its possible defense against ionizing radiation is established (1), the results presented here suggest that for transplantation it will be appropriate to use fetal hepatocytes of 23 – 25 weeks gestation exposed to a UV-B dose of 1500 Jm⁻².

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