Introduction

Maintenance of the red cell volume is a fundamental aspect of ensuring oxygen supply to the tissues. The balance between the very dynamic processes of erythropoiesis and erythrocyte loss is precarious and yet normal individuals experience a remarkably constant haematocrit. This is achieved by a very elegant and sensitive homeostatic mechanism which links tissue oxygen delivery to red cell production. The glycoprotein hormone erythropoietin (EPO) is the principle controller of this process.

Since EPO is essential for normal erythropoiesis, it is clear that minor underproduction of EPO will result in anaemia. The most widespread example of this is the anaemia of end-stage renal failure. The pharmacological use of recombinant human EPO (rHuEPO) in this setting is now well established and has had a dramatic impact on the quality of life of patients with renal disease.

With the more widespread use of EPO in other clinical conditions and the advent of novel therapeutic approaches, this is an opportune moment to review the physiology and patho-physiology of this fascinating and essential hormone.

The discovery of erythropoietin

During 19th Century excursions into the rarefied atmosphere found at altitude, the association between hypoxia and erythropoiesis became firmly established (Jourdanet, 1863; Viault, 1890; Muntz, 1891; Meischer, 1893). Whilst the biological benefit of this relationship was understood, the mechanism which controlled it was not.

In 1906 Carnot and Deflandre became the first to postulate the existence of a humoral erythropoietic factor, however, the experimental evidence used to support their hypothesis, now seems less than definitive.

Through a combination of misconception, prejudice and poor experimental design, almost 50 years were to pass before Alan Ersliev (Ersliev, 1953) published unequivocal evidence of erythropoietin (EPO). In his experiments, Ersliev injected normal rabbits with large volumes of plasma from rabbits which were made severely anaemic by cardiac puncture. The recipient rabbits showed clear, dose dependant erythropoietic responses.

Sites of EPO production

Jacobson et al. (1957) showed that animals subjected to bilateral nephrectomy were unable to mount an EPO response to hypoxia. Evidence that EPO production (in isolated perfused kidneys) could be arrested by puromycin (a protein synthesis inhibitor) proved conclusively that the kidney was indeed the site of EPO synthesis (Ersliev, 1974).

Identification of the cellular production site of EPO has proved more controversial. Using in situ hybridization,
EPO mRNA was found in a subset of peri-tubular endothelial capillary cells (Lacombe et al., 1988). In contrast to these findings two later studies have suggested that EPO originates in tubular cells (Maxwell et al., 1990; Mujais et al., 1999).

**Extra-renal erythropoietin production**

In adults, around 10% of the circulating EPO is produced by the liver (Jacobson et al., 1959) and EPO production is restricted to hepatocytes (Koury et al., 1991). Liver EPO production is however, of greater importance during foetal development (Zanjani et al., 1974). In human studies, the switching of hepatic to renal EPO production has been found to occur at around 30 weeks of gestation (Dame et al., 1998).

**Biochemistry and molecular biology of erythropoietin**

Using HPLC, small amounts of EPO were purified to homogeneity (Miyake et al., 1977; Krystal et al., 1986). This permitted elucidation of the primary amino acid structure (Lai et al., 1986).

EPO has 166 amino acid residues and a molecular weight (MW) of 18 398 D. The overall MW of the EPO molecule is 30 400 D due to three N-linked carbohydrate chains. Two internal disulphide linkages between cysteine residues 7–161 and 29–33 are known to be necessary for biological activity (Sytkowski, 1980).

Jacobs et al. (1985) and Lin et al. (1985) were able to isolate the cDNA encoding for human EPO and express the protein in mammalian cells. There are no physicochemical or biological differences between urinary and rHuEPO (Imai et al., 1990).

The tertiary structure of EPO has been predicted by Bazan (1989) who suggested that the molecule is an antiparallel bundle of four alpha helices analogous (in tertiary structure, but not amino acid sequence) to the growth hormone molecule.

The EPO gene is localized on chromosome 7 (7pter-q22) in humans (Law et al., 1986; Watkins et al., 1986) and is highly conserved among mammalian species (Shoemaker & Mitsock, 1986).

**Control of erythropoietin production**

EPO production occurs by DNA dependent mRNA synthesis (Schooley & Mahlmann, 1972; Erslav, 1974). The oxygen detection system which controls EPO production, responds to changes in venous rather than arterial pO₂ (Kurtz et al., 1988).

In evolutionary terms, the kidney is an excellent ‘choice’ as a site of EPO production (Erslav & Caro, 1986). This is because sodium resorption of the glomerular filtrate consumes oxygen and is directly related to blood flow through it. Thus the venous pO₂ in the kidney is independent of the rate of blood flow.

