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A QUEST FOR ERYTHROPOIETIN OVER NINE DECADES

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ABSTRACT

The major research accomplishments of the author are described from the time of his PhD thesis work on the mechanism of cobalt polycythemia to the present day. His early work on the quest for the cell that produces erythropoietin (Epo) to his current work on oxygen sensing and signal transduction pathways involved in erythropoietin gene expression are reported. He describes his main research interest in the mechanism of cobalt polycythemia between 1954 and 1962 and his research on how hormones such as the glucocorticoids function in the regulation of erythropoiesis (1956–1962). His major findings during this period were the discovery that hydrocortisone and corticosterone stimulated erythropoiesis (1958) and that cobalt increased erythropoietin production in the isolated perfused dog kidney (1961). He describes how he was led astray in some of his early studies on the cells in the kidney that produce erythropoietin, because of the less-developed technology available to him at that time; and how in situ hybridization and other molecular biology techniques enabled him to confirm some of the earlier work in mice by other investigators that interstitial cells in the kidney were the site of production of erythropoietin in the primate. His work in the controversial area of the mechanism of the anemia of end-stage renal disease is described in detail, as it pertains to Epo deficiency and suppressed erythroid progenitor cell response to Epo. He also discusses his recent work on signal transduction pathways (hypoxia, nitric oxide, adenosine, and C kinase) in oxygen sensing and Epo gene expression.

Early Education

I was born in Tucapau (now Startex), South Carolina, in 1925. Tucapau was a textile mill town that enjoyed both hydroelectric and steam (generated by coal-burning furnaces) power to run the mill. My father was attracted to Tucapau to play on their textile league baseball team because he was an outstanding baseball

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catcher. He was offered a nice position in the maintenance of the humidifier systems in the mill, which were required in order to maintain a certain level of humidity for optimal weaving and spinning of cotton cloth. Our family was of modest means, and the textile mills in the Spartanburg County area were hit hard during the great depression of the 1930s. Fortunately, my father was able to maintain his job, with the exception of a brief intervening period when the mill was on strike, with my mother supplementing his salary by being a seamstress and working as a spinner in the textile mill at Tucapau. I was very interested in animals during my childhood, always having pets such as dogs, cats, crows, and owls. I had a great curiosity in biological sciences in high school and college and was an avid bird watcher—often making visits into the forest and along the river in my home town. Being from a family with limited resources, I could not afford to attend a large private university, but instead spent my first year, after graduating from W.L.T. (Welford-Lyman-Tucapau) High School in Welford, South Carolina, at Spartanburg Junior College (1942–1943) before going into the Navy V-12 program in 1943 during World War II. I had remembered meeting Governor (later US Senator) Olin D. Johnston in 1938 on our grammar school graduation trip to the state capital in Columbia and hearing him say that he had grown up in a textile mill town and had gone to school at the Textile Industrial Institute (renamed Spartanburg Junior College and later Spartanburg Methodist College). I was very fortunate to obtain a commission as an Ensign following completion of the Navy V-12 program at the University of South Carolina and the Navy Midshipman School at Northwestern University (Chicago campus). I met my wife Carol while I was in midshipman school in Chicago, and we were married in 1947. We have six children, of whom we are very proud. I returned at the end of World War II after almost a year as a naval officer in the Asiatic Pacific theatre to the University of South Carolina to receive my BS degree in chemistry and biology. After working for a few years in the pharmaceutical industry in the control and development laboratories at Armour Pharmaceutical Company in Chicago and in the pharmacology labs at Lloyd Brothers, Inc. in Cincinnati, Ohio, I decided to return to graduate school at the University of Louisville School of Medicine to pursue a PhD degree in pharmacology. I received my degree in 1958, which was during the heyday of research funding at the National Institutes of Health, when competition for grants was not nearly as great as it is today.

