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Molecular Biology of Erythropoietin

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Abstract

The glycoprotein hormone erythropoietin (EPO) is an essential viability and growth factor for the erythrocytic progenitors. EPO is mainly produced in the kidneys. EPO gene expression is induced by hypoxia-inducible transcription factors (HIF). The principal representative of the HIF-family (HIF-1, -2 and -3) is HIF-1, which is composed of an O_2-labile α-subunit and a constant nuclear β-subunit. In normoxia, the α-subunit of HIF is inactivated following prolyl- and asparaginyl-hydroxylation by means of α-oxoglutarate and Fe^{2+}-dependent HIF specific dioxygenases. While HIF-1 and HIF-2 activate the EPO gene, HIF-3, GATA-2 and NFκB are likely inhibitors of EPO gene transcription. EPO signalling involves tyrosine phosphorylation of the homodimeric EPO receptor and subsequent activation of intracellular anti-apoptotic proteins, kinases and transcription factors. Lack of EPO leads to anemia. Treatment with recombinant human EPO (rHuEPO) is efficient and safe in improving the management of the anemia associated with chronic renal failure. RHuEPO analogues with prolonged survival in circulation have been developed. Whether the recent demonstration of EPO receptors in various non-hemopoietic tissues, including tumor cells, is welcome or ominous still needs to be clarified. Evidence suggests that rHuEPO may be a useful neuroprotective agent.
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Key words: erythropoietin, red blood cells, recombinant drugs, anemia, kidneys, hypoxia-inducible factor

Introduction

Modern recombinant DNA technology enables the manufacture of human proteins in cultured animal cells, yeast and bacteria for use as drugs. More than 20 different blood cell-modulating proteins are currently produced. Some of these act as specific growth factors (Table 1) in the bone marrow and other hemopoietic tissues, where they inhibit the programmed cell death (apoptosis) of the hematopoietic stem and progenitor cells to maintain the growth of young blood cells. Red blood cell production requires the hormone erythropoietin (EPO), a glycoprotein, that is mainly of renal origin. Lack of EPO is the primary cause of the anemia associated with chronic renal failure. Before recombinant human EPO (rHuEPO) became available 15 years ago, about 25% of renal patients on dialysis needed regular transfusions of red cells. Treatment with rHuEPO has proved most useful to increase the quality of life of otherwise anemic patients, and the drug is amongst the top selling pharmaceutical products worldwide. With respect to this success, credit is due to the pioneering work of Miyake et al, who collected and concentrated 2550 l EPO−containing urine from patients with aplastic anemia in Kumamoto City. The material was used to purify the hormone (1) and to partially identify its amino acid sequence. Based on this work the human EPO gene could be characterized and be expressed in host cells (2, 3). Details of the industrial large-scale production of rHuEPO are described elsewhere (4, 5). The present review provides information on the structure of endogenous EPO and the recombinant products, the sites and molecular mechanisms of the hypoxic induction of the EPO gene, the effects of EPO on hematopoietic and non-hematopoietic tissues, and the therapeutic impact of rHuEPO and its analogues.

Structure of Human EPO

EPO is a member of the family of class I cytokines which fold into a compact globular structure consisting of 4 α-helical bundles (6, 7). Its molecular mass is 30.4 kDa (8), although it migrates with an apparent size of 34–38 kDa on SDS-polyacrylamide gels. The peptide core of 165 amino acids (9) suffices for receptor-binding and *in vitro* stimulation of erythropoiesis, while the carbohydrate portion (40% of the total molecule) is required for the *in vivo* survival of the hormone (10). The 4 carbohydrate chains of EPO have been analyzed in detail (11–13). The 3 complex-type N-linked oligosaccharides at asparagines 24, 38 and 83 are important in stabilizing EPO in circulation (14, 15). In contrast, the small O-linked oligosaccharide at serine 126 appears to

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Table 1. Hematopoietic Growth Factors

Factor	Size (kDa)	Sites of formation	Target cells
Erythropoietin*	30	Renal fibroblasts, hepatocytes, neuronal cells	BFU-E, CFU-E
Thrombopoietin	60	Hepatocytes, renal proximal tubular cells	Megakaryocytes
GM-CSF*	14–35	T-lymphocytes, monocytes, endothelial cells, fibroblasts	CFU-GM
G-CSF*	20	Monocytes, endothelial cells, fibroblasts	CFU-G
M-CSF	45–90	Monocytes	CFU-M

*Routinely used as recombinant drugs. BFU: burst-forming unit, CFU: colony-forming unit, CSF: colony-stimulating factor, E: erythroid, G: granulocyte, M: monocyte.

lack functional importance (16, 17).

Human urinary EPO and rHuEPO are identical with respect to their primary and secondary structure. Note, however, that there are minor quantitative differences in the composition of the N- and O-glycans of human urinary EPO (18) and human serum EPO (19), compared to those of rHuEPO. The differences in electrophoretic mobility enable anti-doping laboratories to detect rHuEPO in urinary samples of athletes misusing the drug to enhance their endurance capacity (20). In addition to the approved rHuEPO preparations (Epoetin alfa and Epoetin beta), which are expressed from genetically engineered Chinese hamster ovary (CHO) cells (3, 11, 14, 16), rHuEPO (Epoetin omega) from baby hamster kidney (BHK) cell cultures (21–23) has been applied in clinical trials (24–26). The N-glycans of rHuEPO produced in BHK cells appear to be sulfated to a higher degree (27) which may be relevant regarding the *in vivo* survival of the drug. Other cell lines successfully transfected with the human EPO gene to produce glycosylated rHuEPO included RPMI 1,788 human lymphoblastoid (28), COS African green monkey kidney (2, 29, 30), MDCK canine kidney (31), L929 mouse fibroblast (32) and C127 mouse mammary (33) cells. In view of the relationship between the multiantennary sialic acid—containing carbohydrate chains and the *in vivo* stability of the hormone (34), recently a CHO-cell derived hyperglycosylated rHuEPO analogue (Darbepoetin alfa) has been developed. This compound possesses 2 extra N-linked carbohydrate chains based on site-directed mutagenesis for exchange of 5 amino acids. Compared to the Epoetins, which have a plasma half-life of 6–8 hours, Darbepoetin alfa has a 3- to 4-fold longer plasma half-life (35).

EPO amounts are traditionally expressed in units (U), with 1 U of EPO producing the same erythropoiesis-stimulating response in experimental animals as 5 μmol cobaltous chloride. International reference preparations of human urinary EPO [2nd IRP (36)] and purified DNA-derived human EPO [IS 87/684, rDNA derived (37)] have been established. The specific activity of pure rHuEPO is 130,000 U/mg fully glycosylated protein. Darbepoetin alfa is expressed in gram with the biological activity of 1 μg Darbepoetin alfa peptide core weight corresponding to that of 200 U rHuEPO peptide core weight, both on theoretical grounds (38) and clinical experience (39, 40).

