Original Article

TO INVESTIGATE THE IN-VITRO EFFECT OF ERYTHROPOIETIN ON CELL LINES USING MTT ASSAY

***Sajid Hanif, **Zahur Qayyum, *Muhammad Nauman Sheikh, **Shahid Khan**

*Baqai Dental College, Karachi

**Women Medical College, Abbottabad

ABSTRACT

Objective: *The objective of this study was to investigate the effect of Erythropoietin on cellular proliferation in SCC-25, TR146 and FIBS cells lines.*

Methodology: *This study focuses on the effects of Erythropoietin on 3 cell lines(SCC-25, TR146 and FIBS) when applied for 24 hours respectively. MTT assay was carried out using a range of Erythropoietin concentrations (1, 10, 25 units). Serum free medium was used as a control.*

Results: *In this study Erythropoietin significantly increased the cell proliferation of SCC-25 and FIBS with 1 and 10unit/ml but had no effect on TR146 cells, while 25 unit Erythropoietin showed very little effect in increase cell viability in both SCC-25 and FIBS.*

Conclusions: *This study has confirmed that all the concentration of Erythropoietin used had an effect on SCC-25 and FIBS cell viability but erythropoietin had no effect on TR146 cell viability. .*

Key words: *Erythropoietin, MTT assay, squamous cell carcinoma.*

INTRODUCTION

Erythropoietin is a 34Kda glycoprotein hormone normally produced by kidney. The main function of erythropoietin is to control red blood cell production, by binding to erythropoietin receptor resulting in replication and maturation of red blood cells[1,2].

Erythropoietin promotes erythropoiesis mainly by preventing apoptosis of erythroid progenitor cells. This is due to upregulation of Bcl-2 and Bcl-XL through different intracellular signal transduction pathways like Ras/MAP kinase, phosphatidylinositol 3 kinase and STAT1, 4, 5A, 5B transription factors[3-6].

Recent studies have shown that erythropoietin function goes beyond erythropoiesis. Erythropoietin and erythropoietin receptor have been found in breast tissue[7], brain[8], female genital tract tissue[9] and vascular endothelial cells[10].

Correspondence:
Dr. Sajid Hanif
Assistan Professor
Department of Oral Pathology
Baqai Dental College, Karachi
Cell: 0300-9207166
Email address: drsajidhanif@hotmail.com

It was found that Hematopoietic and endothelial cell lines share common progenitors, it is reasonable to expect that cytokines and growth factors usually associated with hematopoiesis may also have a role in angiogenesis. Previous studies have shown that the human EA. hy926 endothelial cell line expresses erythropoietin receptor and responds to erythropoietin by differentiating into vascular structures when seeded on MatrigelTM[11].

Mammary epithelial cells also contribute to the production of erythropoietin in human milk and erythropoietin in human milk may play a pleiomorphic role in erythropoiesis, neurodevelopment, maturation of gut, apoptosis and immunity in the infant[12].

Human erythropoietin gene cloning was achieved in 1983. After clinical trials with recombinant human erythropoietin (rhu- Erythropoietin) it has been used for more than a decade in the treatment of anaemia in end stage renal failure[13].

Recently recombinant human erythropoietin (rhu- Erythropoietin) has been used to treat patients with anaemia associated with chronic renal failure, AID's patients with anaemia due to treatment with zi-

dovudine, nonmyeloid malignancies in patients treated with chemotherapeutic agents, perioperative surgical patients, and autologous blood donation[5].

In cancer patients, the levels of IL-1, IL-6, TNF, IFN and TGF-β are high which slows down the production of erythropoietin and reduces its proliferative effects on erythroid cell precursors. Erythropoietin treatment stimulates erythropoiesis and reduces the need for blood transfusion in anemic and cancer patients[14].

Erythropoietin and erythropoietin receptor has been studied in a number of diseases. Recombinant human erythropoietin has been used as an adjunct for chemotherapy or radiotherapy but concerns have been raised that it may prolong tumor survival[15]. Erythropoietin and erythropoietin receptor showed a differential expression in oral squamous cell carcinoma and thus the aim of this study was to explore the effect of erythropoietin on cell lines proliferation in vitro. Cell responses were assayed by cellular proliferation (MTT assay).