Using hypoxia responsive, EPO producing cell lines, EPO production was shown to increase (in a dose dependent manner) following exposure to cobalt, nickel and manganese. Furthermore, EPO production by cells cultured with cobalt or nickel was unaffected by carbon monoxide (Goldberg et al., 1988).

These results were explained by reference to the haem molecule. Ferrous-Hb changes its configuration when bound to oxygen, whereas Cobalt-Hb has a very low oxygen affinity and nickel-Hb has no oxygen affinity at all. In the Mn⁺⁺ form manganese-Hb also has low oxygen affinity, but is readily oxidized to the Mn⁺⁺⁺ form which has a high oxygen affinity and is consequently locked in the ‘oxy’ state.

More recent evidence has suggested that oxygen sensing may also rely on a complimentary mechanism which involves detection of reactive oxygen species (Daghman et al., 1999).

At the genetic level, an element within the 3’ region flanking the EPO gene has been shown to enhance the induction of mRNA signal by hypoxia and cobalt (Imagawa et al., 1991).

In addition to the 3’ enhancer element, a promoter element (capable of increasing transcription 50-fold in response to a hypoxic stimulus) has been found within a 117 base pair sequence upstream of the EPO gene (Blanchard et al., 1992).

Hypoxia stimulated interactions between the enhancer element and a 120 kd protein (termed hypoxia inducible factor 1 [HIF 1]) have also been described (Wang & Semenza, 1993).

**Erythropoietin responsive cells**

The first erythroid differentiation stage which can be stimulated by EPO is the BFU.e. Although BFU.e can be formed in the absence of EPO, their proliferation can be stimulated by high concentrations of EPO. Erythroid colony forming units (CFU.e), equate to the pro-erythroblast stage of development and are very sensitive to EPO stimulation, which is a requirement for their proliferation.
The action of erythropoietin on erythroid progenitors

The action of EPO is mediated through mRNA and subsequent protein synthesis, rather than DNA replication and cell division (Gross & Goldwasser, 1971). After EPO stimulation, rapid increases in erythrocyte specific mRNA and proteins are observed. These include, globin mRNA (Terada et al., 1972), transferrin receptor expression (Sawyer & Krantz, 1986), and membrane structural proteins (Koury et al., 1986). Enhanced haem synthesis and nuclear extrusion also feature (Koury et al., 1982).

The viability and maturation of erythroid progenitor cells is greater when they are grown in the presence of EPO (Koury & Bondurant, 1988). Erythroid progenitors cultured in the absence of EPO, generate caspase enzymes with an associated increase in DNA breakage (Gregoli & Bondurant, 1999).

Thus EPO acts as an antiapoptotic agent, causing the proliferation of erythroid cells by erythrocyte protein synthesis through mRNA transcription, rather than by DNA replication and mitogenesis.

The erythropoietin receptor

Erythroid progenitor cells have both low and high affinity EPO receptors (EPO R). A maximum of around 1200 EPO receptors (EPO R) per cell (D’Andrea & Zon, 1990) has been reported. The cDNA of the high affinity EPO specific receptor has been isolated (D’Andrea et al., 1989b) and shown to express a 507 amino acid polypeptide with a single membrane spanning domain.

Similarities between the primary and tertiary structure of the receptors for EPO, IL-2, IL-3, IL-4, IL-6, GM-CSF, growth hormone and prolactin have suggested the occurrence of a cytokine receptor ‘superfamily’ (Bazan, 1989).

Signal transduction mechanisms

Following binding to the EPO R, EPO is internalized by erythroid progenitor cells. Thereafter degenerate and fragmented EPO re-emerges (Mufson & Gesner, 1987).

Membrane protein phosphorylation has been proposed as an initiator of the signal transduction mechanism. One of the membrane proteins affected by EPO activation is associated with the EPO R complex itself (Damen et al., 1992) and is phosphorylated at tyrosine residues by tyrosine kinase (Linnekin et al., 1992). The rapidity of this process (maximal activity being achieved within 30 s of EPO exposure [Komatsu et al., 1992]) suggests that it is the primary event in signal transduction.

Interaction between erythropoietin and other cytokines

The growth factors IL-3 and GM-CSF have been shown to have a co-operative effect with EPO in stimulating early erythropoiesis (Sonoda et al., 1988). However, this effect is limited to the BFU.e stage of development.

