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Research Article

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Expression and immunogenicity of erythropoietin gene cloned in eukaryotic expression vector (pVAX1 vector)

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ABSTRACT

Erythropoietin gene is immunogenic. In the present study the recombinant plasmid pVAX1.epo.hu transfected HeLa cells expressed protein which was confirmed by immunoperoxidase test, 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 pVAX1.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. The in vivo immunological response was studied in mice.

Keywords: HeLa cells, Erythropoietin gene, pVAX1 Vector, Replicase Vector, Gene Expression

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INTRODUCTION

Erythropoietin (epo) is a glycoprotein hormone responsible for the regulation of red blood cell production. This hormone triggers the proliferation, differentiation and maturation of bone marrow erythroid precursors into functional erythrocytes when blood oxygen availability is decreased, such as during hypoxia. epo binds to and activates the receptor on erythroid progenitor cells. The treatment of anemic patients with epo significantly reduces their dependence on blood transfusions and minimizes potential side effects such as iron overload, infections and adverse reactions to leukocyte antigens. In addition to its role in hematopoeisis, epo is neuroprotective in the nervous system and can also protect other organs [1]. Prior to the 1980s, human epo for the treatment of kidney failure and other related blood disorders had to be extracted from donors. However, under normal conditions, the expression of epo is generally low, meaning that many donors are necessary to obtain sufficient material for treatment. Successful cloning of the *epo* gene and subsequent expression in Chinese hamster ovary (CHO) cells led to the production of several types of commercially available recombinant human epo (r.epo.hu) for human use. Keeping the above facts in view, the present work was been undertaken to express the epo.hu gene in replicase based eukaryotic pVAX1 vector.

EXPERIMENTAL SECTION

1.1. Recombinant plasmid (pVAX1.epo.hu)

The recombinant plasmid pVAX1.epo.hu was available in the Biotechnology Laboratory, IBIT, Bareilly. pVAX1 is a 3.0 kb plasmid vector designed for use in the development of DNA vaccines. The vector was constructed to be

consistent with the Food and Drug Administration (FDA) document, "Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications", published December 22, 1996 (see FDA "Points to Consider" below). Features of the vector allow high-copy number replication in *E. coli* and high-level transient expression of the protein of interest in most mammalian cells.

1.2. Cell Line

HeLa cell line was obtained from National centre for Cell Science (NCCS), Pune. HeLa cell line was used in the study for *in vitro* expression analysis of recombinant plasmids (pVAX1.epo.hu) and was maintained in GMEM (Micro lab), supplemented with 10% new born calf serum (Gibco, NY),100 µg/ml penicillin and streptomycin.

1.3. Conjugates

Rabbit anti-mouse HRP conjugated antibody was obtained from Bangalore Genei, Bangalore, (India).

1.4. *In vitro* expression analysis

1.4.1. Transfection of HeLa cells

Cells were trypsinised using trypsin-versenate solution (TVS) and then 4 ml of GMEM containing 10% FCS and penicillin and streptomycin (50 µl/ml) was added, to make cell suspension of 1X105 cells/ml. Harvested exponentially growing cells by trypsinization and prepared cell suspension in growth medium. Prepared the calcium phosphate-DNA coprecipitate as follows: combined 50 µl of 2.5M CaCl2 with 10 µl of plasmid DNA in a sterile microfuge tube, Added 40 µl DW, kept at room temperature. Immediately transferred the calcium phosphate-DNA suspension using 20 µl suspension for each wells of microtitre plate. Added 100 µl of cell culture suspension in each wells of 96 wells microtitter plate. Rocked the plate gently to mix the medium, which will become yellow-orange and turbid. Carried out this step as quickly as possible because the efficiency of transfection declines rapidly once the DNA precipitate is formed. Kept control wells without transfection. Incubated at 37^0C in a humidified incubator with an atmosphere of 5% CO2 for 72 hours. Examined for gene expression by IPT.

1.4.2. Raising primary antibody against pVAX1.epo.hu in mice

Primary polyclonal antibody against the human erythropoietin gene was raised in mouse by hyper immunization of six mice with pVAX1.epo.hu plasmid. 50 µg of rplasmid DNA was injected intramuscularly in lateral region of thigh muscles of each mouse and repeated every week for four weeks consecutively. One week after last injection bled through inner canthus of eyes with a capillary and serum was prepared.