METHODS AND MATERIALS

Observational laboratory based study design was used to carry out the experiment. This study was conducted at Department of Oral Pathology Barts and the London Queen Mary School of Medicine and Dentistry Queen Mary, University of London in the year 2004-2005.

Cell Culture

The 3 cell lines were used for the in vitro study of cell proliferation by methyl-thiazoldiphenyl tetrazolium (MTT).

Transformed oral Keratinocytes (TR146)

The human oral Keratinocyte cell lines, TR146 were derived from a buccal carcinoma[16]. The cells were grown in T 75 flask using Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% FBS, 1% penicillin, streptomycin and fungizone in 5% CO2 and 95% humidity.

Human Gingival Keratinocytes (FIBS)

The FIBS Cell line was derived from human gingival keratinocytes and was obtained from Dr. Supriya Kapas, Department of Clinical and Diagnostic Oral Sciences (CDOS), Barts and the London UK. The cells were grown in T75 flask using Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% FBS, 1% Penicillin, Streptomycin and fungizone in 5% CO2 and 95% humidity.

Squamous Cell Carcinoma (SCC-25)

The SCC-25 cell line (ATCC, Number CRL-1628) was derived from a squamous cell carcinoma of tongue[17]. The cells were grown in T75 flasks using RM plus medium [D-MEM F:12(1:1), L-Glutamine, 15 Mm Hepes, Gibco, 1% RM plus ready mix, 10% FBS, 1% Pencillin and Streptomycin] in 5% CO2 and 95% humidity.

Cell Maintenance

Both the cell lines were grown in respective media in T75 flasks in 5% CO2 and 95% humidity at 37°C. The medium was changed every third day.

A confluent T75 flask was rinsed in 10-15ml PBS (Ca++ and Mg++ free-Dulbecco, phosphate buffered saline) and 3ml of trypsin (Trypsin – EDTA1x, Gibco, UK) was added to detach cells from the flasks. The flask was then agitated to expedite the cell detachment, 6ml of serum medium was then added to neutralize trypsin. The cell suspension was then harvested into new T75 flask containing 15ml of respective media. For smaller flask or plates the cell suspension was diluted and an appropriate volume of growth media was added accordingly. Medium was changed every 3rd day. Cells were incubated at 37°C in 5% CO2 and 95% humidity.

Erythropoietin Treatment

Concentration of Erythropoietin

50µg of rhu-erythropoietin (45kDa; Santa Cruz Biotechnology) was dissolved in 1ml sterile PBS to give a stock solution of 600 unit/ml.

The working solutions of Erythropoietin were prepared as follows -

a. 1unit/ml = 1.6µl stock-1ml Serum Free Medium

b. 10unit/ml = 16.7µl stock-1ml Serum Free Medium

c. 25unit/ml = 41.67µl stock-1ml Serum Free Medium

d. Serum free medium alone was used as a control

Methyl-thiazoldiphenyl tetrazolium (MTT Assay)

The viability of exposed cultures was measured by the quantification of mitochondrial dehydrogenase activity using a modified methyl-thiazoldiphenyl tetrazolium (MTT assay)18. MTT assay involves the use of a colour reaction as a measure of cell activity. MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a pale yellow substrate, which is reduced to a dark blue insoluble formazan product, when incubated with living cells. The amount of formazan uptake is measured using densitometry.

RESULTS

The effects of erythropoietin on 3 cell lines when applied for 24 hours respectively. Tissue proliferation was assessed using a MTT assay. The results are quoted as (mean + standard deviation).

Transformed oral Keratinocytes (TR146)

Compared to control culture no significant proliferation was observed in TR146 cells treated with erythropoietin (Figure 1 and Table 1).

FIBS

1 unit (191.26 + 29.50) and 10 units (178.60 + 65.00) concentration erythropoietin caused a significant increase in the proliferation of FIBS cells compared to serum free control. P<0.05. (Figure 2 and Table 2).