In contrast, the inflammatory cytokines IL1-α, IL1-β and IFN-γ have been reported to markedly inhibit the action of EPO on erythroid progenitor cells (Schooley et al., 1987; Means & Krantz, 1992). Furthermore, the same cytokines have been shown to inhibit EPO production (Jelkmann et al., 1994; Faquin et al., 1992; Vannucchi et al., 1994) and limit iron supply to erythroid tissues (Hirayama et al., 1993).

Taken together, the concerted inhibitory effects of some inflammatory cytokines suggest that a reduction in erythropoiesis may be an active part of the inflammatory response. Thus during infection, it may be advantageous to an organism to undertake a short-term reduction in erythropoiesis in order to generate greater numbers of immunologically active cells.

Long-term suppression of erythropoiesis caused by inflammatory cytokines may partly explain the aetiology of anaemias associated with chronic inflammatory diseases.

Clearance of erythropoietin

The clearance of intravenous rHuEPO by renal failure patients has been widely studied. The mean T½ clearance rates reported by 15 study groups range from 4 to 11.2 h. (Macdougall et al., 1991).

Clearance appears to be via three potential routes. These are, excretion through the kidneys, metabolism by the liver or consumption by the erythron.

EPO is present in urine, however, the contribution of the kidneys to clearance is less than 10% (Rosse & Waldemann, 1964).

The liver rapidly metabolizes desialated EPO (Fukuda et al., 1989). This is consistent with the observation that removal of the sialic acid residues of EPO abrogates its bioactivity in vivo but not in vitro (Sytkowski, 1980).

Evidence, suggests that the clearance of EPO alters with the size of the erythron and that this might be a significant route for EPO clearance. Thus patients with aplastic anaemia have higher EPO levels than patients with erythroid hyperplasia and an equivalent degree of anaemia (De Klerk et al., 1981; Fukuda et al., 1989; Jelkmann & Widemann, 1990).

Further evidence has shown that myeloid ablative therapy (Birgegard et al., 1989; Grace et al., 1991)
increases circulating EPO levels prior to development of anaemia. In addition, anaemic patients treated with the appropriate haematinics (B12/Folate [Kendall et al., 1992] or iron [Cazzola & Beguin, 1992]), show a marked fall in circulating EPO levels prior to a corresponding rise in Hb levels.

Assays for erythropoietin

Bioassay of erythropoietin

The first bioassays for EPO involved the measurement of $^{59}$Fe uptake into the RBCs of animals injected with test material. These crude assays could be rendered more sensitive by suppressing endogenous EPO production by test animals. This could be achieved by the use of hypophysectomised, starved or transfused rats (Fried et al., 1957). A more practical assay was developed by Cotes & Bangham (1961) who proposed the use of ex-hypoxic polycythaemic mice.

Despite optimization and standardization of the in vivo bioassay, many problems remained. Not least was the insensitivity of the assay which at best was not sufficient to detect the EPO levels in normal plasma.

Cultured erythroid cells increase haem synthesis in an EPO dose dependent manner (Krantz et al., 1963) and this phenomenon was therefore used as a means of assaying EPO in vitro. The sensitivity of such assays was greatly improved by using the spleen cells removed from mice after programmed induction of haemolytic anaemia using phenylhydrazine (Krystal, 1983).

Whilst the in vitro bioassays were more sensitive, the assays were technically demanding and suffered non specific interferences, which included variance in the iron and transferrin concentration in test plasma (De Klerk et al., 1978) as well as (C$^+$) fixing IgM antibodies (Kazal & Erslev, 1975).

With the advent of sensitive and specific EPO immunoassays, the need for bioassays of EPO in biological fluids declined. However, their use persists for determination of the potency of rHuEPO for pharmaceutical use.

Immunoassays for erythropoietin

In 1979, Sherwood and Goldwasser used human urinary EPO which was purified to homogeneity to develop a radioimmunoassay (RIA). The limit of detection of the assay was 2 mU of EPO, this was achieved by adopting a biphasic disequilibrium method in which the anti-EPO antibodies were incubated with test material prior to addition of the radiotracer.

A similar format was employed by Cotes (1982) who rigorously characterized EPO RIA and demonstrated appropriate changes in serum EPO levels in response to altered physiological conditions. Thus anaemic patients were shown to have an inverse relationship between their EPO levels and haematocrit. When transfused, the EPO levels of anaemic patients decreased appropriately.

Following cloning of the EPO gene, rHuEPO became available and was used to replace native EPO for production of antibodies and radiotracer (Egrie et al., 1987). The results obtained using this technique were identical to those of well characterized assays based on native EPO derived reagents.