Early Work on Erythropoietin

I was intrigued by the work of Carnot & DeFlandre published in 1906 (1) in which they postulated that a humoral factor, which they called hemopoietin, regulated red blood cell production. They carried out experiments in rabbits in which plasma from a donor rabbit following a bleeding stimulus produced a prompt reticulocytosis when injected into a normal recipient rabbit. Several

years later in 1936, Erling Hjort (2) published confirmation of Carnot & DeFlandre's work in Norwegian journal. He removed plasma from rabbits following bleeding, injected this plasma into recipient rabbits, and produced a marked reticulocytosis. He carried out 18 experiments with serum from anemic rabbits and included 5 controls. His paper did not come to my attention until the International Erythropoietin Conference in Lubeck, Germany, in 1991 (3), when Svere Halvorsen, a former postdoctoral research fellow in my lab and at that time Chairman of Pediatrics at the University of Oslo, apprised me of these findings. Hjort died in 1997 at 98 years of age. I had so hoped to meet Erling Hjort before his death, but this was not possible. In 1943, Krumdieck published very similar findings in the *Proceedings of the Society for Experimental Biology and Medicine* (4), in which he demonstrated that erythropoietically active plasma from bled rabbits produced a reticulocytosis in recipient rabbits. It was in 1953 that Allan Erslev injected large volumes of plasma from donor rats following bleeding into normal recipients, which produced a reticulocytosis (5). Erslev's paper has been the most publicized, having been published in the journal *Blood*, which is more widely circulated than the journal in which Hjort (2) published his article. Allan Erslev has been one of the pioneers in erythropoietin (Epo) research and is to be given much credit for his work.

The existence of Epo was not generally accepted for several years; in fact, a physiology friend of mine at the University of Tennessee in Memphis would often ask me, "Do you think there is such a thing as erythropoietin, or are you just chasing a ghost?" One of the most important papers confirming the existence of Epo was by Kurt Reissmann, who in 1950, published a paper in *Blood* (6) in which he demonstrated in parabiotic rats that when one partner is exposed to hypoxia, and the other partner breathed an atmosphere at normal oxygen tension, both partners developed erythroid hyperplasia in their bone marrow. These data indicated that the hypoxic partner produced an erythropoietic substance that passed into the circulation of the partner breathing air at a normal atmospheric pressure and stimulated erythroid cell proliferation in the bone marrow. In 1948, Bonsdorff & Jalavisto, two Finnish investigators, named this humoral substance erythropoietin (7). A major advance in Epo research was the purification of Epo to homogeneity by Miyake et al (8) in Gene Goldwasser's lab at the University of Chicago. This made it possible for the Amgen company to develop a transfected cell line in the golden hamster ovary cell (9) and thereby produce large amounts of human recombinant Epo for clinical use in anemia.

Early Work on Cobalt

My interest in cobalt polycythemia was triggered by LJ Klotz, Director of Research, Lloyd Brothers Pharmaceutical, Inc., in Cincinnati in 1954, who encouraged me to enter graduate school to complete a PhD program in pharmacology.

My early interest in the regulation of erythropoiesis was stimulated by my curiosity about the mechanism of cobalt polycythemia. Around 1929, Waltner & Waltner (10) found that cobalt induced a polycythemia in rats and other animals. Cobalt is one of the 99 elements. It was discovered in about 1735 and first studied chemically in 1802. Chemically, cobalt resembles iron and nickel in its atomic structure and differs from iron only by the position of one electron in one of the orbits. Early studies in rats revealed that the daily administration of oral doses of elemental cobalt in the range of 0.125 to 1.0 mg or from the continued parenteral administration of doses of cobalt salts providing 0.125 mg of elemental cobalt or more per day produced polycythemia (10). Weissbecker (11) reported pronounced erythroid hyperplasia of the bone marrow in hematologically normal human subjects following the oral administration of cobalt salts. Lloyd Brothers actually marketed cobalt under the brand name Roncovite®, which was used for several years during the 1950s for the treatment of the anemia of chronic renal disease and other anemias in adults and children. However, the toxicity of cobalt, especially in producing thyroid suppression, necessitated its removal from the market during the late 1950s. It was apparent that cobalt did not directly affect the bone marrow but initiated the production of some erythropoietic factor that then affected the bone marrow. This stimulated my interest in pursuing this problem. In early studies it was found that cobalt did not alter the rate of bone marrow cellular respiration and, in concentrations of 10^{-2} to 10^{-8} M, did not stimulate heme synthesis or oxygen consumption in bone marrow preparations in vitro.