Table-1: Methyl-thiazoldiphenyl tetrazolium (MTT) Results after 24 hours Erythropoietin treatment on TR146

Treatment	Mean Proliferation %	Standard Deviation	No of samples
1 unit	83.27	8.94	4
10 unit	121.54	3.49	4
25 unit	106.73	12.81	4
Serum free(Control)	100	6.71	4

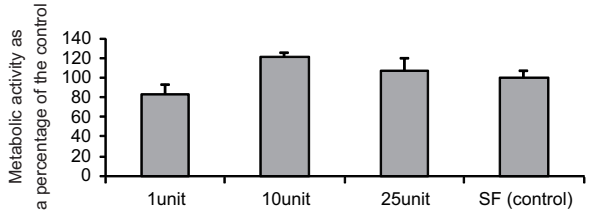

Figure-1: The effects of erythropoietin on TR146 cells viability

Table-2: Methyl-thiazoldiphenyl tetrazolium (MTT) Results after 24 hours erythropoietin treatment on FIBS Cells

Treatment	Mean Proliferation %	Standard Deviation	No of samples
1 unit	191.26	29.50	4
10 unit	178.60	65.00	4
25 unit	119.96	46.60	4
Serum free (control)	100	4.17	4

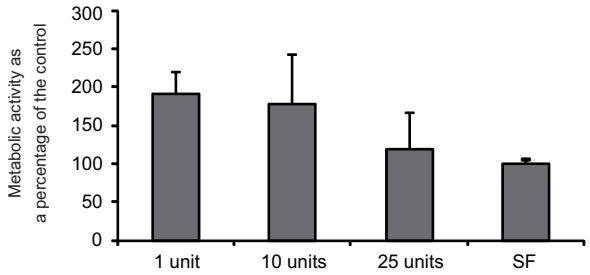

Figure-2: The effects of Erythropoietin on FIBS cells viability

Table-3: Methyl-thiazoldiphenyl tetrazolium (MTT) Results after 24 hours erythropoietin treatment on SCC-25 Cells

Treatment	Mean Proliferation %	Standard Deviation	No of samples
1 unit	213.09	11.72	4
10 unit	217.45	15.03	4
25 unit	178.78	12.27	4
Serum free (control)	100	28.41	4

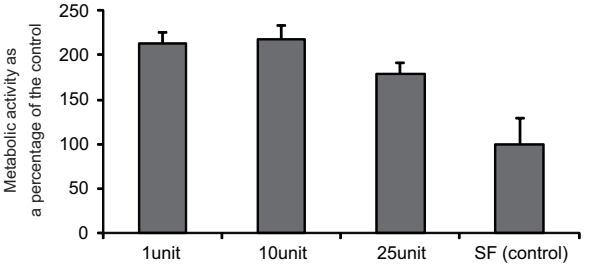

Figure-3: The effects of Erythropoietin on SCC-25 cells viability

Squamous Cell Carcinoma (SCC-25)

1 unit (213.09 + 11.72) and 10 unit (217.45 + 15.03) and 25 unit (178.78 + 12.27) concentration caused a significant increase in the proliferation of SCC-25 cells compared to serum free control. p<0.05. (Figure 3 and Table 3).

DISCUSSION

The aim of this study has been to investigate the effect of erythropoietin on cellular proliferation in cells lines. In a previous study it was found that erythropoietin (epoetin β) could improve cancer control and survival of patients irradiated for head and neck cancer. They found no advantage for locoregional progression – free survival, compared with the patients given placebo significantly better than those given epoetin β[15]. Furthermore concern has been expressed that it may make the tumour more aggressive. In the light of this, we used SCC-25, TR146 and FIBS cells to see the effect of erythropoietin on cellular proliferation. In our study erythropoietin significantly increased the cell proliferation of SCC-25 and FIBS with 1 and 10 unit/ml but had no effect on TR146 cells. The finding that erythropoietin receptor stimulate proliferation is supported by previous study of endothelial cells[19,20] and erythroid precursors[21] but the reason TR146 cells did not respond is unclear. It may suggest that cellular differences in responsiveness exist or reflect variation in erythropoietin receptor expression and activation. The presence of the receptor could be investigated by immunocytochemistry or western blotting. In a study found that erythropoietin and its mRNA were produced in uterus as E2 – dependant manner, when they cultured uterus from ovx mouse invitro. When they inject erythropoietin into the ovx mouse – uterine cavity promoted blood vessel formation in the uterine endometrium. It is suggested that erythropoietin is an important factor for the E2 – dependant cyclical angiogenesis in uterus[9]. In a study found that expression of erythropoietin receptor in breast cancer and erythropoietin induced proliferation of breast cancer cell lines in vitro[22]. Expression of erythropoietin and erythropoietin receptor has been recently reported in renal carcinoma cells that also exhibited increase erythropoietin mediated proliferation[23]. Our data presented here and the result of these previous studies suggest functional significance for erythropoietin and erythropoietin receptor expression in cancer cells with respect to cancer cell proliferation.