Relatively few enzyme linked immunosorbant assays (ELISA) have been developed for EPO. These rely on the use of at least one monoclonal anti-EPO antibody or an affinity purified polyclonal antibody (Wognum et al., 1989). ELISAs which have been developed are reported to be highly sensitive and take less time to perform than RIA.

Erythropoietin levels in health

The EPO levels of normal subjects are log normally distributed with a mean of around 15 mU EPO ml$^{-1}$ and 95% confidence limits between 10 and 30 mU/ml (Sherwood & Goldwasser, 1979; Cotes, 1982). No sex related differences in EPO estimates have been shown (Cotes, 1982; Kendall et al., 1991) and EPO levels are unaffected by the menstrual cycle (Cotes et al., 1983). Some individuals exhibit diurnal variation in EPO levels (Cotes, 1989). During pregnancy EPO levels increase from around the 70th gestational day through to delivery.

At 1 month old, normal (full term) infants have statistically lower EPO levels than normal adults, however, by three months normal adult levels are achieved and maintained throughout childhood (Hellebostad et al., 1988). Premature neonates have significantly lower EPO levels than adults (despite having much less available oxygen) and the EPO response to hypoxia is lowest in the most immature infants (Brown et al., 1984).

Erythropoietin levels in disease

Erythropoietin levels in patients with primary and secondary erythrocytosis

The secondary polycythaemias may be subcategorized (Erslev, 1983) into those with an appropriate rise in EPO levels in response to hypoxia (caused by cardiac or pulmonary insufficiency or high affinity haemoglobinopathies) and those with an inappropriate rise in EPO in the
absence of systemic tissue hypoxia (for example, hypersecretion from tumours, cysts and kidneys with impaired vasculature).

Patients with polycythaemia rubra vera have EPO levels which are normal and significantly lower than patients with nonmyeloproliferative secondary polycythaemia (Koeffler & Goldwasser, 1981).

Measurement of EPO in patients with erythrocytosis has proved valuable, as 93% of primary and secondary polycythaemias can be differentiated.

**Erythropoietin levels in anaemia**

An inverse linear relationship has been shown between the haematocrit and log\(_10\) plasma EPO levels in anaemic nonuraemic patients (Erslev et al., 1980).

Patients with chronic renal failure have been shown to have inappropriately low EPO levels for their degree of anaemia (Adamson et al., 1968; Caro et al., 1979). Interestingly the oxygen sensing mechanisms of these patients remains intact. Thus they can be shown to produce an appropriate EPO response to haemorrhage and blood transfusions (Walle et al., 1987). However, they exhibit no inverse relationship between the Hb and EPO levels.

Patients with renal failure due to polycystic kidney disease have significantly higher Hb and EPO levels than other patients on haemodialysis (Chandra et al., 1985). In acute renal failure, the plasma EPO levels are demonstrably low during the anaemic phase but recover after re-establishment of renal function (Lipkin et al., 1990).

**Erythropoietin levels in hypoplastic anaemia**

EPO levels are generally found to be higher in patients with hypoplastic anaemia than equally anaemic patients with active erythropoiesis (De Klerk et al., 1981; Jelkmann & Widemann, 1990). Following cytotoxic chemotherapy, rapid increases in the EPO level are found to occur prior to any fall in the Hb level (Grace et al., 1991).

**Erythropoietin levels in anaemia associated with rheumatoid arthritis**

Patients with rheumatoid arthritis have been variously reported to have normal (Cotes, 1982; Birgegard et al., 1987; Nielsen et al., 1990a) or inappropriately low (Baer et al., 1987; Hochberg et al., 1988; Vreugdenhil et al., 1990; Kendall et al., 1993) EPO levels for their degree of anaemia. The degree to which the EPO response is ‘blunted’ appears small and therefore unlikely to be the sole explanation of the anaemia seen in these patients.

**Erythropoietin levels in patients with acquired immune deficiency syndrome**

The EPO response to anaemia in patients with AIDS who are not receiving zidovudine (AZT) therapy is ‘blunted’. Following AZT treatment, the patients became more anaemic and the EPO response for a given degree of anaemia is greater than that of a control group of iron deficient patients (Spivak et al., 1989).

**Erythropoietin levels in patients with solid tumours**

Immunoreactive EPO levels in patients with a variety of solid tumours (Miller et al., 1990) have been shown to be inappropriately low for their degree of anaemia.

Some tumours however, are associated with hypersecretion of EPO. These include hepatocellular carcinoma (Kew & Fisher, 1986), renal carcinoma (Da Silva et al., 1990), cerebellar haemangioblastoma (Rosenlof et al., 1985) and uterine fibromyoma (Naets et al., 1977).

