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EXPRESSION OF HUMAN ERYTHROPOIETIN GENE IN HELA CELLS

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ABSTRACT

The cloned human erythropoietin (pSin.epo.hu) gene in pSin vector was transfected in HeLa cells. The protein expression was assayed by immuno assay, SDS-PAGE and western blotting. The cells were found to express the protein with development of purple color precipitate in Immunoperoxidase test. Intense purple coloration of cells was observed in HeLa cells transfected with pSin.epo.hu. The vector alone and untransfected healthy cells failed to show any coloration confirmed no expression of proteins. By SDS-PAGE and western blotting, the expressed erythropoietin (34 kDa) was confirmed.

KEYWORDS: HeLa cells, Erythropoietin gene, pSin Vector, Replicase Vector, Gene Expression.

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INTRODUCTION

Erythropoietin (epo) is the principal hormone involved in the regulation and maintenance of a physiological level of circulating erythrocyte mass[1, 2]. The hormone is produced primarily by the kidney in the adult and by the liver during fetal life[3, 4, 5] and is maintained in the circulation at a concentration of 15-30 milliunits/ml of serum [6, 7, 8] or about 0.01 nM under normal physiological state. Construction of epo is stimulated under conditions of hypoxia[9]. epo is proposed to exert its biological effect by attaching to specific binding sites on erythroid progenitor cells to stimulate their differentiation into mature erythrocytes [2, 10]. Human erythropoietin is one of the hematopoietic growth factors that promote erythropoiesis, which is the proliferation and differentiation of erythroid progenitor cells into erythrocytes (red blood cells) [11]. The epo is produced as a response to low oxygen concentration in blood known as hypoxia [12]. Other than in kidney and liver, epo is also produced by neuroblastoma cells induced by hypoxia[13] and in brain after oxidative or nitrosative stress. Sakanaka reported that epo protects neuron against ischemia-induced cell death and suggested that epo could have neuroprotective effect by reducing the nitric oxide-mediated formation of free radicals or antagonizing their toxicity [14]. In anemic patients the endogenous epo is very low due to chronic renal failure. Recombinant human epo (repo.hu) is used in the treatment of anemia resulting from reduced production of endogenous epo in renal failure, and in the treatment of another chronic anemia, such as those due to severe infections[15]. The epo gene was first cloned in 1985[16, 17]. This sialoglycoprotein hormone consists of 165 amino acids that form a single polypeptide chain containing two intra chain disulfide bonds (Cys7-161 and Cys 29-33) and four potential glycosylation sites. It has three *N*-linked (Asn24, Asn38 and Asn83) and one *O*-linked (Ser126) glycosylation sites[17, 15]. The molecular mass of epo is 30-34 kDa. About 40% of its molecular weight is due to carbohydrate moieties. Glycosylation may affect protein stability [18].Recombinant epo.hu had been expressed in mammalian cells, such as CHO, BHK and COS cells[16, 17, 19]. It had also been expressed in various hosts, such as Tobacco plant cells [20, 21], *Spodoptera frugiperda* insect cells [22] and in yeasts such as *Saccharomyces cerevisiae*[23] and *Pichia pastoris* [24].

MATERIALS AND METHODS

Materials

Cell line: HeLa cell lines were available in Biotechnology Laboratory of IBIT, Bareilly.

Plasmid DNA: pSin.epo.hu. Recombinant plasmid was constructed in Biotechnology laboratory of IBIT, Bareilly.

Methods

Transfection in HeLa cells

Cell culture was trypsinised using TVS. 100 µl of cell culture suspension was plated in 96 well microtitre plate. Calcium phosphate-DNA co-precipitate was prepared by combining 50 µl of 2.5 M CaCl2 with 10 µg of rplasmid (pSin.epo.hu) and 40 µl distilled water in a sterile microfuge tube. Immediately transferred the calcium phosphate-DNA suspension into 96 well microtitre plate containing the cell suspension. Transfected cells were incubated at 37^0C in a humidified chamber with an atmosphere of 5% CO_2 for 72 hours. After 72 hours of incubation, the cells were assayed for expression of transfected gene.

Immunoassay

Cells were washed twice with PBS and fixed with 100 µl of chilled acetone. After washing the fixed cells with PBS, cells were treated with, 2% H_2O_2 in PBS for 10 minutes and again washed with PBS twice for 5 minutes each. Cells were incubated with mouse anti epo.hu hyper immune sera for 1 hour at 37 ºC and washed thrice with PBS, 5 minutes each.

