IN-VITRO EFFECT OF ERYTHROPOIETIN ON CELL LINES AND MEASUREMENT OF GROWTH FACTOR RELEASE

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ABSTRACT

Objective: *The objective of this study was to investigate the effect of Erythropoietin on SCC-25, TR146, and FIBS cells lines and measurement of growth factor release, using the ELISA technique.*

Materials and Methods: *ELISA was performed to see if Erythropoietin (1, 10, 25 units) treatment in SCC-25, FIBS, and TR146 cells had any effect on the release of growth factor VEGF and EGF. Serum-free was used as a control.*

Results: *It was found that 10- and 25-unit concentration of Erythropoietin showed the maximum release of EGF in SCC-25. The effect on VEGF release was slightly different where 1 unit of Erythropoietin showed maximum VEGF release in FIBS and had no effect on the other cell types.*

Conclusion: *This study has confirmed that 10- and 25-unit concentration of Erythropoietin showed the maximum release of EGF in SCC-25. The effect on VEGF release was slightly different where 1 unit of Erythropoietin showed maximum VEGF release in FIBS.*

Keywords: *Erythropoietin, Angiogenesis, EGF, VEGF, ELISA*

INTRODUCTION

Erythropoietin is a hormone produced by the kidney that promotes the formation of red blood cells. Erythropoietin is a 34-kDa glycoprotein hormone physiologically produced by cells of peritubular capillary endothelium of the kidney. The primary functions of Erythropoietin are to promote the differentiation and development of red blood cell production by binding to Erythropoietin Receptors resulting in replication and maturation of red blood cell.[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 three kinase, and STAT1, 4, 5A, 5B transcription factors.[3,4,5]

Tissue Hypoxia is the primary stimulus of the production of Erythropoietin.[6] Hypoxia stimulates the release of HIF which regulates the expression of EPO by binding to a specific region in the DNA called Hypoxia Responsive Element (HRE), HRE becomes activated. This results in an increased expression of various downstream gene targets like vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) in vitro. Almost all cells respond to hypoxia by an up-regulation of HIF. The HIF molecule is a protein heterodimer composed of an α and β submit. The degradation rather than

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the production of HIF α is oxygen regulated and controls the abundance and function of HIF. In the presence of oxygen, HIF α submit is hydroxylated. This facilitates the binding of HIF α submit to a protein known as the VHL protein. The HIF-VHL product is ubiquitinated, and this is degraded by Proteasomes.[7,8,9] Erythropoietin acts on the later stages of the development of erythroid progenitor cells. Primarily Erythropoietin acts on colony-forming unit erythroid (CFU – E) to induce these cells to proliferate and mature through the normoblasts into reticulocytes and mature erythrocytes.[10]

It has recently been shown that the Erythropoietin function goes beyond erythropoiesis. Erythropoietin and Erythropoietin Receptor have been found in non-hemopoietic organs like breast tissue, brain, female genital tract tissue and vascular endothelial cells.[11,12,13,14]

Erythropoietin works by binding to specific receptor Erythropoietin Receptor. The intracellular signaling pathway is established to enable erythroid differentiation.[6] The Erythropoietin Receptor is expressed primarily on erythroid cells between the CFU-E and the pronormoblast stage of erythroid cell development.[15]

Erythropoietin and its receptor are found in various areas of the brain, including glial cells, neurones, cortex, hippocampus, midbrain and brain endothelial cells and astrocytes. Hypoxia and ischaemia have been recognised as critical driving forces of Erythropoietin expression in the brain. Erythropoietin has neuroprotective properties in vivo and in vitro. Brain-derived Erythropoietin can protect neurons by direct and indirect mechanism, direct mechanism involves inhibition of Hypoxia/ischemia-induced apoptosis.[16,17] One proposed mechanism for direct intervention that Erythropoietin represses apoptosis in neurons by either maintaining expression of Bcl-2 and Bcl-xl, as it is the case in erythroid precursor cells, or by inactivation of Caspases.[18,19] Since 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. 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 Matrigel™.