**Erythropoietin levels in patients with myeloma**

The EPO levels of anaemic nonuraemic patients with myeloma or Waldenstrom’s macroglobulinaemia have been found to be appropriately raised. However, myeloma patients with demonstrable renal impairment showed a reduced EPO response to anaemia (Nielsen et al., 1990b).

**Erythropoietin levels during tissue transplantation**

Following renal transplantation, an immediate and unexplained rise in the serum EPO level has been demonstrated which peaks within 5 days of transplantation. A second and more sustained rise in EPO then occurs, followed by a decline as the Hb levels and renal function normalized (Sun et al., 1989). During cytotoxic conditioning for autologous and allogeneic bone marrow transplantation EPO levels rose precipitously prior to any demonstrable fall in Hb levels. During the hypoplastic phase, EPO levels remained relatively high for the patients degree of anaemia (Grace et al., 1991). During the recovery phase, EPO levels have been variously reported to be either appropriate (Grace et al., 1991) or low (Ireland et al., 1990).

Children who have normocytic normochromic anaemia which persists beyond the immediate peri-operative period after heart or heart lung transplantation are known to have inappropriately low EPO levels for their degree of anaemia (Blackburn et al., 1992).
Pharmacological use of recombinant human erythropoietin

The efficacy of rHuEPO in treatment of the anaemia associated with end stage renal failure was first shown by Winears et al. (1986). rHuEPO caused a significant rise in Hb levels and eliminated the need for blood transfusion in many patients. The physical fitness of patients treated with rHuEPO was also significantly improved (Grutzmacher et al., 1988). Not all patients have a good response to rHuEPO therapy, the causes of failure include iron deficiency (both functional [Macdougall et al., 1989] and absolute), infection, inflammation and aluminium toxicity (Scigalla et al., 1990).

Several side-effects in treated patients have been seen, the most important of which is hypertension which can lead to encephalopathy and seizures (Brown et al., 1989). However low dose EPO therapy and the more gentle attainment of target Hb levels has largely overcome this phenomenon. Subcutaneous injection of EPO has proved to be the most satisfactory route for administration (Macdougall et al., 1989).

Whilst EPO therapy has become a standard therapy in patients with renal failure, the utility of EPO in other settings remains less consistent. Whilst EPO has proved efficacious in the treatment of many patients with nonrenal anaemia, its use remains far from universal. Reports of the successful use of EPO in the anaemias associated with AIDS (Garnick et al., 1991), rheumatoid arthritis (Birgegard et al., 1991), neonatal prematurity (Obladen et al., 1991), cancer (Henry & Abels, 1994), myeloma (Ludwig et al., 1990), myelodysplastic syndromes and aplasia (Hirashima et al., 1991) have all been published. EPO has been used to enhance the amount of blood that can be collected during autologous transfusion programmes (Goednough et al., 1989), it has also proved beneficial in treating the persistent anaemia which occurs in children postheart transplantation (Blackburn et al., 1992).

New horizons

Mainly through the advent of molecular biology, our understanding of the physiology and pathophysiology of EPO has accelerated dramatically in the past 15 years. Having become one of the first recombinant proteins to be made available for pharmacological use, attention is now being turned to how EPO therapy might be delivered in a more convenient and effective manner.

New approaches have included the development of delayed release delivery sytems to provide sustained EPO supply over prolonged periods (Pistel et al., 1999). Such systems commonly rely on the loading of inert and biodegradable microsperes with recombinant HuEPO. Others have shown that hybrid molecules of GM-CSF and EPO have greater erythropoietic activity than EPO alone Coscarella et al. (1997).

Successful attempts have also been made to develop non-EPO molecules which are capable of EPO like bioactivity. Whilst some of these EPO mimetic substances are formed from polypeptides (Johnson et al., 1998), others are not (Qureshi et al., 1999). Such molecules may provide better pharmacodynamic properties than true EPO and in the future may even be effective in an oral form.

A more permanent resolution for patients with long-term EPO insufficiency may be achieved through gene therapy. This has already been successfully attempted in mice (Bohl & Heard, 1997). Control of EPO production was achieved through a tetracycline resistance gene fused to the mouse EPO gene inserted into myoblasts. Using this approach the haematocrit of the animals increased and EPO production was controlled by administration of tetracycline. More recent studies suggest that intramuscular injection of naked DNA can achieve similar results. (Rizzuto et al., 1999).

These exciting developments bode well for the prospects of long-term, relatively inexpensive therapies to ameliorate a wide variety of anaemias.

References


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