1.4.3. Immunoperoxidase test (IPT)

After 72 hours, transfected HeLa cells were washed with 1XPBS twice and fixed with 80% chilled acetone at 4^0C for 10 min and air-dried. Put a few drops of mouse anti epo.hu hyperimmune serum and incubated at 37^0C for 1 hr and again washed with PBS, added a few drops of horseradish peroxidase (HRPO) conjugated rabbit anti-mouse antibody to wells and incubated at 37^0C for 1 hr in humid chamber. The cells were again washed with PBS thrice and incubated with 2-3 drops of Nadi reagent for 5 min. After the development of color, cells were washed with PBS, dried in air and observed under microscope and photographed, protocol was carried out as per [2].

1.4.4. SDS-PAGE

HeLa cells were transfected in 96 well plates with pVAX1.epo.hu rplasmid and pVAX1 vector. After 48 hours of transfection cells were processed following the method [3].

Gel preparation: The gels consist of a lower resolving gel and an upper stocking gel that concentrate the sample before its entry into the resolving gel. The buffer system was the discontinuous system of laemmli. Stored all solution in brown bottle at 4°C and SDS solution at room temperature. The volumes below were for slab gels of 15 ml vol with a 6 ml stacking gel. Volumes are gives in ml.

The gel plates were assembled and sealed against leakage. 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 acryl amide. The gel was then assembled in the running apparatus and running buffer 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 min to ensure denaturation of the samples. Gels were typically run at 40 mA for the marker dye reaches the end of the gel. The gel apparatus was dissembled and the gel removed. The gel was

immediately dried on Whatman No.1 filter paper and stained for direct visualizing of polypeptide bands. Placed gel in a staining container with lid. Added 50 ml staining solution. Covered and placed at room temperature for 15 min. Poured off solution, replaced with 50ml staining solution + 5ml stain. Covered and placed at room temperature for overnight. The gel was turned blue at that time. Poured off the staining solution, added 100ml destaining solution covered and returned to room temperature for 15min. Gentle mixing improved the destaining process. Poured off the destaining solution and replaced. Continued to repeat this step many times. Completed destaining to produce a clear gel with blue polypeptide bands depends on gel thickness and required 24 h.

Table 1: Component of resolving and stacking gel

Reagents	Resolving gel (10%)	Stacking gel (4%)
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 persulphate	0.05	0.02
TEMED	0.07	0.004

1.4.5. Western blot analysis

After completion of Sodium dedecyl sulfate-polyacrylamide gel electrophoresis, the epo.hu protein bands from unstained gel were blotted on to nitrocellulose membrane using SNAP i.d. system. This Millipore‟s patent pending SNAP i.d. Protein Detection System provides a fast and convenient method for the detection of immune reactive proteins on western blots and the Millipore‟s protocol was followed.

System Set-up

Placed the SNAP i.d. system on a level bench top and attached the vacuum tubing to the back of the system by pushing the coupling epo.hu insert on the end of the tubing into the quick disconnect fitting at the back of the system base. Connected the other end of the tubing to a vacuum source, used a one-liter vacuum flask as a trap and a Millex-FA50 filter to protect the vacuum source from contamination.

Rolled gently with blot roller to remove any air bubbles trapped between the blot holder and the blot. Wet the inner white face of the blot holder with Milli-Q water, until it turns gray. Removed excess liquid using the blot roller, was preventing movement of the blot during assembly. Placed the pre-wet blot membrane in the center of the blot holder and the protein side down Open the blot holder lid.

Blot Assembly

Before starting the blot assembly procedure, prepared the antibody, blocking, and wash buffers solutions. Placed the spacer on to top of the blot, making sure it completely covers all edges. Rolled blot again to ensure completed contact of blot spacer with blot membrane, Closed the blot holder lid Squeezed firmly at the base of the tab area to secure lid. Opened lid of system by squeezing latch between thumb and forefingers and lifting upwards Placed blot holder in system chamber with the well side up, aligning the blot holder tabs with notches of chamber. Repeated the assembly procedure for all blots being processed Closed and latch the system lid.