Sites and Mechanisms of EPO Production and Degradation

EPO is mainly produced by hepatocytes during the fetal stage. After birth, almost all circulating EPO originates from peritubular fibroblast-like cells located in the cortex of the kidneys (41–43). Transcription factors of the GATA-family may be important in the control of the time- and tissue-specific expression of the EPO gene (44). In adults, minor amounts of EPO mRNA are expressed in liver parenchyma, spleen, lung, testis and brain (45, 46). In brain, EPO exerts neurotrophic and neuroprotective effects, which are separate from the action of circulating EPO on erythropoietic tissues (47, 48). Tissue hypoxia is the main stimulus of EPO production [for references, see (49)]. In persons with intact renal function plasma EPO levels increase exponentially with decreasing blood hemoglobin (Hb) concentration. Values may rise to 10,000 U/*l* compared to the normal value of about 15 U/*l* [for references, see (50)]. EPO gene expression is not only stimulated when the O_2 capacity (corresponding to the Hb concentration) of the blood decreases, but also when the arterial pO_2 decreases (f.e. at high altitude residence) or when the O_2 affinity of the blood increases. Like other plasma glycoproteins EPO circulates as a pool of isoforms that differ in glycosylation, molecular mass, biological activity and immunoreactivity (51, 52). There is a diurnal fluctuation of the concentration of circulating EPO, with values being about 40% higher at midnight than in the morning (53).

The mechanisms of the degradation of circulating EPO are still incompletely understood. To a minor degree, EPO may be cleared by the liver and the kidneys. However, there is evidence to assume that EPO is mainly removed from circulation by uptake into erythrocytic and other cells possessing the EPO receptor (38). Accordingly, new rHuEPO formulations are presently tested which contain methoxy-polyethylene glycol to prevent internalization of the drug, thus resulting in prolonged biological half-life (54).

Control of EPO Gene Expression

Based on experiments with human EPO-producing hepatoma cell cultures major progress has been made in

understanding the nature of the O_2 sensor controlling the rate of the expression of the EPO gene and other hypoxia-inducible genes (55–59). There are several regulatory DNA sequences in the neighborhood of the EPO gene. The key sequence is located within the so-called hypoxia response element (HRE). It is composed of the nucleotides [A/G] CGTG, to which the hypoxia-inducible transcription factors (HIF) can bind. HIF are dimers composed of an α- and a β-subunit, the latter of which is identical with aryl-hydrocarbon nuclear translocator (ARNT). They belong to the family of basic helix-loop-helix (bHLH) proteins with a PAS domain (according to its presence in the Drosophila Period, ARNT and the Drosophila Single-minded proteins). There are at least three subtypes of the HIF-α subunit (-1α, -2α, -3α). Of these, only HIF-1α and HIF-2α , but not HIF-3α possess a C-terminal transactivation domain (C-TAD). HIF-1α/β is generally considered the primary mediator of hypoxia-induced gene expression (60, 61). The role of HIF-2α/β is only beginning to be understood (62) and HIF-3α/β may actually be a suppressor of hypoxic gene induction (63).

Both the 100–120 kDa HIF-1α and the 91–94 kDa HIF-1 β are continuously produced, with their mRNA levels being essentially unaltered by the induction of hypoxia (64, 65). However, HIF-1α is usually not detectable in normoxic cells (66), while HIF-1β is constantly present in the nucleus. HIF-1α possesses two oxygen-dependent proteolytic degradation domains (ODD) and two TADs. In the presence of O_2 , HIF-1α is hydroxylated at the proline residues 402 (67) and 564 (68, 69) in the ODDs. This reaction is catalyzed by specific HIF-1α prolyl-hydroxylase domain (PHD) containing enzymes that belong to the group of α-oxoglutarate- and Fe (II)-dependent dioxygenases (70–72). On binding to the Fe^{2+} (a non-heme iron), O_2 molecules are split with one atom being transferred to the proline residues and the other forming CO_2 (and succinate) with α-oxoglutarate. Ascorbate prevents the inactivation of the PHDs due to oxidation of Fe^{2+} (73). The K_m values of the three PHDs for O_2 are essentially identical and close to atmospheric O_2 concentrations (74). Prolyl-hydroxylated HIF-1α is tagged by the von-Hippel-Lindau gene product pVHL which forms a complex with the E3 ubiquitin ligase (75, 76). Polyubiquitinated HIF-1α is immediately degraded by the proteasome (77, 78). Only under hypoxic conditions, HIF-1α is enabled to enter the nucleus and to heterodimerize with HIF-1β. The binding of pVHL is a prerequisite for the degradation of HIF-1α. Thus, the mutation of pVHL in patients suffering from congenital Chuvash polycythemia is associated with increased transcription of the EPO gene (79).

In addition, HIF-1α is hydroxylated at asparagine in position 803 in the C-TAD in the presence of O_2 which results in a reduced ability of HIF-1α to bind the transcriptional coactivator p300/CBP (80–82). Thus, there are at least 4 specific hydroxylases which function as cellular O_2 sensors (Fig. 1). Interestingly, studies utilizing transfected human cells overexpressing these hydroxylases have revealed differences in their subcellular localization. PHD-1 is exclusively present in the nucleus, PHD-3 occurs in both nucleus and cytoplasm, while PHD-2 and the asparaginyl-hydroxylase (also called FIH, factor inhibiting HIF) are mainly located in the cytoplasm (83, 84).

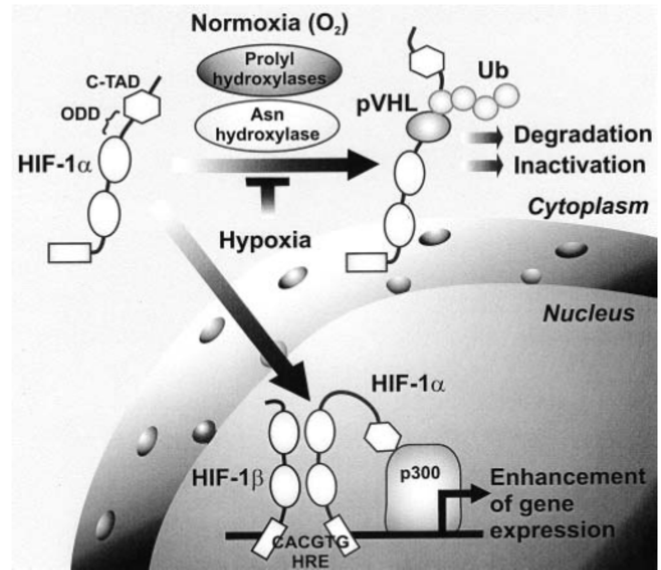

Figure 1. HIF-1α prolyl- and asparaginyl-hydroxylases as cellular O_2 sensors. In normoxia, (a) prolyl hydroxylation in the O_2-dependent degradation domain (ODD) results in binding of the von-Hippel Lindau protein (pVHL) and the ubiquitin ligase complex (Ub) with subsequent proteasomal degradation and (b) asparaginyl hydroxylation in the C-terminal transactivation domain (C-TAD) prevents binding of the p300/CBP transcriptional coactivator. In hypoxia, HIF-1α enters the nucleus to form the active transcription complex with p300/CREB and HIF-1β. Modified from Ref. (197).

The HIFs are not only the key regulators of EPO production. They are involved in most pathways of the adaptation of genes to hypoxia (85) and therefore considered attractive candidates for pharmacologic manipulation (86). For example, the earlier observation that Fe-chelating agents such as desferrioxamine increase EPO production (87–89) may be explained by inhibition of the activity of the Fe (II)-requiring HIF prolyl- and asparaginyl-hydroxylases (Fig. 2). On the other hand, since HIF-1 is associated with angiogenesis and tumor progression, oncologic investigations aim at identifying compounds that specifically inhibit HIF-1 driven gene expression (90).