I am most grateful to Lloyd Brothers for understanding my decision to return for my PhD and for providing some financial support for my graduate training. Peter K Knoefel, then Professor and Chairman of Pharmacology at the University of Louisville, sponsored me for one of the first National Institutes of Health predoctoral fellowships in the mid-1950s. I had commitments to my wife Carol and three young children at that time, and no way would I have ever been able to afford to return to graduate school without Carol's support and funding from a fellowship. Carol still remembers the call, while I was out of town on a trip for Lloyd Brothers, from Ronald Scantlebury, who was at that time Director of the NIH fellowship programs at the National Institutes of Health, informing me that I had been awarded a predoctoral fellowship to return to graduate school to complete my research studies on the mechanism of cobalt polycythemia. During that time we were convinced that the adrenal cortex had an influence on the mechanism of cobalt polycythemia, primarily because cobalt produced adrenal hypertrophy. I was also told by an investigator at the University of Pittsburgh that adrenalectomy suppressed cobalt polycythemia in the dog, which I later confirmed in my PhD thesis work. However, we learned later that this was only a permissive effect of the glucocorticoids in cobalt

polycythemia. We at the University of Louisville thought that cobalt polycythemia might be related to enhancement of adrenocortical steroids. It had been known from occasional clinical observations in patients receiving corticosteroids for inflammatory diseases that adrenocortical steroids that affect glucose metabolism (glucocorticoids) stimulated erythropoiesis. However, the mechanism for this effect had not been clearly elucidated. We found in earlier work that we had carried out on hydrocortisone and corticosterone injections for 60 days in normal rats that these glucocorticoids produced a significant increase in red cell volume (12). It appears that low sustained doses of glucocorticoids for long periods of time stimulate erythropoiesis, whereas high doses inhibit erythropoiesis. Our report that hematocrit, erythrocytes, hemoglobin and total red cell volume were significantly increased after 60 days of injections of hydrocortisone or corticosterone in normal rats was a landmark paper in support of an effect of adrenal corticoids on erythropoiesis (12). The adrenal cortex played a permissive role in the effects of cobalt polycythemia, as concluded from our later adrenalectomy experiments. However, our work with Svere Halvorsen[1,2] indicated that low sustained doses of glucocorticoids are capable of increasing erythropoietin production (13).

The University of Tennessee (1958–1968)

After completing my PhD degree in pharmacology at the University of Louisville under the mentorship of Peter K. Knoefel, William F. Cantrell, and Kee C. Huang I was fortunate to obtain a full-time faculty position as an instructor in the Department of Pharmacology at the University of Tennessee Medical Units in Memphis. I am very grateful to Robert A Woodbury, who was Professor and Chairman of the department when I joined it, for giving me the opportunity for a position in academic pharmacology. I was fortunate in having several medical students work with me in the continuation of my quest for the mechanism of action of cobalt polycythemia. While they were students, Ben Birdwell[3], Joanne Sivadon[4], and James Langston[5] worked with me in developing an isolated dog kidney perfusion system in 1960. We were stimulated to work on this problem by the publication in *Nature* (14) and *Science* (15) of work by Leon Jacobson, Gene Goldwasser, Wally Fried, and Louis Plzak. These studies conducted at the University of Chicago demonstrated that bilateral nephrectomy in rats almost completely abolished the effects of cobalt in

[1]Indicates a former research fellow with James W Fisher; (. . .) indicates years as fellow.

[2]Sverre Halvorsen, MD, (1966–1967), Professor and Chair, Department of Pediatrics, University of Oslo, Norway.

[3]Benjamin Birdwell, MD, (1960–1961), Practicing medicine, Hermitage, TN.

[4]Joanne Sivadon, MD, (1960–1961), Medical practice, Memphis, TN.

[5]James W Langston, MD, (1966–1967), Radiologist, Memphis, TN.