Immunodetection Protocol

Added the appropriate volume of blocking solution to each well being used after the well(s) had emptied completely (20 seconds), turn vacuum off using the vacuum control knob(s). Antibody collection trays were sold separately. Added the appropriate volume (100 µl) of primary antibody (mouse anti-epo Ab) to each well being used incubated the primary antibody (mouse anti-epo Ab) for 10 minutes at room temperature, with the vacuum off. Turned the vacuum on and wait 20 seconds to make sure that antibody solution had been completely emptied from the blot holder the solution was absorbed into the blot holder and the surface appeared dry. With vacuum running continuously, washed the blot with 10 ml of wash buffer (PBS), three sequential washes were required for optimal performance. Each washed had been taken 20 seconds to complete and the blot holder was empty, turn vacuum off. With the vacuum off, applied the appropriate volume (100µl) of secondary antibody (rabbit anti-mouse HRP conjugate) evenly across the entire surface of the blot holder Incubated the secondary antibody (rabbit anti-mouse HRP conjugate for 10 minutes at room temperature with vacuum off. Again, the antibody solution was absorbed into the blot holder and the surfaces appear dry. Turned on the vacuum and waited 20 seconds to make sure that antibody

solution had been completely emptied from the blot holder. With vacuum running continuously, washed the blot with 10 ml of wash buffer, three sequential washes were required for optimal performance Removed blot holder from the system, placed it on the bench with the well-side down, and opened the lid. With forceps, removed and discarded the spacer. Removed blot and incubated with the appropriate detection reagent such as Immobilon® Western HRP Substrate, visualize. Discarded the single-use blot holder.

1.6. Immune response studies

1.6.1. Assessment of hematopoietic activity *in vivo*

Female mice were used to test the hematopoietic activity of r.epo.hu. Each mouse was injected subcutaneously with 0.25 mL of sample (acetone-precipitated supernatant of r.epo.hu cultures) for three consecutive days. Three mice (6-8 weeks old) were used for each treatment and two groups of mice were used as controls (one of these two groups was treated with PBS and the other received no treatment). Blood samples were collected into 5% sodium EDTA on the fourth day after treatment. An equal volume of blood was mixed with new methylene blue and incubated at 37 °C for 1 h. Seven microlitres of this blood-dye mixture was then used to prepare smears on glass slides. Five slides were prepared for each mouse (total of 15 slides per treatment since there were three mice per group). Reticulocytes were counted with the aid of a microscope (at 100X magnification) in five randomly selected areas of each slide and their number expressed as a relative to the total number of red blood cells observed.

1.6.2. Statistical analysis

The results were expressed as the mean ± SEM, where appropriate. Statistical comparisons were done using Students *t*-test, with a value of $p < 0.01$ indicating significance.

RESULTS AND DISCUSSION

The expression of rplasmid (pVAX1.epo.hu) was checked by immuno assay in HeLa cell line and cells were found to express the epo.hu protein by development of the purple color precipitate. Intense purple coloration of cells was observed in which HeLa cells were transfected with pVAX1.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 pVAX1.epo.hu plasmid. In this expression technique, 90% cells were found to express epo.hu protein.