Truly, however, with respect to the renal production of EPO it must be conceded that the role of HIF-1 has not been well explored. EPO mRNA expression in renal cells has been reported to follow an all-or-nothing fashion rather than to be a graded process (91). In addition, attempts have failed to establish renal cell cultures for study of O_2-dependent EPO production. Studies in EPO transgenic mice (92) have shown that the HREs are located at opposite sites of the gene in the kidney (between 9.5 and 14 kb 5′ to the gene) and the

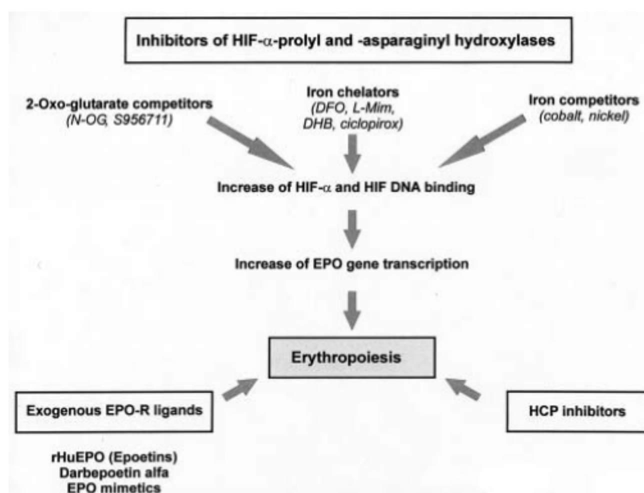

Figure 2. Novel pharmacological approaches to increase erythropoiesis. (a) Endogenous EPO production may be stimulated by compounds stabilizing HIF-α and enhancing HIF transcriptional activity. (b) Exogenous ligands of the EPO receptor (EPO-R) can be administered (clinically approved drugs are the Epoetins and Darbepoetin alfa). (c) Inhibitors of the hemopoietic cell phosphatase (HCP) may prolong the action of EPO. N-OG: N-oxalylglycine, S956711: 6-chloro-3-hydroxychinoline-2-carbonic acid-N-carboxymethylamide, DFO: desferrioxamine, L-Mim: L-Mimosine, DHB: 3, 4-dihydroxybenzoate.

liver (within 0.7 kb 3′ to the gene). Instead of HIF-1α the closely related HIF-2α may control EPO gene expression in the kidney, because HIF-2α was detected in the EPO producing renal fibroblasts of hypoxic rats (93). In contrast to HIF-1 and -2, HIF containing the 3α-subunit is thought to suppress the expression of hypoxia-responsive genes (63).

Finally, it has to be remembered that there are other transcription factors which can modulate EPO gene transcription. Imagawa et al (94, 95) have demonstrated that GATA-2 inhibits EPO gene transcription by binding to the EPO promoter under normoxic conditions. For example, the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) lowers EPO production by increasing GATA-2 DNA-binding (96). L-NMMA is considered one of the candidate substances that suppress EPO synthesis in patients with chronic renal failure (97). Furthermore, the EPO promoter and the 5′ flanking region contain binding sites for nuclear factor κB (NFκB) (98). Evidence suggests that both GATA-2 and NFκB are involved in the inhibition of EPO gene expression in inflammatory diseases. The pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) activate GATA-2 (99) and NFκB (99, 100). IL-1 and TNF-α are thought to contribute to the anemia of chronic disease partly by suppressing EPO production (101). Recent studies have shown that the GATA-specific inhibitor K-7174 restores EPO production in IL-1, TNF-α or L-NMMA treated human hepatoma cell cultures and experimental mice (102). Cyclic AMP has also been shown to antagonize the inhibition of EPO production by IL-1 and TNF-α (103), but the precise role of protein kinase A in the control of EPO mRNA expression still needs to be elucidated (104).

Mechanism of Action of EPO

Erythrocytic progenitors in the bone marrow are the principal targets of EPO. The normally low concentration of the hormone enables only a small percentage of progenitors to survive and to proliferate while the remaining progenitors undergo apoptosis. Thus, the primary mechanism by which EPO maintains erythropoiesis is the prevention of programmed cell death (105, 106). The most primitive EPO-responsive progenitor is the burst-forming unit-erythroid (BFU-E), which gives rise to several colony-forming units-erythroid (CFU-E). BFU-Es are devoid of transferrin receptor and express little GATA-1, whereas CFU-Es possess transferrin receptors and exhibit abundant levels of GATA-1 (107). GATA-1 is an important transcription factor in erythrocytic development (108). The balance between GATA-1 and caspase activity largely determines the rate of proliferation and differentiation of erythrocytic progenitors (106). GATA-1 induces the anti-apoptotic protein bcl-x_L (109). Erythrocytic progenitors may have the potential to produce small amounts of EPO to maintain basal rates of erythropoiesis (110). However, when the concentration of EPO rises in blood, either endogenously or following the administration of rHuEPO, many more BFU-Es and CFU-Es escape from apoptosis and proliferate to result in the growth and maturation of morphologically identifiable proerythroblasts and normoblasts. At the stage of the polychromatic normoblast hemoglobin is accumulated. Subsequently, the nucleus becomes pyknotic and is excluded from the cell. The time from the CFU-E to the reticulocyte is about 7 days and involves 4–6 cell divisions. Significant reticulocytosis becomes apparent about 3–4 days after an acute increase in plasma EPO.

CFU-Es are the most EPO-sensitive cells with the highest density of EPO receptors on their surfaces. The mature EPO receptor is a 484 aminoacid glycoprotein which is a member of the cytokine class I receptor superfamily (111, 112). It possesses a single hydrophobic transmembrane sequence, a variable cytoplasmic domain and an extracellular domain with conserved cysteines and a WSXWS-motif (112). Two of the membrane-spanning EPO receptor molecules form a dimer to which one EPO molecule binds. By means of light scattering, sedimentation equilibrium and titration calorimetry it has been shown that the EPO dissociation constants (K_d) are 1 nM and 1 μM for the two EPO receptor binding sites (113). Crystal structure analysis of the EPO receptor binding residues has already been carried out (114). With respect to novel pharmacological compounds used for therapy it is noteworthy that the degree of receptor binding depends on the carbohydrate content of EPO. Affinity for the receptor

decreases with glycosylation (35). Apparently, the carbohydrate portion of a glycoprotein ligand prevents receptor binding through electrostatic forces (115). Reduced receptor binding and internalization may provide an explanation for the prolonged *in vivo* half-life of hyperglycosylated EPO analogues such as Darbepoetin alfa (38) which has a 4-fold reduction in EPO receptor binding affinity compared to rHuEPO (35).

Figure 3 shows a scheme of EPO signalling. Ligand binding induces a conformational change and a more tighter connection of the two receptor molecules (116–118). As a result, two Janus kinase 2 (JAK2) tyrosine kinase molecules, which are in contact with the cytoplasmic region of the EPO receptor molecules, are activated (118, 119). Thereupon, several tyrosine residues of the EPO receptor are phosphorylated and exhibit docking sites for signalling proteins containing SRC homology 2 (SH2) domains (120, 121). As a result, several signal transduction pathways are channeled, including phosphatidyl-inositol 3-kinase (PI-3K/Akt), JAK2, STAT5, MAP kinase and protein kinase C (122–124). However, the specific roles of the various enzymes and transcriptional cofactors is only beginning to be understood with respect to the fate of the different erythrocytic progenitors in terms of survival, proliferation and differentiation (125–127). In addition, many observations have been derived from studies with cell lines, which may differ in response from primary EPO-responsive cells (128). Interestingly, EPO receptor signalling is inhibited by the cytokine-inducible SH2 protein 3 (CIS3; also known as SOCS-3, for suppressor of cytokine signalling), which can bind to phosphorylated EPO receptor and JAK2 (129). The effect of EPO is terminated by the action of the hemopoietic cell phosphatase (HCP) which catalyses JAK2 de-phosphorylation (130, 131). *In vitro* studies have shown that the EPO-induced signalling pathways return to nearly basal levels after 30–60 minutes (132). Apparently, the EPO/EPO-receptor complex is internalized following dephosphorylation of the receptor. The proteasome controls the duration of EPO signalling by inhibiting the renewal of cell surface receptor molecules (132, 133). Mutations of the cytoplasmic C-terminal regions of the EPO receptor and functional deficiencies of HCP may lead to familial erythrocytosis (134). On the other hand, inhibitors of HCP have been developed to prevent JAK2 de-phosphorylation and to prolong the action of EPO (135).