increasing Epo titers. Jacobson and his colleagues had developed an assay for Epo that involved radioactive iron incorporation in red cells of starved rats and found that cobalt's erythropoietic effect was largely due to the effect of cobalt in increasing Epo titers in plasma (14, 15). This was my first entry into the Epo field, and I read with increased vigor all of the earlier work on Epo as it was evident that cobalt's effect was through enhanced production of Epo (14, 15). Leon Jacobson is to be given considerable credit for his seminal finding that spleen shielding in mice protected the mice from the lethal effects of irradiation (16). This finding led to the pioneering studies showing that stem cells emanate from the shielded spleen to repopulate the bone marrow. Jacobson thought that the spleen might produce a hormone that stimulated hematopoiesis. After reading Erslev's paper (5) describing his confirmation of Carnot & DeFlandre's work (1) on a humoral factor that controls erythropoiesis, Jacobson became interested in the possibility that the spleen produced Epo. Jacobson's group persevered in elucidating the site of Epo production, having removed the pituitary, spleen, 90% of the liver, adrenals, and the gonads before finding that only bilateral nephrectomy prevented the response to cobalt and bleeding in increasing Epo titers in plasma (14, 15). It is my understanding from discussions with Wally Fried that he and Louis Plzak decided to do the nephrectomy experiments following a discussion with Sandy Krantz, a medical student at the University of Chicago at the time. Sandy had been reading about patients with erythrocytosis in association with renal tumors (hypernephroma) and suggested that since patients with renal failure are anemic and those with renal tumors are occasionally polycythemic, perhaps the kidney produces Epo.

We took our cue to perfuse the isolated dog kidney to determine whether cobalt triggers production of Epo in the kidney as well as to determine whether the kidney produced Epo based on the bilateral nephrectomy experiments by Jacobson's group. Extirpation experiments such as bilateral nephrectomy abolishing enhanced Epo production following bleeding and cobalt could have been due to a permissive action of the kidney. To investigate this point further Ben Birdwell and Joanne Sivadon, two medical students working in my lab at that time, and myself first carried out isolated perfused kidney studies in the dog by using cobalt as the stimulus. During a visit to the Argonne Cancer Research Hospital at the University of Chicago in early 1960, Cliff Gurney and Leon Jacobson invited me to spend a few months working with them to clarify the role of the kidney and glucocorticoids on Epo production. It was Cliff Gurney who asked Leon Jacobson (known to his friends as "Jake") if I might come there for several months to carry out studies on kidney production of Epo and particularly to assay all of the isolated perfused dog kidney perfusates from our studies carried out at the University of Tennessee. I am most grateful to Jacobson for giving me the opportunity to work at the Argonne Cancer Research

Hospital to carry out these studies. The samples of perfusates were assayed in the starved rat using radioactive iron incorporation in newly formed red cells as the assay for measuring Epo titers. It was of course necessary that we remove the cobalt from the perfusates in order to be sure that the iron incorporation in the starved rats was due to the Epo in the sample and not due to cobalt triggering endogenous production of Epo in the starved rat. I am also grateful to Gene Goldwasser for allowing me to work in his laboratory to carry out the starved rat assays, for teaching me the technique of removing cobalt by a dialysis procedure, and for teaching me the chemical assay for cobalt to be sure that the perfusate samples were free of cobalt.

When we finished up the assay of the perfusates from the isolated perfused dog kidney at the Argonne Cancer Research Hospital, we submitted our manuscript to *Nature.* However, *Nature* declined the paper, indicating that the audience for such a paper on the kidney as the site of production of Epo was too narrow. We presented the work in the spring of 1961 at the Federation (FASEB) Meetings in Atlantic City (17). Our manuscript was then accepted for publication in *Acta Haematologica* (18) and appeared in June 1961. We demonstrated that cobalt enhanced production of Epo in the isolated perfused kidney in the dog. A few months later in 1961, Zofia Kuratowska and her husband Bohdan Lewartowski, in collaboration with E Michalski at the University of Warsaw, Poland, published their article in *Blood* (19), showing that the isolated rabbit kidney perfused with hypoxemic blood enhanced Epo production. They used a reticulocyte assay for their assessment of Epo activity. We were stunned to read four years later in the *American Journal of Physiology* an article by Allan Erslev and his group at the Cardeza Foundation at Jefferson Medical School in Philadelphia showing that hypoxemic perfusion of the isolated dog kidney failed to elevate perfusate levels of Epo in their samples (20). For this reason, we immediately repeated our isolated perfused kidney work in further work carried out by myself and James Langston, and found in fact that Epo titers were significantly elevated in the blood perfusates from isolated dog kidneys perfused with blood at reduced oxygen tension or blood containing cobalt (21). In studies of the kidney histology from the perfusion systems (cobalt or low oxygen tension) most of the kidneys had varying degrees of congestion, but no correlation was found between erythropoietin elaboration and renal congestion or any other degenerative cellular changes in the kidneys. Erslev had contended that erythropoietic material is released only from injured and disintegrating renal tissue but not from well-preserved and metabolically active kidneys (20). Our finding (18) and the work of Kuratowska et al (19) were confirmed by Reissmann & Nomura (22) and Zangheri et al (23) and were most helpful in expelling doubts by some investigators that the kidney produced Epo. Interestingly, a few years later, Erslev also reported that isolated rabbit kidneys

perfused with serum-free medium alone triggered kidney production of Epo (24).