Furthermore, recombinant human Erythropoietin (rhu- Erythropoietin) induced a potent angiogenic response in the chick embryo chorioallantoic membrane, strongly suggesting that Erythropoietin acts directly as a bona fide angiogenic factor.[20] Angiogenesis is mediated mainly by VEGF. In the case of Erythropoietin, expression of the VEGF gene and increased VEGF production is controlled by the transcription factor HIF-1.[21] However, recent studies provided evidence that Erythropoietin can also elicit an angiogenic response in endothelial cells in vitro and in vivo, and thus, like VEGF, is an effective angiogenic factor.[20] Erythropoietin and its mRNA were produced in uterus as E_2 – dependent 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 E_2 – dependant cyclical angiogenesis in uterus.[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 zidovudine, nonmyeloid malignancies in patients treated with chemotherapeutic agents, perioperative surgical patients.[5] Erythropoietin and Erythropoietin Receptor has been studied in a number of diseases. The recombinant Erythropoietin is useful to treat anemia in cancer patients, particularly as an adjunct to chemotherapy.[22] The harmful results to Erythropoietin related to cancer cell survival, proliferation, angiogenesis and promotion of tumour growth is a challenge its use in solid tumours.[23, 24, 25, 26] Therefore, the aim of this study was to investigate the effect of Erythropoietin on 3 cell lines in vitro. Measurement of growth factor release, using the ELISA technique.

MATERIALS AND METHODS

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

Cell Culture

The three cell lines were used for the in vitro study for growth factor (EGF, VEGF) release using ELISA.

Transformed oral Keratinocytes (TR146)

The human oral Keratinocyte cell lines, TR146, were derived from a buccal carcinoma [27]**.** 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% CO_2 and 95% humidity.

Human Gingival Keratinocytes (FIBS)

The FIBS Cell line was derived from a human gingival keratinocyte 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% CO_2 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 the tongue [28]. 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% Penicillin and Streptomycin] in 5% CO_2 and 95% humidity.

Cell Maintenance

Both the cell lines were grown in respective media in T75 flasks in 5% CO_2 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 a 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. The medium was changed every 3rd day. Cells were incubated at 37°C in 5% CO_2 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

ELISA for Growth Factor

In practice, growth factor release was measured by collecting the culture medium from the treated cultures, and supernatants were frozen at -20ºC until use. Supernatants were defrosted (mixed on a vortex mixer) and growth factor measured using the Elisa kit as follows. The effect of Erythropoietin on the production of VEGF and EGF by the three cell lines was assessed on a commercially available assay kit. (DUOSET R and D system). The procedure was as follows.

RESULT

The effect of Erythropoietin on growth factor release, using the ELISA technique.

Release of VEGF from TR146, FIBS, and SCC-25:

Results for VEGF release on TR146 are listed in Table 1. Results are illustrated in graphical format in figure 1. Erythropoietin had no significant effect on VEGF release compared to SF control. Results for VEGF release on FIBS are listed in the table1 figure 1. Erythropoietin had a significant effect on increased VEGF release when compared to SF control. ($p<0.05$). Results for VEGF release on SCC-25 are listed in table 1 figure 1. Erythropoietin had no significant effect on VEGF release compared to SF control.

Release of EGF from TR146, FIBS, and SCC-25:

Results for EGF release on TR146 are listed in table 2. Results are illustrated in graphical format in figure 2. Erythropoietin had no significant effect on EGF release compared to SF control. Results for EGF release on FIBS are listed in table 2 figure 2. Erythropoietin had no significant effect on EGF

Table 1: VEGF results after Erythropoietin treatment on TR146, FIBS, and SCC-25 cells.

Treatment	TR 146		FIBS		SSC-25		Samples
	Pg/ml	St-dev	Pg/ml	St-dev	Pg/ml	St-dev	4
1 Unit	1326.79	277.47	468.43	114.53	17.63	2,67	4
10 unit	1398.48	217.88	325.51	87.16	17.11	1.72	4
25 unit	1487.11	204.90	301.18	60.81	16.99	6.50	4
Serum free	1218.38	126.72	191.62	65.82	9.66	6.58	4

Table 2: EGF results after Erythropoietin treatment on TR146, FIBS, and SCC-25 cells.