EPO was earlier thought to act exclusively on erythrocytic progenitors. However, recent studies have shown that EPO is a more pleiotropic hormone [for references see (136–138)]. For example, EPO receptor mRNA and/or protein have been shown to be present in endothelial cells (139, 140), epicardium and pericardium (141), renal mesangial and epithelial cells (142), pancreatic islets (143), placenta (144), and defined areas of brain (145–148). Based on these findings it has been proposed that EPO fullfills angiogenic and neurotrophic functions (48, 137, 138, 149). However, the physiological role of the EPO/EPO receptor system in non-erythrocytic tissues requires further clarification. Transgenic mice expressing EPO receptor exclusively in hematopoietic cells develop normally, are healthy and fertile. They do not display neurologic disturbances (150).

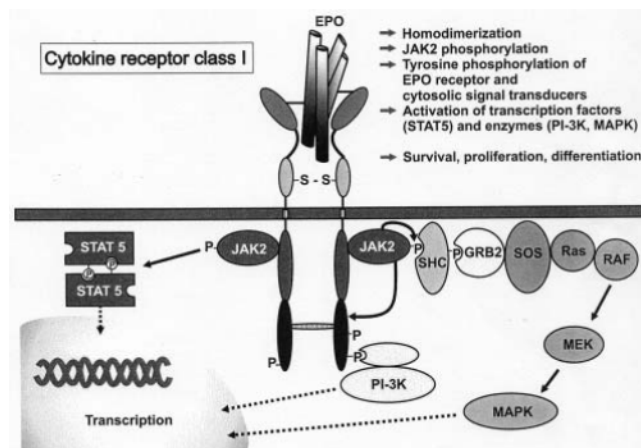

Figure 3. Simplified scheme of EPO signalling, involving autophosphorylation of JAK2 (Januse kinase 2), phosphorylation of the EPO receptor, homodimerization of STAT5 (signal transducer and activator of transcription 5), activation of PI-3K (phosphatidyl-inositol-3-kinase), phosphorylation of the adapter protein SHC (SrC-homology and collagen) to form a complex with GRB (growth factor receptor binding protein), SOS (son of sevenless) and the G-protein Ras, and the sequential activation of the serine-kinase RAF, MEK (syn. MAPKK) and MAPK (mitogen activated protein kinase). The signalling cascade results in survival, proliferation and differentiation of erythrocytic progenitors. The EPO/EPO-receptor complex is internalized and degraded. In addition, the action of EPO is terminated by HCP (hemopoietic cell phosphatase) which catalizes the dephosphorylation of JAK2.

An intriguing question is whether or not tumor cells express the EPO receptor and whether EPO promotes tumor growth. Initial studies utilizing a number of different tumor cell lines *in vitro* failed to show growth-modulating effects of rHuEPO when tested over a wide dose range (151–153), even when EPO receptor-positive cell lines were tested (154). Neither was rHuEPO found to stimulate the growth of freshly explanted human cancers in primary culture (155). On the other hand, EPO-binding sites and EPO receptor protein, respectively, were detected in biopsies of human lung carcinoma (156), human breast carcinoma (157–159), and human uterus cervical carcinomas (160). Furthermore, EPO receptor mRNA and/or protein have been shown in Hep3B human hepatocarcinoma (161), renal carcinoma (162), various breast carcinoma cell lines (157) and malignant tumors of female reproductive organs (163). Moreover, these recent studies indicate that EPO can indeed stimulate the proliferation of the tumor cells *in vitro* (157, 162) and in nude mice *in vivo* (164). Tumor regression can be induced by inhibition of EPO signalling produced by the local injection of anti-EPO antibody or soluble forms of the EPO receptor into

tumor tissue (163, 165). It will be an important issue to clarify the reasons between the earlier negative and the more recent positive results regarding the potential of EPO to stimulate the growth of tumor cells.

Clinical Implications and Future Directions

Therapy with recombinant human EPO has become a standard for correction of renal and non-renal anemias. Manufacturers of biogenerics may immediately step into the market when the patents of the original products expire. Current pharmaceutical attempts aim at developing follow-on biologics with an increased *in-vivo* survival. Thus, in addition to the conventional Epoetins, which have a plasma half-life of 6–8 hours, the hyperglycosylated Darbepoetin alfa has been approved, which has a plasma half-life of 24–26 hours (35). The most novel agent CERA which contains a polyethylene glycol polymer is still under investigation (54). While EPO is normally internalized and degraded following receptor-binding, evidence suggests that CERA may escape degradation by dissociating from the receptor. The development of erythropoietic drugs with a sustained efficacy compared with current therapies may allow less frequent clinical dosing. An alternate possibility of increasing the potency of rHuEPO could be the use of dimers or trimers of the protein (166). Another approach, which has apparently not yet been tested clinically, may be to administer EPO mimicking cyclic peptides that show no sequence homology to EPO but bind to the EPO receptor and enhance erythropoiesis in experimental animals (167). Non-peptidic ligands of the EPO receptor have also been described (168, 169). In addition, inhibitors of the hemopoietic cell phosphatase (HCP) may prove useful to prolong the action of EPO (135). EPO gene transfer is another alternative to the administration of rHuEPO (170). However, there is still lack of knowledge of the efficacy, stability and tissue-specificity of such transgenes. Present investigations focus on the effects of inhibitors of HIF-α prolyl- and asparaginyl-hydroxylases. Iron chelators or competitors such as desferrioxamine and cobalt have already been shown to stimulate EPO gene expression *in vivo* (87–89). In addition, HIF-dependent EPO gene expression may be enhanced by competitive inhibitors of prolyl- and asparaginyl-hydroxylases with respect to 2-oxoglutarate (74, 171).

Given intravenously or subcutaneously the Epoetins or Darbepoetin alfa are routinely administered to patients on hemodialysis or continous ambulatory peritoneal dialysis as well as to many predialysis patients (172–174). Overall, there seems to be an underutilization of rHuEPO during the predialysis period, although the correction of anemia allows the patients to enter dialysis later than without rHuEPO therapy and prevents left ventricular hypertrophy and congestive heart failure (175–177). RHuEPO can correct the anemia in practically all patients with renal failure. Reasons for rHuEPO resistance may be iron deficiency, inflammatory of infectious disease, aluminium overload, hyperparathyroidism and osteitis fibrosa. Iron deficiency is reflected by a proportion of hypochromic red cells >10%, a transferrin saturation <20% and a serum ferritin concentration <100 μg/*l* (178). A recent report indicates that the transferrin saturation is a better clinical marker for iron supplementation, although the reticulocyte hemoglobin content reflects the iron status more accurately (179). Most nephrologists set the target hematocrit value at 0.33–0.36 (hemoglobin 110–120 g/*l*). This level of anemia correction leads to an acceptably restored quality of life, exercise capacity, cardiac performance and cognitive function. The question is whether increasing the doses of rHuEPO to attain normal hematocrit values is beneficial. Unfortunately, a major randomized prospective long-term multicenter study on 1,233 patients with cardiac disease showed that the mortality rates were somewhat higher in the normal-hematocrit (0.42) than in the low-hematocrit (0.30) group (180).