Rabbit anti mouse globulin HRP conjugate antibody was added to the wells and incubated for 1 hour at ºC. The cells were washed with PBS thrice and incubated with Nadi reagent containing freshly added H_2O_2 for 5 minutes at room temperature. After the development of color, cells were washed with PBS, dried in air and observed under microscope [25].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

HeLa cells were transfected with pSin.epo.hu rplasmid and pSin vector separately in 96 well microtitre plates. After 48 hrs of incubation, the cells were processed following the method of Rodriguez and Tail [26].The gel consisted of a lower resolving gel and an upper stacking gel that concentrate the sample before its entry into the resolving gel. The buffer system was the discontinuous system of Laemmli.

Table1
Component of resolving and stacking gel

Reagents	Resolving gel (10%) ml	Stacking gel (4%)ml
Lower gel buffer	3.75	------
Upper gel buffer	--------	1.25
Acrylamide stock	5.0	0.80
Distilled water	6.05	3.87
10% SDS	0.15	0.06
10% ammonium persulfate	0.05	0.02
TEMED	0.07	0.004

The gel plates were assembled and sealed. The resolving gel was poured and covered. After polymerization, the butanol was removed by flushing briefly with water, then the stacking gel poured, and a comb inserted to form sample wells. Following polymerization of the stacking gel, the comb was removed and the wells rinsed to remove unpolymerized acrylamide. The gel was then assembled in the running apparatus and running buffer was added. epo.hu protein samples (25 µl) were prepared by adding an equal volume, i.e. 25 µl of 2X SDS sample buffer and heating at 100°C for 5 minutes to ensure denaturation of the samples. Gels were typically run at 40 mA till the marker dye reached near the end of the gel. The gel apparatus was dissembled, and the gel was removed. The gel was immediately dried on Whatman No.1 filter paper and stained for direct visualizing of polypeptide bands. The gel was placed in a staining container having 50 ml staining solution. It was covered and placed at room temperature for 15 minutes. The solution was poured and replaced with 50 ml staining solution and kept at room temperature for overnight. Poured off the staining solution and added 100 ml distaining solution, covered and returned to room temperature for 15 minutes. Poured off the destaining solution and replaced. Continued to repeat this step till a clear gel with blue polypeptide bands appeared.

Western blot analysis

After completion of electrophoresis, the epo.hu protein bands from the unstained gel were blotted onto nitrocellulose membrane following Millipore protocol using SNAP i.d. system. This Protein Detection System provides a fast and convenient method for the detection of immune reactive proteins on western blots.10 ml BSA as blocking solution was added to each well of triple well plate. Emptied the wells, added 100 µl primary antibody mouse anti-epo Ab, to each well and incubated for 10 minutes at room temperature. Turned the vacuum on to empty the antibody solution. Washed the blot with 10 ml of wash buffer three times and then added 100 µl secondary antibody rabbit anti mouse HRP conjugate and incubated for 10 minutes at room temperature. Emptied the antibody solution and washed thrice with wash

buffer. Removed the blot holder and took at the blot and incubated it with DAB H_2O_2 (Bangalore Genei) substrate solution to develop color. Then the blot was washed with water and photographed.

RESULTS

The expression of rplasmid (pSin.epo.hu) was checked by immuno assay in HeLa cell line and cells were found to express the epo.hu protein by developmentofthpurplecolor precipitate. Intense purple coloration of cells was observed in which HeLa cells were transfected with pSin.epo.hu (Fig. 1). The vector alone and untransfected healthy cells (Fig. 2) failed to show any color indicating that the epo.hu gene was expressed in cells due to the presence of rpSisn.epo.hu plasmid. In this expression technique, 90% cells were found to express epo.hu protein.The SDS-PAGE analysis of expressed epo.hu protein indicated a specific isolated thick band (Blue color) of 34 kDa (Fig. 4).The western blot analysis of expressed protein indicated a specific isolated band seen in nitrocellulose membrane of 34 kDa size (Fig. 5).

Fig.1: HeLa cells with pSin.epo.hu. rplasmid showing positive IPT test, 100X

Fig 2: Healthy Control HeLa cells showing no color reaction, 100X

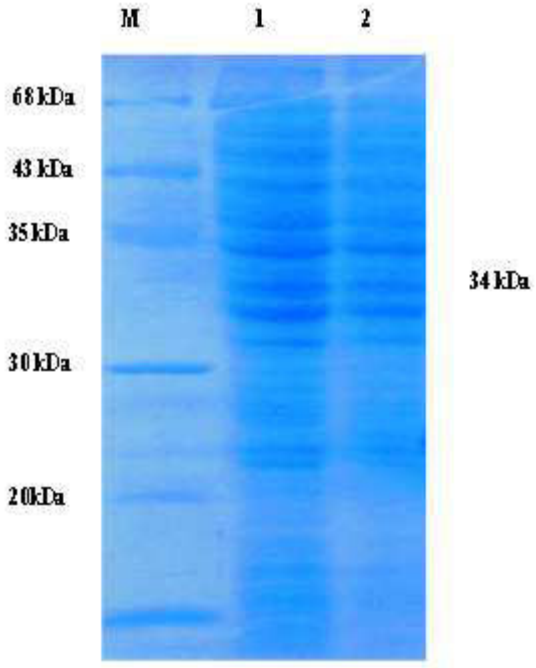

Fig. 3: sds-page of HeLa cell extract for epo expression.