Treatment	TR 146		FIBS		SSC-25		Samples
	Pg/ml	St-dev	Pg/ml	St-dev	Pg/ml	St-dev	4
1 Unit	12.84	3.68	6.11	2.73	10.52	5.98	4
10 unit	11.87	2.16	6.36	1.17	32.24	5.44	4
25 unit	9.25	2.88	20.38	11.98	88.81	6.41	4
Serum free	10.20	5.92	11.14	5.37	10.28	3.50	4

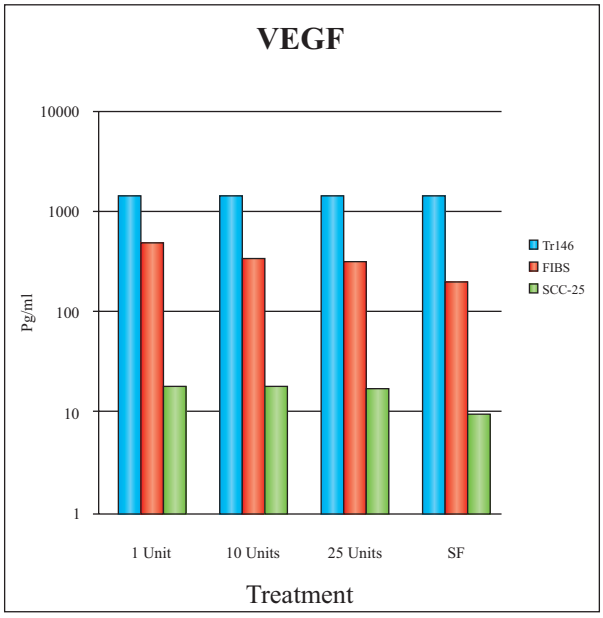

Figure 1: VEGF releases after Erythropoietin treatment on TR146, FIBS, SCC-25 cells.

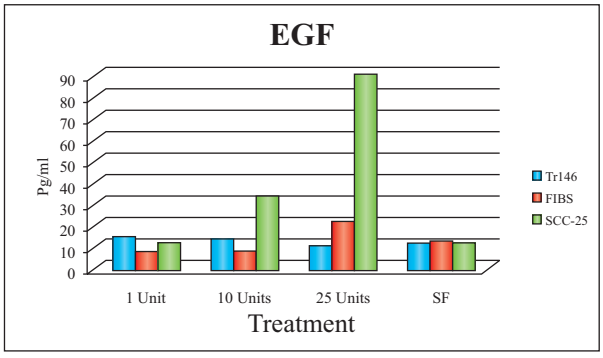

Figure 2: EGF releases after Erythropoietin treatment on TR146, FIBS, SCC-25 cells.

release compared to SF control. Results for EGF release on SCC-25 are listed in table 2 figure 2. In 10 and 25 units of Erythropoietin had a significant effect on EGF release compared to SF control. ($p<0.05$).

DISCUSSION

This project was designed to investigate the in-vitro effect of erythropoietin on cell lines. Furthermore, an ELISA was carried out to see if Erythropoietin induced growth factor stimulation by using supernatant from Erythropoietin treated cells. Erythropoietin was used in three different concentrations. 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.[29] Furthermore, concern has been expressed that it may make the tumor more aggressive. In light of this, we used SCC-25, TR146 and FIBS cells to see the effect of erythropoietin on growth factor release.

Keratinocytes also produce vascular endothelial growth factor VEGF, which promotes endothelial cell growth and angiogenesis, and keratinocyte derived VEGF is a potent mitogen for dermal endothelial cells.[30] VEGF plays an important role in the angiogenesis of many solid tumors,[31] and erythropoietin has been shown to upregulate VEGF in HUVECS.[32] Erythropoietin, however, did not affect

TR146 and SCC-25 cells VEGF production, but there was a slight increase in a release by FIBs cells after 1unit/ml Erythropoietin.

Although the tumor cell lines did not increase VEGF release after Erythropoietin treatment, there was a dose-dependent increase in EGF release by SCC25 cells but not TR146. EGF and transforming growth factor – β (TGF – β) family provide keratinocyte growth via the proliferation stimulating the effect of EGF, and the proliferation is inhibiting the effect of TGF – β.[33] In a previous study, it had been revealed that the overexpression of EGF by hepatocytes is associated with the promotion of hepatocellular carcinogenesis.[34] These results raise an exciting possibility that Erythropoietin also plays an essential role in tumor promotion via the release of EGF. Whether this is true requires further investigation.