Potential non-renal indications for rHuEPO administration include the anemias associated with cancer (primarily chemotherapy-associated anemia), autoimmune diseases, AIDS, bone marrow transplantation and myelodysplastic syndromes [for references see (181)]. In contrast with the high response rate in renal anemia, rHuEPO resistance (hemoglobin increase <10 g/*l* in 4 weeks) is often seen in patients with non-renal anemias. In tumor patients rHuEPO therapy aims at maintaining the patients' hemoglobin values above the transfusion trigger, increasing the exercise tolerance and improving quality of life parameters. A recent review has noted methological deficiencies in most reports claiming improved quality of life in rHuEPO treated patients (182). Clearly, hemoglobin concentrations in cancer patients should not be raised into the normal range. Such overtreatment caused a poorer survival rate in randomized, double-blind placebo-controlled trials on patients with metastatic breast cancer (183) and head and neck cancer patients under radiotherapy (184). Evidence-based clinical practice guidelines have been provided by the American Society of Clinical Oncology and the American Society of Hematology (185). Accordingly, the use of Epoetin is recommended as a treatment option for patients with chemotherapy-associated anemia with a hemoglobin concentration below 100 g/*l*. However, the dosage of Epoetin should be titrated to maintain a hemoglobin concentration of 120 g/*l* to avoid cardiovascular disorders. During over 15 years of the use of recombinant EPO in renal patients, no clinical evidence has been provided to assume that exogenous EPO induces or promotes tumor growth.

A fascinating finding of potential clinical relevance has been the demonstration of functional EPO receptors by neuronal cells (186, 187). Both EPO receptor mRNA and protein are expressed in defined areas of the mammalian brain, primarily in the hippocampus, capsula interna, cortex and midbrain (145, 146, 148). EPO exerts neuroprotective effects *in vivo* as first demonstrated in 1998 (188, 189), when rHuEPO was infused in the lateral ventricles of Mongolian gerbils with experimental cerebral ischemia. Similar to its effects on erythrocytic progenitors, EPO up-regulates the ex-

pression of bcl-x_L, an anti-apoptotic protein, in neuronal cells (190). Brines et al (191) first investigated the efficacy of systemically administered rHuEPO in rodent models of focal brain ischemia, concussive brain injury, experimental autoimmune encephalomyelitis and cainate-induced seizures. Presumedly mediated by EPO receptor-mediated transfer across the blood brain barrier, about 1% of systemically administered rHuEPO became detectable in the cerebrospinal fluid in rats after 3–4 hours (192). Details of the experimental studies on the neuronal effects of EPO have been summarized elsewhere (137, 149, 193–195). In view of the neuroprotective action of EPO in animal studies, Ehrenreich et al (196) recently performed a clinical trial with rHuEPO in patients suffering from acute stroke. In a double-blind randomized proof-of-concept study, 40 patients received either rHuEPO or saline. The trial resulted in a strong trend for reduction in infarct size in the rHuEPO treated patients, compared to the untreated controls as assessed by magnetic resonance imaging. This reduction was associated with a markedly improved neurological recovery and clinical outcome as determined one month after stroke. Progress is expected in understanding the value of rHuEPO for use as a neuroprotective drug in cerebral ischemia, brain trauma, inflammatory diseases and neural degenerative disorders.

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EXHIBIT 14

Title: **FDA Clears Epogen For Treatment Of Anemia In Children On Dialysis**
URL: http://www.pslgroup.com/dg/142036.htm
Doctor's Guide
November 4, 1999

THOUSAND OAKS, CA. -- November 4, 1999 -- The U.S. Food and Drug Administration has approved Amgen's Epogen® (Epoetin alfa) for the treatment of anemia in children with chronic renal failure who are currently undergoing dialysis therapy. Epogen elevates or maintains the red blood cell level (the hematocrit) and virtually eliminates the need for maintenance blood transfusions in both adult and pediatric patients. FDA's action came almost on the 10th anniversary of the initial approval of Epogen.

"Epogen is one of the most important improvements in the care of children with chronic renal failure in the last 20 years. Safety is of the utmost importance in treating children; the clinical trials demonstrated that Epogen is safe in children, as it is in adults," said Kathy Jabs, M.D., medical director of Dialysis and Renal Transplantation at the Children's Hospital of Philadelphia, and co-principal investigator of the pivotal pediatric clinical trial.

"Anemia associated with dialysis is often more severe in children than in adults. Prior to the availability of Epogen, the majority of children with chronic renal failure were dependent on repeated blood transfusions which can lead to adverse effects such as iron overload, a potentially serious disorder, and the development of antibodies that can preclude patients from later receiving a successful kidney transplant," Dr. Jabs said.

In the United States, the incidence of chronic renal failure in children varies from 10 to 20 new cases per year per million children, according to the United States Renal Data System. The incidence is generally higher in older children and is slightly higher in boys than in girls.

"We are proud to make Epogen available to children with chronic renal failure on dialysis," said Gordon Binder, Amgen's chairman and chief executive officer. "Amgen has made a commitment to the nephrology community for more than a decade, and children are an important part of that community.

We feel it is our responsibility to obtain comprehensive clinical information regarding Epogen's proper use and dosage for pediatric dialysis patients so that they can also experience the benefits of Epogen."

Concurrent with today's approval, Amgen is making available a new children's book "Justin's Journey: My Life as a Kid With Kidney Disease" to provide children with kidney disease and their parents a peer-to-peer resource to help them understand the disease and enjoy a high quality of life.

Efficacy Of Epogen

The approval of Epogen for children with kidney disease was based on the results of four clinical trials conducted at 19 U.S. sites involving 128 pediatric dialysis patients, ages two months to 19 years old. In the trials, Epogen demonstrated efficacy in alleviating the anemia of End Stage Renal Disease (ESRD) and markedly reducing blood transfusion dependency.

A total of 113 patients were enrolled in the phase 3 clinical trial, a randomized, multi-center, double-blind, placebo-controlled 36-week study of Epogen therapy. Patients treated with Epogen experienced clinically significant increases in hematocrit levels (attainment of a hematocrit of at least 30 percent, or a six-point increase above baseline levels). Ninety-six percent of the patients who were randomized to receive Epogen attained the target hematocrit.

Furthermore, there was a dramatic decrease in patients' transfusion dependency. 92.3 percent of the patients who received Epogen were transfusion independent after three months of treatment.

Safety Of Epogen

In the clinical trials, Epogen was well tolerated, and no patients were withdrawn from therapy due to adverse events. The

most frequently reported adverse events were hypertension, abdominal pain and headache. Only myalgia (muscle pain) occurred with a statistically significantly higher frequency in patients treated with Epogen.

Epogen's Effects On Anemia

Although dialysis compensates for many kidney functions, it cannot duplicate the production of a naturally-occurring protein called erythropoietin. Produced by the normally functioning kidney, erythropoietin signals the bone marrow to manufacture red blood cells, which are responsible for transporting energy-producing oxygen to all cells of the body. Failure of the kidney to produce this protein leads to anemia in 90 percent of all dialysis patients and can leave patients feeling fatigued and exhausted, impairing their ability to perform even routine tasks.