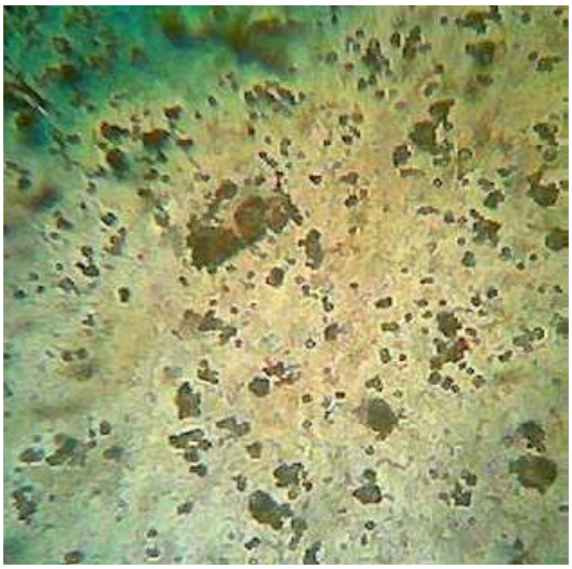

Fig.1: HeLa cells with pVAX1.epo.hu. rplasmid showing positive IPT test, 100X

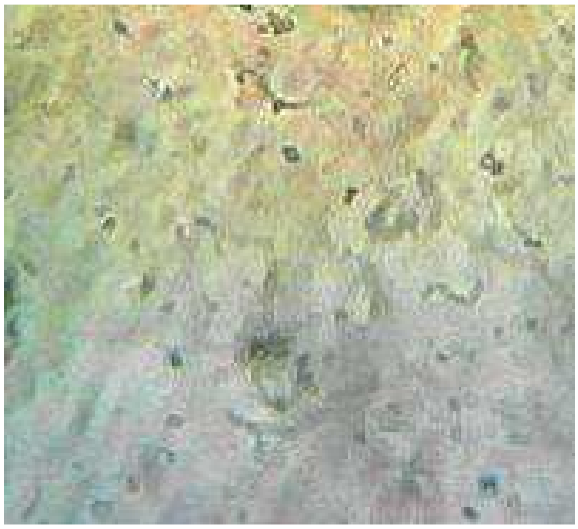

Fig.2: Healthy Control HeLa cells showing no color reaction, 100X

The SDS-PAGE analysis of expressed epo.hu protein indicated a specific isolated thick band (Blue color) of 34 kDa (Fig. 3).The western blot analysis of expressed protein indicated a specific isolated band seen in nitrocellulose membrane of 34 kDa size (Fig. 4). 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 trasfection 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 hypoxia27. Hoc and Viet expressed epo.hu in *E. coli* cells, the expression of the epo.hu was analyzed by SDSPAGE 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.

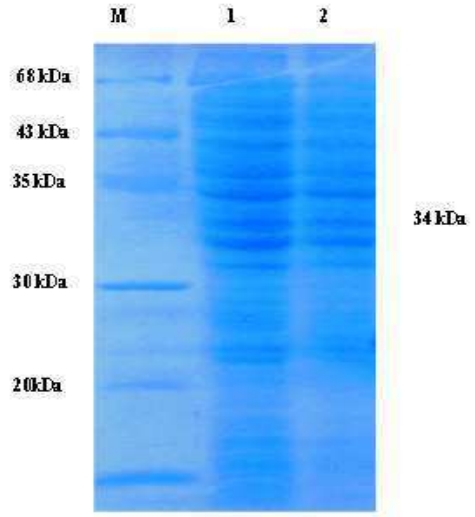

Fig.3: SDS-PAGE of HeLa cells extract for epoexpression
Lane M- Protein marker
Lane 1-transfected cells showing expressed protein band
Lane 2-mock transfected cells extract showing no expression

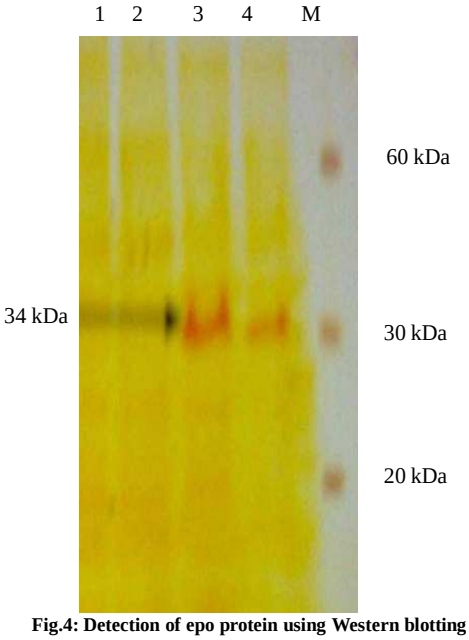

Fig.4: Detection of epo protein using Western blotting
Lane : M 1 kDa protein marker
Lane : 1 Mock transfected
Lane : 2 Vector control
Lane : 3 & 4 epo.hu protein

In mice injected with r.epo.hu the number of reticulocytes increased by 3.16 ± 0.64 (Fig. 5). These increases were significantly ($p < 0.01$) higher than those observed in mice injected with PBS and the non-treated negative control. This increase in reticylocytes showed that the r.epo.hu produced in this study was functionally active despite variations in glycosylation when compared to native human epo and CHO-expressed epo.

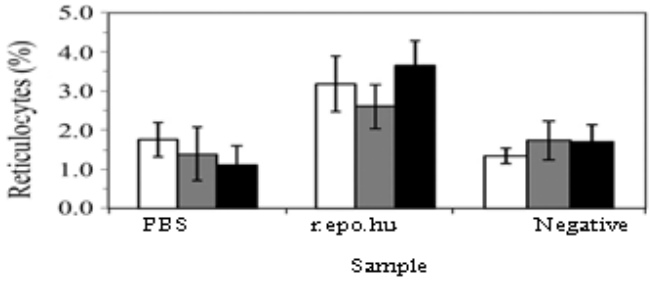

Fig. 5: Increase in the number of reticulocytes after injections of r.epo.hu.

CONCLUSION

The expression of rplasmid (pVAX1.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. In mice injected with r.epo.hu the number of reticulocytes increased by 3.16 ±0.64. These increases were significantly ($p < 0.01$) higher than those observed in mice injected with PBS and the non-treated negative control.

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