CONCLUSION

The ELISA assay was performed to see if Erythropoietin (1, 10, 25 units) treatment in SCC-25, FIBS, and TR146 cells had any effect on the release of growth factor VEGF and EGF. Serum-free medium was used as a control. It was found that 10 and 25 unit concentration of Erythropoietin showed the maximum release of EGF in SCC-25.The effect on VEGF release was slightly different where 1 unit of Erythropoietin showed maximum VEGF release in FIBS and had no effect on the other cell types.

REFERENCES

1. Thuc-Nghi Duc Pham, Weili Ma, David Miller, Lidia Kazakova, and Samuel Benchimol, corresponding author. Erythropoietin inhibits chemotherapy-induced cell death and promotes a senescence-like state in leukemia cells. Cell Death Dis. 2019 Jan; 10(1): 22
2. Nasri H.Renal Cell Protection of Erythropoietin beyond Correcting The Anemia in Chronic Kidney Disease Patients. Cell J. 2014 Winter;15(4):378-80.
3. Andrea Zivot, Jeffrey M. Lipton, Anupama Narla, Lionel Blanc. Erythropoiesis: insights into pathophysiology and treatments in 2017.Mol Med. 2018; 24: 11.
4. Zhang Y, et al. Erythropoietin action in stress response, tissue maintenance, and metabolism. Int J Mol Sci. 2014;15:10296–10333.
5. Socolovsky M., Nam H., Fleming M.D., Haase V.H., Brugnara C., Lodish H.F. Ineffective erythropoiesis in STAT5a(−/−)5b(−/−) mice due to decreased survival of early erythroblasts. Blood. 2001;98:3261–3273. doi: 10.1182/blood.V98.12.3261.
6. Obara N., Suzuki N., Kim K., Nagasawa T., Imagawa S., Yamamoto M. Repression via the GATA box is essential for tissue-specific erythropoietin gene expression. Blood. 2008;111:5223–5232. doi: 10.1182/blood-2007-10-115857.
7. Veronica L. Dengler, Matthew Galbraith, and Joaquín M. Espinosa.Transcriptional Regulation by Hypoxia-Inducible Factors.Crit Rev Biochem Mol, Biol. 2014 Jan-Feb; 49(1): 1–15.
8. Bryan L. Krock, Nicolas Skuli, M. Celeste Simon.Hypoxia-Induced Angiogenesis.Genes Cancer. 2011 Dec; 2(12): 1117–1133.
9. Agnieszka Zimna,Maciej Kurpisz.Hypoxia-Inducible Factor-1 in Physiological and Pathophysiological Angiogenesis: Applications and Therapies.Biomed Res Int. 2015; 2015: 549412.
10. Satish K. Nandakumar, Jacob C. Ulirsch, Vijay G. Sankaran.Advances in Understanding Erythropoiesis: Evolving Perspectives.Br J Haematol. 2016 Apr; 173(2): 206–218.
11. Ka Kui Chan, Kyle B. Matchett, Jonathan A. Coulter, Hiu-Fung Yuen, Cian M. McCrudden, Shu-Dong Zhang, Gareth W. Irwin, Matthew A. Davidson, Thomas Rülicke, Sophie Schober, Ludger Hengst, Heidelinde Jaekel, Angela Platt-Higgins, Philip S. Rudland, Ken I. Mills, Perry Maxwell, Mohamed El-Tanani,Terence R. Lappin. Erythropoietin drives breast cancer progression by activation of its receptor EPOR.Oncotarget. 2017 Jun 13; 8(24)
12. Naoki Oshima, Hiroshi Onimaru, Akira Yamagata, Seigo Itoh, Hidehito Matsubara, Toshihiko Imakiire, Yasuhiro Nishida, Hiroo Kumagai. Erythropoietin, a putative neurotransmitter during hypoxia, is produced in RVLM neurons and activates them in neonatal Wistar rats. Am J Physiol Regul Integr Comp Physiol. 2018 May 1;314(5).
13. Yasuda Y, Masuda S, Chikuma M, Inoue K, Nagao M, Sasaki R. Estrogen-dependent production of erythropoietin in the uterus and its implication in uterine angiogenesis. J Biol Chem.1998; 273. 25381–25387.
14. Farrell, F and Lee A. The Erythropoietin Receptor and Its Expression in Tumor Cells and Other Tissues. Oncology.2004; 9: 1830.
15. Wickrema A, Krantz SB, Winkelmann JC, Bondurant MC.Differentiation and erythropoietin receptor gene expression in human erythroid progenitor cells. Blood. 1992;80:1940–1949.
16. **Marti HH. Erythropoietin and the hypoxic brain.** *J Exp Bio*.2004;207: 3233-324.
17. **Marti HH, Bernaudin M, Petit E, and Bauer C.** Neuroprotection and Angiogenesis: Dual Role of Erythropoietin in Brain Ischemia. News in Physiological Sciences, (2000) Vol. 15, No. 5, 225-229.Lipton P.Ischemic cell death in brain neurons. Physiol Rev.1999;79:

1431–1568.

18. 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. Blood.1996; 88:1576-1582.
19. 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.1999;93:2627–2636.
20. **Semenza GL.**Hypoxia, clonal selection, and the role of HIF-1 in tumor progression Crit Rev Biochem Mol Biol.2000; 35: 71-103.
21. Cella D, Zagari MJ, Vandoros C, Gagnon DD, Hurtz HJ, Nortier JW. Epoetin alfa treatment results in clinically significant improvements in quality of life in anemic cancer patients when referenced to the general population.J Clin Oncol. (2003) Jan 15;21(2):366-73.
22. Batra S, Perelman N, Luck LR, Shimada H, Malik P.Pediatric tumor cells express erythropoietin and a functional erythropoietin receptor that promotes angiogenesis and tumor cell survival. Lab Invest. (2003) Oct;83(10):1477-87.
23. Westenfelder C, Baranowski RL. Erythropoietin Stimulates Proliferation of human renal carcinoma cells. Kidney International. 2000;58(2): 647 –657.
24. Yasuda Y, Fujita Y, Matsuo T, Koinuma S, Hara S, Tazaki A, Onozaki M, Hashimoto M, Musha T, Ogawa K, Fujita H, Nakamura Y, Shiozaki H, Utsumi H, Erythropoietin regulates tumor growth of human malignancies. Carcinogenesis. 2003 Jun;24(6):1021-9.
25. Regulates tumor growth of human malignancies. Carcinogenesis.2003;24:1021-9.
26. Henke M, Laszig R, Rube C, Schafer U, Haase KD, Schilcher B, Mose S, Beer KT, Burger U, Dougherty C, Frommhold H. Erythropoietin to treat head and neck cancer patients with anemia undergoing radiotherapy: a randomised, double-blind, placebo-controlled trial. Lancet. 2003;362(9392):1255-60.
27. Rupniak HT, Rowlatt C, Lane EB, Steele JG, Trejdosiewicz LK, Laskiewicz B, Povey S, Hill BT. Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. J Natl Cancer Inst. Oct; 1985;75(4):621-35. PMID: 2413234.
28. Rheinwald JG, Beckett, MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultures from human squamous cell carcinomas.Cancer Res.May 1981;41(5):1657-63. PMID: 7214336
29. Henke M, Laszig R, Rube C, Schafer U, Haase KD, Schilcher B, Mose S, Beer KT, Burger U, Dougherty C, Frommhold H. Erythropoietin to treat head and neck cancer patients with anemia undergoing radiotherapy: a randomised, double-blind, placebo-controlled trial. Lancet.2003; 362(9392):1255-60.
30. Detmar M, Yeo KT, Nagy JA, Van de Water L, Brown LF, Berse B, Elicker BM, Ledbetter S, Dvorak HF. Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. J Invest Dermatol.1995; 105:44-50.
31. Kleespies A, Guba M, Jauch KW, Bruns CJ. Vascular endothelial growth factor in esophageal cancer. J Surg Oncol.2004; 87:95-104. Review.
32. Hashimoto K. Regulation of keratinocyte function by growth factors.J Dermatol Sci.2000 .24 suppl.1-46-50.
33. Borlak J, Meier T, Halter R, Spanel R, Spanel-Borowski K. Epidermal growth factor-induced hepatocellular carcinoma: gene expression profiles in precursor lesions, early-stage and solitary tumors. Oncogene.2005; 24:1809-19.