Epogen, a recombinant form of erythropoietin, supplements the failing kidney's inadequate supply of erythropoietin and stimulates production of red blood cells to correct the anemia associated with chronic renal failure (ESRD). Healthy adults have a red blood cell count (hematocrit) of 37-52 percent. Healthy children older than one year have hematocrits of 35-39 percent. The approved target hematocrit range for dialysis patients using Epogen therapy is 30-36 percent. Today, a majority of patients undergoing dialysis receive Epogen as part of their treatment regimen.

Epogen is manufactured by Amgen. Amgen is a global biotechnology company that discovers, develops, manufactures and markets cost-effective human therapeutics based on advances in cellular and molecular biology.

Related Links: Amgen.

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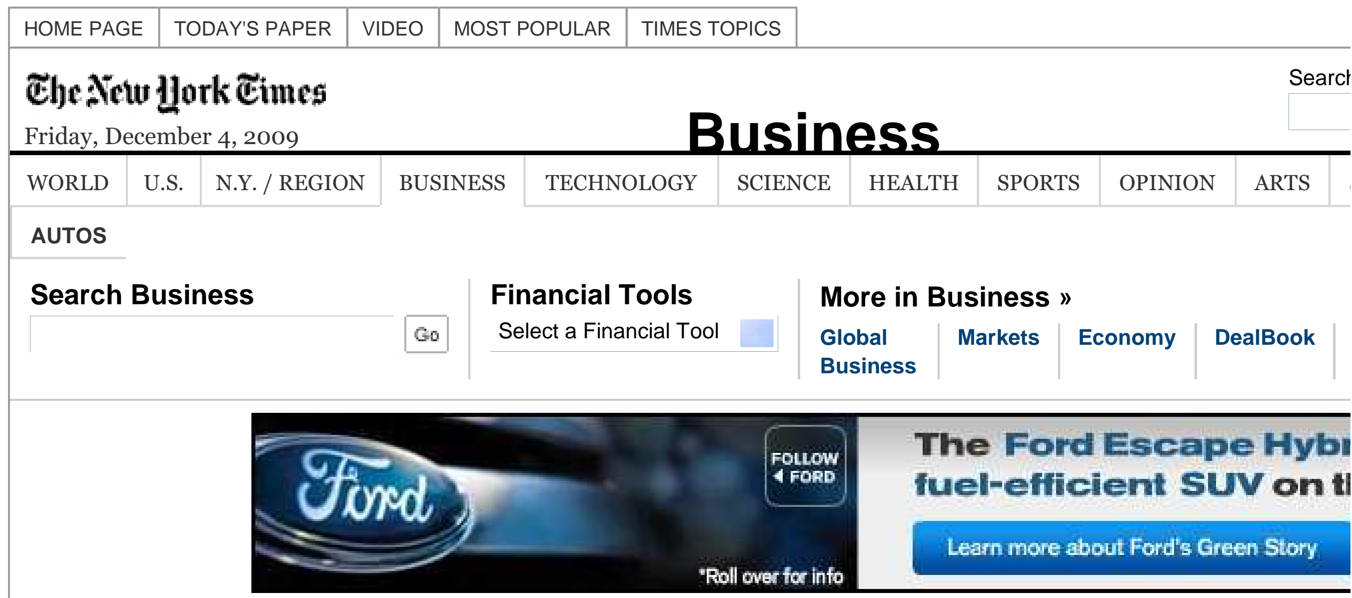
EXHIBIT 15

Welcome to TimesPeople
Get Started



TECHNOLOGY BRIEFING: BIOTECH; AMGEN SHARES RISE ON RULINGS

Published: Tuesday, January 23, 2001

Shares of Amgen, the world's largest biotechnology company, surged yesterday after a federal judge ruled that its rival, Transkaryotic Therapies, had infringed on three Amgen patents on its anemia drug, Epogen. Amgen sold about $4 billion of the drug worldwide in 1999. The company's stock rose $7.63, to $67.63. Transkaryotic Therapies shares tumbled $10.88, to $23.13, making it the day's worst performer.

Graph tracks Amgen share price.

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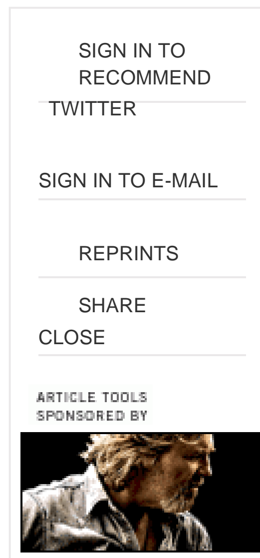

A version of this article appeared in print on Tuesday, January 23, 2001, on section C page 5 of the New York edition.

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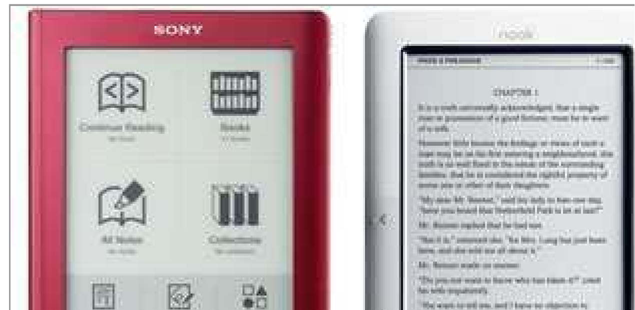

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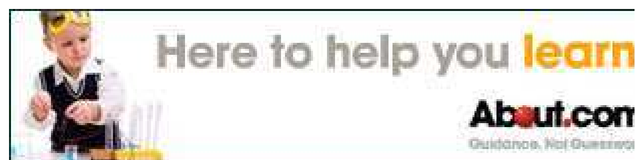

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Exhibit 16

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Clinical Medical Research Award

Hepatitis C Virus and eliminating post-transfusion hepatitis

HARVEY J. ALTER & MICHAEL HOUGHTON

You'll wonder where the yellow went: A 30-year perspective on the near-eradication of post-transfusion hepatitis

HARVEY J. ALTER

The story I will relate here traces the near-total eradication of transfusion-associated hepatitis over the course of three decades. I am perhaps the thread that links these events, but the story is a fabric woven by many collaborators who played essential parts and by the conducive environment of the National Institutes of Health (NIH) intramural program that has nurtured these clinical investigations. The story and my research career began in the early 1960s when, as an NIH Clinical Associate, I investigated the non-cellular causes of febrile transfusion reactions by screening multiply transfused patients for antibodies against serum proteins using agar gel diffusion. Ouchterlony plates were piled high on my lab bench, the way unread manuscripts are today. One day Richard Aster told me that he had heard an interesting lecture by Baruch Blumberg, who was using similar methodology to investigate protein polymorphisms. I visited Blumberg and began a collaboration that within a year uncovered an unusual precipitin line resulting from the reaction between sera from a patient with hemophilia and an Australian Aboriginal person (Fig.1). The line was unusual in that it stained only faintly with lipid dyes, in contrast to the lipoprotein polymorphisms that were then being studied. Because it stained red with the azocarmine counter-stain, we initially called this the red antigen, then debated calling it the Bethesda antigen and ultimately called it the Australia antigen, based on the evolving nomenclature for new hemoglobin discoveries. Subsequent investigations showed the prevalence of Australia antigen to be only 0.1% in the donor population, but very high (10%) in patients with leukemia. The first publication on the Australia antigen[1] cited this association with leukemia in the title. We considered that the antigen might be a component of the long-postulated leukemia virus. In retrospect, the antigen merely reflected the high transfusion exposure and the immunocompromised status of these patients. My initial first-author publication was the biophysical characterization of the Australia antigen[2].