My friend and colleague Cliff Gurney, of the University of Chicago, was taking a sabbatical in 1961–1962 at the Churchill Hospital in Oxford, England, with Lazlo Lajtha. During a visit with them in Oxford in 1962, I asked Lajtha if it would be possible for me to take a sabbatical with him to work on problems related to regulation of the production and action of Epo. I had a Research Career Development Award from the National Institutes of Health at the time that took care of my salary, making it much easier for the University of Tennessee to allow me a paid sabbatical. Lajtha invited me to come to Oxford to carry out my proposed studies on the regulation of kidney production of Epo, to begin in September 1963. A few months later, he was invited to become Director of the Paterson Laboratories at the Christie Hospital and Holt Radium Institute in Manchester, England, and informed me that he would be moving some time during the spring and would like to have me join him for my sabbatical in Manchester. We were first a little hesitant about going to Manchester because we had heard that it was an industrial city in the north with heavy rainfall. After some reflection, we decided to accept Lajtha's invitation to join him in Manchester. We arrived in Manchester in August 1963, and unfortunately, the home owned by the hospital in which we were to live for a year was occupied by radiation biologist and radiotherapist Tony Nias, who was away on vacation in Sweden and had not moved out of the house. It was necessary that we spend the first four weeks in Manchester in a guest house, having our meals in a central dining room, which was chaos with our six children. To add fuel to the fire, I had accepted an invitation to present a paper at the European Society of Hematology, which was meeting in Lisbon, Portugal, during the last week of August 1963. I had also been invited to give a presentation on our work at the Second International Pharmacology Congress in Prague, Czechoslovakia, also in August 1963. Therefore, it was necessary that I leave my wife Carol alone for three weeks with all six children in this guest house in Manchester near Christie Hospital. Thanks to her strength and patience, she persevered and managed the children during my absence.

I was very interested to work with Lajtha to learn more about the mechanism by which Epo stimulated erythroid cells in the bone marrow as well as to learn more about the mechanism of hypoxia and cobalt-induced kidney Epo production. Fortunately, Lajtha had the transfusion polycythemic mouse assay for Epo ongoing in his laboratory; it was being coordinated by Donald Porteous from Yorkshire, Kunitaki Hirashima from Tokyo, and SC Tso from Hong Kong. It was an excellent environment to work in without any pressures from administration and teaching responsibilities, which were very heavy at the University of Tennessee at that time. Lajtha had an open-door policy, and any time that

I had data to discuss with him, his office was open. Lajtha's greatest strength was when you presented the data from your recent studies to him and asked, "Where do we go from here?" He was brilliant in the analysis of the results and in suggesting further experiments. Most of the work at the Paterson Laboratories was focused on radiation biology because this was the international center for research on dosimetry in radiotherapy, and most of the research was supported by the British Cancer Campaign. Ralston Paterson, the former director, distinguished radiotherapist, and for whom the labs were named, had just retired and was raising sheep in Moffat, Scotland.