Lane M: Protein marker

Lane 1: Transfected cells showing expressed protein band.

Lane 2: Mock transfected cell extract showing no expression.

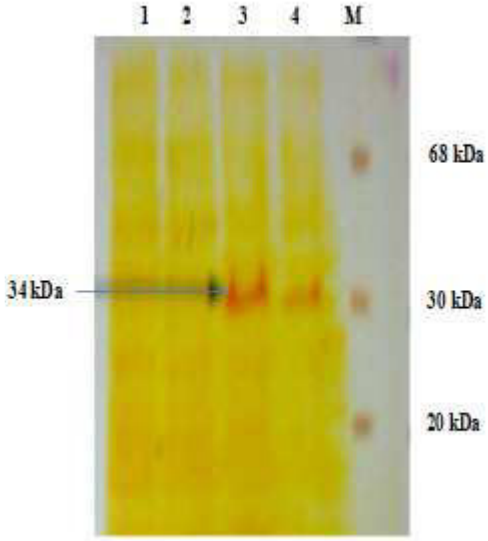

Fig. 4 : Detection of epo protein using western blotting

Lane M : 1 kDa protein marker

Lane 1 : Mock transfected

Lane 2 : Vector control

Lane 3 & 4 : epo.hu protein

DISCUSSIONS

The expression of recombinant plasmid pSin.epo.hu was studied in HeLa cell culture using immuno assay, SDS-PAGE and western blotting. The expression of plasmid was detected by Immuno assay in HeLa cell line and 90% of the cells were found to express the epo.hu protein by development of a purple color precipitate. The SDS-PAGE analysis of expressed protein indicated a specific thick band of 34 kDa (Blue color) and western blot analysis indicated a specific band of 34 kDa on nitrocellulose membrane. Adaptive responses to hypoxia occur in many biological systems. A well-characterized example is the hypoxic induction of the synthesis of erythropoietin, a hormone which regulates erythropoiesis and hence blood oxygen content. The restricted expression of the erythropoietin gene in subsets of cells within kidney and liver has suggested that this specific oxygen-sensing mechanism is restricted to specialized cells in those organs. Using transient transfection of reporter genes coupled with a transcriptional enhancer lying 3' to the erythropoietin gene, it was shown that an oxygen-sensing system similar, or identical, to that controlling erythropoietin expression is widespread in mammalian cells. The extensive distribution of this sensing mechanism contrasts with the restricted expression of erythropoietin, suggesting that it mediate other adaptive responses to hypoxia[27]. Hoc and Viet expressed epo.hu in *E. coli* cells, the expression of the epo.hu was analyzed by SDS-PAGE and confirmed by western Blotting using anti epo.hu antibody. The *Pichia pastoris* expression system was used to produce recombinant human erythropoietin. The entire recombinant human erythropoietin (repo.hu) gene was constructed, cloned and expressed through the secretary pathway of the *Pichia* expression system. Recombinant erythropoietin was successfully expressed in *Pichia pastoris* [28]. The estimated molecular mass of the expressed protein ranged from 32 kDa to 75 kDa, with the variation in size being attributed to the presence of repo.hu glycosylation analogs. A crude functional

analysis of the soluble proteins showed that all of the forms were active *in vivo* [29]. Evidence from cell culture and animal experiments suggested a neuroprotective and neurotrophic function of erythropoietin. They had quantified the distribution of epo mRNA expression in the developing the human central nervous system (CNS) [30]. Compared to the EAC control animals, PEEAP treatment showed significant tumour inhibition resulted in appreciable improvement in hemoglobin content and RBC count. These observations assume great significance as anemia is a common complication in cancer and the situation aggravates further during chemotherapy since a majority of antineoplastic agents exert suppressive effects on erythropoiesis and thereby limiting the use of drugs [31].

CONCLUSION

The expression of rplasmid (pSin.epo.hu) was checked by immuno assay in HeLa cell lines and cells were found to express the epo.hu protein by development of a purple color precipitate. The expression of rplasmid was again checked by using SDS-PAGE and western blot analyses. The expressed epo.hu protein (34 kDa) was confirmed by SDS-PAGE and western blot analysis.

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