CONCLUSIONS

In conclusion, the result in this study suggests that erythropoietin significantly increased the cell viability in SCC-25 and FIBS with 1 and 10 unit concentrations. 25 unit Erythropoietin showed very little effect in increase cell viability in both SCC-25 and FIBS. It was also seen that all the concentration of erythropoietin used had no effect on TR146 cell viability.

REFERENCES

1. Faruki H, Kiss JE. Erythropoietin. Transfusion medicine Update. 1995.
2. Lacombe C, Mayeux P. The Molecular biology of Erythropoietin. 1999 Nephrol. Dial Transplant..14 (Suppl. 2): 22-8.
3. Lacombe C, Mayeux P. Biology of erythropoietin. 1998. Haematological..83: 724-32
4. Silva M, Grillot D, Benito A, Richard C, Nunez G, Fernandez-Luna JL. Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-xL and Bcl-2. 1999.Blood. 88:1576-82.
5. Fisher JW. Erythropoietin: Physiology and Pharmacology update. Exp Biol Med 2003; 228: 1-14
6. Mulcahy L. The erythropoietin Receptor. Semin oncol. 2003; 2 (Suppl 8): 19-23.
7. Acs G, Xhang PJ, Rebbeck TR, Acs P, Verma A. Immunohistochemical expression of erythropoietin and erythropoietin receptor in breast carcinoma. Cancer. 2002; 95(5): 969-81
8. Marti HH. Erythropoietin and the hypoxic brain. J Exp Bio. 2004; 207: 3233-42
9. Yasuda Y, Masuda S, Chikuma M, Inoue K, Nagao M,Sasaki R. Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. J Biol Chem. 1998; 273. 25381–7.
10. Farrell F, Lee A . The Erythropoietin Receptor and Its Expression in Tumor Cells and Other Tissues. Oncolo. 2004; 9 : 1830
11. Ribatti D, Presta M, Vacca A et al. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Blood. 2003; 93:2627–36.
12. Semba RD and Jull SE. Erythropoietin in human milk: physiology and role in infant health. J Hum Lact. 2002; 18: 252-61.
13. Bommer J, Kugel M, Schoeppe W et al. Dose-related effects of recombinant human erythropoietin on erythropoiesis. Results of a multicenter trial in patients with end- stage renal disease. 1998.Contrib Nephrol. 66:85–93.
14. Buemi M, Aloisi C, Cavallaro E, Corica F, Floccari F, Grasso G et al. Recombinant human erythropoietin (rHuEPO): More than just the correction of uremic anemia. J Nephrol. 2002; 15: 97-103.

15. Henke M, Laszig R, Rube C, Schafer U, Haase KD, Schilcher B et al. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. Lancet. 2003; 362(9392):1255-60.

16. Rupniak HT, Rowlatt C, Lane EB, Steele JG, Trejdosiewicz LK, Laskiewicz B et al. Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. J Natl Cancer Inst. 1985; 75(4):621-35.

17. Rheinwald JG, Beckett MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultures from human squamous cell carcinomas. Cancer Res. 1981; 41(5):1657-63.

18. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 1983; 65:55-63.

19. Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M. Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc Natl Acad Sci U S A. 1990; 87:5978-82

20. Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, Kessimian N et al. Erythropoietin receptor mRNA expression in human endothelial cells. Proc Natl Acad Sci USA. 1994 91: 3974-8.

21. Krantz, S.B. Blood. 1991;77:419-434.

22. Acs G, Acs P, Beckwith SM, Pitts RL, Clements E, Wong K, Verma A. Cancer Res. 2001; 61(9):3561-5.

23. Westenfelder C, Baranowski RL. Kidney Int. 2000;58(2):647-57.