I spent a few days at the Radcliffe Infirmary in Oxford working with Bill Cook in Sir George Pickering's lab to determine whether the juxtaglomerular cells in the kidney produced Epo. We isolated rat renal glomeruli by using Cook's technique. We were never able to obtain any convincing evidence that juxtaglomerular cells contained Epo. I was fortunate to be able to obtain a sheep kidney from the slaughterhouse in Manchester and partially purified Epo from the National Institutes of Health in Bethesda. This partially purified sheep Epo was prepared by the Armour Company under a contract from the US Atomic Energy Commission, in which Leon Jacobson, Director of the Argonne Cancer Research Hospital, was the Principal Investigator. We produced antibodies to this partially purified sheep plasma Epo in rabbits to be used in some planned fluorescent antibody studies. Lajtha introduced me to Geoffrey Taylor, who was an immunologist and faculty member in the Department of Microbiology at the University of Manchester. I explained to Geof Taylor my interest in localizing the cells in the kidney that produce Epo. Remember, these were the days before the gene for Epo had been cloned, and in situ hybridization techniques were not available at that time for doing work on mRNA for Epo. Geof Taylor and I carried out studies in his laboratory at Manchester, using a fluorescent antibody technique to localize Epo in the kidneys of sheep. We were very surprised to see strong fluorescence in the glomerular epithelial cells. This was an exciting finding for me at that time because these kidney glomeruli stood out like lamppost lights under the fluorescence microscope. We did not know why Epo was seen in these cells. We were able to block this staining with partially purified sheep plasma Epo. Admittedly, we knew that it was still possible that these cells did not produce Epo, but only sequestered or trapped Epo flowing in the blood-perfused glomeruli. We were also well aware of the fact that our antiserum was raised to partially purified sheep plasma Epo. We prepared a manuscript, which was published in *Nature* (25), in which we postulated that the glomerular epithelial cells produced Epo. Unfortunately, we did not give proper significance to cells outside the glomerular tuft, which were fully visible between proximal tubules that also showed fluorescent staining. In fact, one of these cells can even be seen just outside the glomerular tuft in the figure

published in our *Nature* paper (25). Until the gene for Epo was cloned by Lin et al (9) at Amgen and Jacobs et al (26), Epo was thought to be produced in the glomerular epithelial cells. The ability to clone made it possible to prepare cDNA probes for in situ hybridization studies on kidneys for the determination of Epo mRNA. We found later that these cells outside the glomerular tuft were interstitial cells, which were the site of production of Epo; Epo mRNA was found in these interstitial cells but not in glomerular epithelial cells (discussed below).

I was pleased to be able to work with a very close friend, Albert Gordon at New York University, in organizing a New York Academy of Sciences Symposium in New York in 1966 (27). At this symposium, which took place at the Waldorf-Astoria Hotel, we were able to assemble over 400 investigators from all over the world working on Epo. Al Gordon was just recovering from a heart attack but was still able to actively participate in the meeting. Al was recognized as one of the major contributors and pioneers in Epo research for his work carried out from the 1930s. Gordon and his former student Esmail Zanjani (28) postulated at one time that the kidney produced a renal erythropoietic factor ("Erythrogenin") that was secreted into plasma to interact with a plasma protein to produce Epo. This concept was of course dropped when Erslev reported that the isolated rabbit kidney perfused with serum-free perfusate produced Epo (24) and when Koury et al (29) and Lacombe et al (30) found that the kidneys from anemic mice contained high levels of Epo mRNA. The monograph (27) published in 1968 on this symposium contained much of the then-current ongoing research on Epo.

Tulane University (1968–Present)

I came to Tulane University School of Medicine as Professor and Chairman of the Department of Pharmacology in August 1968. This was an interesting period at Tulane University School of Medicine because during the same month, August 1968, Nick Diluzio came as Chairman of the Department of Physiology, John Walsh came as Dean of the Medical School, and Ted Drapanos came as Head of the Department of Surgery. This was a good time at Tulane. I had anticipated being Chairman for 10 years and then returning to my research program, but found myself still in the Chair until July 1, 1996.

After arriving at Tulane, I immediately started to work further on the cells in the kidney that produced Epo. A first-year medical student named Ronald Busuttil[6] came to work with me and B. L. Roh[7] for a combined MS/MD degree,

[6]Ronald Busuttil, MD, PhD, (1971–1975), Professor of Surgery, UCLA School of Medicine, Los Angeles, CA.

[7]Byung Lim Roh, MD, PhD, (1965–1972), Staff Physician, Clinical Pharmacology Service, VA Medical Center, Wood, WI.

and he found Epo in the glomerular epithelial cells of both hypoxic dog (31) and anemic human (32) kidneys, using a fluorescent antibody technique. Busuttil removed a kidney in the middle of the night immediately after death from a patient who had died at Charity Hospital from a gastrointestinal bleeding episode (32). He won first place in the national competition of the Student American Medical Association Research Forum in Galveston, Texas, in 1971, for this work on the anemic human kidney. Unfortunately, our antiserum was again raised to partially purified human Epo, making it difficult to interpret the results. Using cDNA probes and in situ hybridization to detect mRNA for Epo, Koury et al (29) and Lacombe et al (30) had shown in 1988 that high levels of Epo mRNA occurred in the interstitial cells of the mouse kidney following a bleeding stimulus. No mRNA for Epo was detected in glomerular tuft cells. This stimulated our interest further in elucidating the cells in the kidney that produce Epo. Recently, we were fortunate to complete collaborative studies with Stephen Koury at the University of Buffalo, where we exposed rhesus monkeys to hypoxia in our hyperbaric chamber at Tulane and compared the mRNA levels in the hypoxic monkey kidney with those of a kidney from a normal monkey. This work was published in 1996 in the *British Journal of Haematology* (33).