In 1964, I left the NIH to complete my training in internal medicine and hematology, and Blumberg moved to the Institute for Cancer Research in Philadelphia, where he continued to pursue the importance of the Australia antigen. Both serendipity and good science led, by 1968, to the linkage of this antigen to viral hepatitis, a link that transformed the study of hepatitis, protected the blood supply, led to a hepatitis B vaccine and culminated in the Nobel prize.

I returned to the NIH in 1969 to investigate the causes and prevention of post-transfusion hepatitis and to pursue clinical investigations of hepatitis B and its associated antigens. I had no premonition that this would be a lifetime endeavor. My first function was to continue and expand on prospective studies of post-transfusion hepatitis initiated by John Walsh, Bob Purcell, Paul Holland and Paul Schmidt. Walsh and colleagues had already demonstrated the inordinately high hepatitis risk of blood transfusion and, particularly, the risk of paid-donor blood. In 1970, Holland, Schmidt and I, in a still-memorable meeting that would later influence national blood policy, decided that the continued use of paid donors could not be tolerated and also concluded that we should introduce donor screening for what was by then called the hepatitis-associated antigen. I then simultaneously did a retrospective analysis that demonstrated the value of hepatitis B antigen testing and initiated a new prospective study to assess the effect of this dual change in the donor supply. The result was substantial: hepatitis incidence among patients undergoing open-heart surgery plummeted from 33% to 9.7% (ref. 3) (Fig. 2). We calculated that the main determinant of this reduction of about 70% was the exclusion of paid donors. Indeed, retrospective testing for hepatitis B virus (HBV) markers showed that only 20% of the hepatitis found before antigen screening was related to HBV. The recognition of transfusion-associated non-B hepatitis therefore evolved. Improved hepatitis B antigen assays brought hepatitis B transmission to near zero by 1977. In 1973, Steve Feinstone, who had the unenviable task of sifting through stool specimens in the bowels of NIH building 7, used immune electron microscopy to discover the hepatitis A virus, in collaboration with Al Kapikian and Bob Purcell[4]. We immediately delved into our repository of non-B hepatitis cases and were surprised to find that not a single case was due to hepatitis A virus[5]. In a less-than-brilliant foray into nomenclature, we designated these cases non-A, non-B (NANB) hepatitis. Bob Purcell, in particular, felt that we should not call the agent the hepatitis C virus until we had proved transmissibility and until we established the number of agents that might be involved. In our optimism, we did not suspect that the designation non-A, non-B would persist for 15 years before its specific etiology could be defined.

Fig.1 An Australian Aborigine (left) and the precipitin line formed between the aboriginal serum and that of a multiply transfused patient with hemophilia (right). The precipitin failed to stain for lipid, but stained red with the azocarmine counter-stain for protein.

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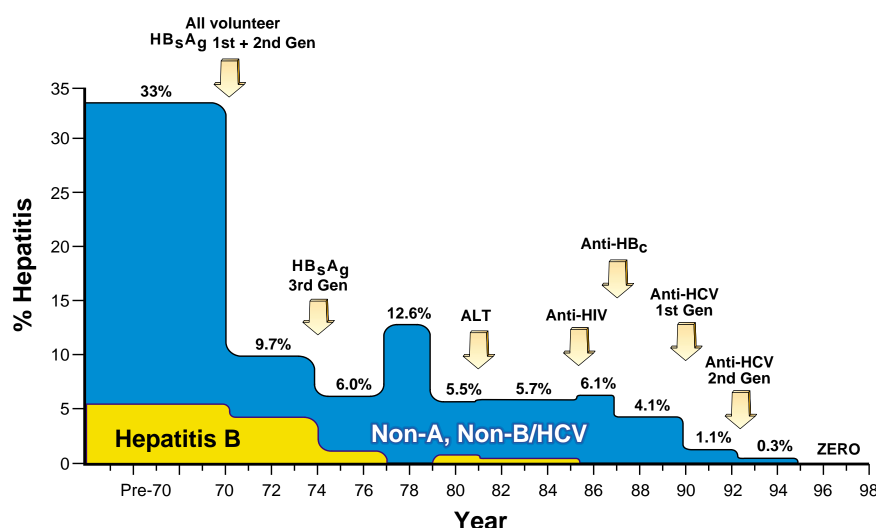

Fig. 2 The decreasing incidence of transfusion-associated hepatitis in blood recipients monitored prospectively. Incidence, traced from 1969 to 1998, demonstrates a decrease in risk from 33% to nearly zero. Arrows, main interventions in donor screening and selection that effected this change.

In 1975, as the prospective studies continued, my attention was directed at proving NANB hepatitis transmission in the chimpanzee model. Earlier attempts at chimpanzee transmission had failed, but I reasoned that we could use the highly pedigreed samples from our prospective studies and use inocula in volumes equivalent to blood transfusion in humans. We achieved success in our first attempt, as five of five chimpanzees developed increases in alanine aminotransferase (ALT) at appropriate intervals after inoculation[6]. We were later able to use this same approach and accomplish the first experimental animal transmission of human immunodeficiency virus[7]. In the absence of a tissue culture system, an observed particle or a serologic assay, the availability of an animal model was essential to further study of the NANB agent. Throughout these prospective studies, my main collaborator was Bob Purcell of the National Institute of Allergy and Infectious Diseases, who provided the basic research arm necessary to propel the investigations of NANB. From among the 50 NANB hepatitis cases identified at that time, I selected a patient (H) who had a particularly severe acute NANB hepatitis and from whom we had obtained apheresis units while his ALT levels were increasing. Purcell established a titration series of the H plasma and then did infectivity studies in the chimp. H had an infectivity titer of $10^{6.5}$ CID_{50}/ml (chimp infectious $doses_{50}$), allowing us to then undertake a series of manipulations of the virus and to test their effect on infectivity. Such studies by Feinstone[8], He[9] and others showed that the NANB agent was sensitive to chloroform and less than 30 nm in diameter, based on filtration. The agent of NANB hepatitis thus seemed to be small, lipid-enveloped, blood-transmissible and responsible for most residual cases of transfusion-associated hepatitis. Subsequently, Patrizia Farci from the University of Cagliari, in collaboration with Purcell and I, did a series of chimpanzee studies[10,11] in which she mixed chronic phase serum from patient H with the acute-phase infectious inoculum and studied the infectivity of the mixture in the chimp model. These studies made the important observation that neutralizing antibodies against hepatitis C virus (HCV) develop, but are very strain-specific and, in most cases, incapable of preventing the emergence of viral variants that lead to persistent infection.

As these virologic and immunologic studies were proceeding in the late 1970s and early 1980s, my main focus was to define the clinical consequences of NANB virus infection and to establish an assay that might be amenable to blood screening. The former proved easier than the latter. The entity NANB hepatitis initially met with considerable skepticism, and some believed it caused only an irrelevant transaminitis, because few patients had recognized clinical illness. However, as we monitored patients long-term and did liver biopsies in collaboration with Jay Hoofnagle and the NIH Liver Service, it became apparent that most NANB-infected patients had biochemical evidence of chronic hepatitis, and that 20% progressed to cirrhosis over the course of one to two decades. Later, after the discovery of HCV, we expanded these natural history studies in both asymptomatic donors[12] and transfusion recipients[13], the latter in collaboration with Leonard Seeff, and confirmed that 20–30% of HCV-infected individuals have severe histologic outcomes. However, equally important, these studies showed that about 20% of HCV-infected individuals undergo spontaneous recovery and that most have an indolent, perhaps non-progressive, course. In collaboration with Farci and Purcell, we have also shown the considerable viral diversity (‘quasispecies’) of HCV infection and that the extent of diversity in the acute phase of illness predicted whether chronic infection would ensue[14].