We are very grateful to Fu Kuen Lin at Amgen Company, who was one of the first to clone the gene for Epo (9), for preparing a cDNA probe for us in the region of the human Epo gene that was homologous with the monkey Epo gene. A 645–base pair Kpnl-BgIII arrangement of monkey Epo cDNA, which was subcloned into the plasmid vector pGEM4Z, was used in this work to generate both sense and antisense RNA probes, which were labeled with [α-^{33}P] uridine 5-triphosphate in collaboration with Steve Koury. We found extremely high levels of Epo mRNA in the interstitial cells in the kidney of the hypoxic rhesus monkey. The normal monkey kidney showed only an occasional interstitial cell with high levels of Epo mRNA. The glomerular cells showed no mRNA for Epo. We were fortunate to have a large supply of purified recombinant human Epo, to which we had raised antibodies in rabbits. Using this Epo antibody and an immune peroxidase method, we, in collaboration with Ili Chen in the Department of Anatomy at Tulane, were able to show that these interstitial cells also contained high levels of Epo. Not surprising to us, we also found that the glomerular epithelial cells also contained immune peroxidase–positive material. However, no mRNA for Epo was found in these glomerular epithelial cells, and we concluded that the Epo was not produced in these cells but perhaps was sequestered there when high levels of Epo were contained in blood-perfusing glomeruli. We also did in situ hybridization on the liver, skeletal muscle, brain, spleen, and lung in the hypoxic monkey and did not find any detectable levels of Epo mRNA in any of these organs. We were very pleased to finish this story on the cells in the kidney that produce Epo, which was begun at the

Paterson Laboratories, Christie Hospital in Manchester, England, back in 1964 (25).

We were interested in whether reducing oxygen delivery to the kidney via renal artery constriction was effective in increasing Epo production by the kidney, because our early work, in collaboration with Jerry Crook[8], showed that angiotensin increased radioactive iron incorporation in red cells of hypophysectomized rats (34). Robert Noveck[9] also found that 5-hydroxytryptamine enhanced Epo production that could have been due to renal ischemia. Working with a former postdoctoral fellow, Luis Malgor[10], we infused angiotensin II intravenously, carefully monitoring renal blood flow in dogs, and found a relationship between reduction in renal blood flow and Epo production (35). Angiotensin infusions produced a brisk increase in plasma levels of Epo, but when the reduction in renal flow was antagonized by hydralazine, no increase in plasma Epo titers occurred (35). Alan Samuels[11] was able to produce an increase in plasma Epo levels in the dog following reduction in renal blood flow by mechanical constriction of the renal artery or angiotensin infusion (36). Thus, the mechanism of increased Epo production by angiotensin is most likely due to ischemic hypoxia due to the intense vasoconstriction in the kidney. The effects of angiotensin on Epo production was clarified further by Anagnostou et al (37) when they found that angiotensin II also increased extrarenal Epo production.

We began work on the mechanism of the anemia of end-stage renal disease (ESRD) shortly after my arrival at Tulane. Jack Stuckey, who was then Chief of Hematology at Tulane, approached me about carrying out collaborative studies with him in anemic uremic patients to study the mechanism of the anemia of ESRD. We had developed a hypothesis that the anemia of ESRD was due to the inability of the kidneys, with compromised function, to produce sufficient amounts of Epo to meet the increased demands in uremic patients for new red cell production. The mechanism of this anemia was controversial. We knew in fact that the primary cause of anemia of ESRD was due to the lack of sufficient amounts of Epo to maintain steady state erythropoiesis. However, we were impressed by the fact that in many of these patients, the levels of Epo in plasma, using an immunoassay for Epo, were almost five times higher than those in normal patients (38). For example, serum Epo levels in normal human

[8]Jerry J. Crook, MD, (1961–1962), Internist, Cartersville, GA.