The histologic studies that documented progression to cirrhosis made it even more imperative to develop a blood-screening assay. In the decade from 1978 to 1988, we attempted every permutation of serologic approaches to assay development. Despite using ‘highly pedigreed’ infectious specimens, ‘presumed convalescent’ sera, eluted fractions, purified gamma globulins and the most-sensitive radio-immune assay approaches, we were unable to develop a specific serologic test for this elusive agent. In the absence of a specific assay, we looked for ‘surrogate’ markers that might identify NANB carriers. The most logical approach was measuring ALT. Although a retrospective analysis of our prospective collections showed that increases in ALT in the donor correlated with hepatitis transmission[15], we were unable to show the efficacy of this ‘surrogate’ assay in a subsequent prospective study. We then sought other measures of donor intervention and reasoned that donors who had been exposed to HBV might also be more likely to have been exposed to NANB; such donors were likely to have recovered from HBV infection and pass the donor screen, but might be persistent carriers of NANB. Thus, we used antibody against hepatitis B core antigen (HBc) as an index of past HBV infection, and showed in a retrospective analysis of our cohort that donors with antibody against HBc were four times more likely to transmit NANB hepatitis and that their exclusion might prevent 30% of such transmissions[16]. These data and those from a multicenter collaborative transfusion-transmitted viruses study[17] convinced the main blood organizations to implement testing for antibodies against HBc and for ALT in routine donor screening in 1987. It was difficult to measure the specific effect of these ‘surrogate’ assays because the threat of transfusion-associated AIDS had

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emerged and the 'surrogate' assays were introduced in concert with more-intensive questioning of donors regarding high-risk behavior and by a lessened use of allogeneic blood. Nonetheless, we could show that these combined measures served to decrease hepatitis incidence to 4.5% by 1989 (Fig. 2). Efforts to develop a specific NANB assay continued throughout the 1980s, although the main effort by Chiron was kept well concealed.

During this time, I had developed a panel of sera consisting of duplicate coded samples that had been proved to be infectious in the chimp or non-infectious in humans. By 1989, many different laboratories claimed to have developed a NANB assay and asked to test the panel. None was able to break the code and by 1989, the score was viruses, 20; investigators, zero. At that time, I received a call from George Kuo at Chiron, saying that they too felt they had a NANB assay. I sent George the remnants of the now-dwindling panel and within days received their results followed by several anxious calls asking if I had yet broken the code. When I did, I was excited to find that Chiron had detected all but two of the infectious sera and had properly found all the non-infectious sera to be negative. Further, the two samples that they missed were acute-phase sera, and subsequent samples from these same patients proved to be positive for what Chiron now called the hepatitis C virus. Michael Houghton will describe the events that preceded this discovery.

Using the newly developed assay for antibodies against HCV, we again delved into our repository and were able to rapidly show that 88% of NANB hepatitis cases seroconverted for antibody against HCV, that the development of antibody was in temporal relationship to the course of hepatitis and that infected patients could be linked to infected donors[18]. Thus, by 1990 it was clear that HCV was the principal agent of NANB hepatitis, and universal donor screening was initiated. We established a new prospective study to measure the effect of such testing and to define the extent of residual hepatitis unrelated to HBV or HCV. The first-generation assay for antibody against HCV resulted in a further 70% decrease in hepatitis incidence to a residual rate of 1.5%, and a more-sensitive second-generation assay, introduced in 1992, nearly eliminated HCV transmission (Fig. 2). Although mathematical modeling indicates that antibody-screened blood might still transmit HCV to 1:100,000 to 1:200,000 recipients, the observed decrease from 33% in 1970 to nearly zero in 1997 stands as a testament to the cumulative effectiveness of a series of donor screening interventions that were evidence-based. Viral nucleic acid testing of donors and improved viral inactivation technologies will soon bring transmission of hepatitis and human immunodeficiency virus from near-zero to absolute zero. I am now looking for another line of work.

Acknowledgments

Throughout almost the entire course of these clinical investigations, my right arm, and sometimes my left as well, has been my dedicated assistant, J. Melpolder. There is no way to adequately acknowledge the substantial contribution she has made in coordinating these studies that have involved thousands of patients. My gratitude is without bounds. I would also like to gratefully acknowledge the manifold contributions of my long-term associate James W. Shih, Ph.D. who so ably supervised the diverse laboratory aspects of these prospective studies.

The hepatitis C virus: A new paradigm for the identification and control of infectious disease

MICHAEL HOUGHTON

Identification of the hepatitis C virus

The problem of non-A, non-B (NANB) hepatitis emerged in 1975, after serological tests for hepatitis A virus (HAV) and hepatitis B virus (HBV) were developed. It then became evident that most hepatitis cases after transfusion were not due to either HAV or HBV, and that the risk of NANB hepatitis after blood transfusions was as high as 10% or even greater[5]. Later, it also became evident that NANB hepatitis occurred frequently in the form of sporadic, community-acquired infections. A frustrating period of 13 years followed, in which the methods successfully used to identify HAV and HBV all failed to result in the molecular identification of the etiological agent(s) of NANB hepatitis. No NANB-hepatitis-specific antigen, antibody or cell culture system was identified, and this lack of a molecular 'handle' limited progress in identifying the causative agent(s) of NANB hepatitis[19]. However, a chimpanzee model successfully developed by several groups[6,20,21] was exploited to show that one NANB hepatitis agent induced characteristic membranous tubules within the endoplasmic reticulum of infected chimpanzee hepatocytes[22]. Known as the tubule-forming agent, it was later shown to be filterable and to lose infectivity after treatment with organic solvents, consistent with its being a lipid-enveloped virus, possibly a toga-like or flavi-like virus[9,23]. Other data supported the existence of an HBV-like NANB hepatitis agent[19] as well as a chloroform-resistant (non-enveloped) NANB hepatitis virus[23] and possibly other NANB hepatitis agents[22].

Eventually, what turned out to be the main form of parenterally transmitted NANB hepatitis was identified using a 'blind' immunoscreening approach applied to recombinant λgt11 (ref. 24) cDNA libraries prepared from total RNA and DNA extracted from infectious chimpanzee plasma[25]. Serum from a patient diagnosed with NANB hepatitis was used as a presumed (but unproven) source of NANB-hepatitis-specific antibodies to identify just one viral cDNA clone from a complex cDNA library constituting one million other cDNAs. Formal proof of its etiological origin came from the demonstration that the clone was not derived from the host genome, that it bound a large RNA molecule of around 10,000 nucleotides found only in NANB-hepatitis-infectious materials, and that it was derived from a positive-stranded RNA encoding a protein that induced antibodies only in NANB hepatitis-infected individuals[25,39]. The RNA genome also encoded a large polyprotein of about 3,000 amino acids that had distant primary sequence identity with members of the Flaviviridae family[26]. HCV was therefore identified by direct molecular cloning of its genome in the relative absence of knowledge concerning the nature of the infectious agent and the immune response. This 'blind' method could be of value in the future in unearthing other unknown infectious agents involved in disease. The molecular identification of HCV was the culmination of a team effort[25] spanning 7 years, during which hundreds of millions of bacterial cDNA clones were screened for a putative NANB hepatitis origin using many different approaches. The successful approach involved Qui-Lim Choo in my Laboratory at Chiron and the laboratories of George Kuo (Chiron) and Daniel Bradley (CDC). I accept the award on behalf of these collaborators (Fig. 3).

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