[9] Robert J. Noveck, MD, PhD, (1968–1972), Instructor in Medicine and Pharmacology, Tulane University School of Medicine, Associate Director, Clinical Research Center, New Orleans, LA.

[10]Luis Malgor, MD, (1967–1968), Former Dean and Chairman, Department of Pharmacology, Universidad Nacional del Nordeste Corrientes, Argentina.

[11]Alan Samuels, PhD, (1967–1975), Private Consulting, Pharmacology, Tuckaho, NY.

subjects range between 1 and 27 mu/ml (mean 6.2 ± 4.3 mu/ml, $N = 53$), whereas serum Epo levels in patients with ESRD were between 4.2 and 102 mu/ml (mean 29.5 ± 4.0 mu/ml, $N = 36$) (38). We were not entirely in agreement with John Adamson and Joe Eschbach (39) as to the cause of the anemia of ESRD. Their contention was that chronic uremia does not alter the responsiveness to Epo (39), whereas our position has been that even though Epo deficiency is the primary cause of the anemia of ESRD, the uremic state suppresses the bone marrow response to Epo. ESRD patients had Epo titers higher than normal, yet they were still anemic. There were several possibilities, such as inhibitors of the effects of Epo on the marrow (40–45) or a refractoriness of the marrow to Epo (46). During the early 1970s, Yoshioka Moriyama[12], Juan Lertora[13], and Arvind Rege[14] provided some of the first data supporting the role of inhibitors of erythropoiesis in the mechanism of the anemia ESRD (40). They demonstrated a suppressed response of human bone marrow erythroid cells to Epo in the presence of plasma from uremic patients (41).

Two nephrologists—Richard McGonigle[15], from England, and Heinz Radtke[16], from Germany—and Juan Lertora were most important in unraveling this puzzle on the mechanism of the anemia of ESRD. McGonigle et al (42) found further support for the existence of inhibitors of the effects of Epo on the erythroid progenitor cells in the marrow of children with renal disease. Radtke et al (41) provided some support for polyamines as at least one of several uremic toxins that could be the cause of the blunted effect of Epo on bone marrow erythroid cells in uremic patients. David Kushner[17], who worked with me during this period, provided further support for polyamines as inhibitors of erythroid progenitor cells in uremic patients (44, 45). An important recent study by Allan Erslev & Anatole Besarab (46) addressed the rate and control of baseline red cell production in hematologically stable patients and anemic patients with uremia, and helped to further clarify the role of inhibitors in the mechanism of the anemia of ESRD. Despite the same or higher Epo titers, the erythrokinetic rates in the anemic uremic patients were about half the rate in

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[13]Juan J. L. Lertora, MD, PhD, (1970–1974), Chief of Clinical Pharmacology, Professor of Medicine and Pharmacology, Tulane University School of Medicine, New Orleans, LA.

[14]Arvind Rege, PhD, (1973–1982), Assistant Professor, Department of Pharmacology, Tulane University School of Medicine, New Orleans, LA (deceased).

[15]Richard McGonigle, MD, (1981–1983), Chief of Nephrology, Derriford Hospital, Plymouth, England.

[16]Heinz Radtke, MD, (1978–1979), Vice President for Research, Byk Gulden Pharmaceuticals, Konstanz, Germany.

[17]David S. Kushner, MD, (1987–1989), Neurologist, Miami Beach, FL.

normal hematologically stable individuals. They suggested that the anemia of uremia is caused, in part, by a decreased bone marrow response to endogenous Epo. The essential cause remains to be defined. Nonetheless, for the ESRD patient who is not well dialyzed, the bone marrow may show some resistance to the effects of Epo. Interestingly, ESRD patients undergoing ambulatory peritoneal dialysis maintain a significantly higher hematocrit than those on hemodialyses (47, 48). Pharmacological doses of Epo in patients with ESRD may overwhelm the bone marrow and correct both the Epo deficiency and the suppression of the erythroid cell response to Epo.

Signal Transduction Pathways in Epo Production

We became interested in oxygen sensing and the signal transduction pathways in Epo mRNA expression in 1991 after finding that L-NAME (a nitric oxide synthase inhibitor) partially blocked the increase in plasma levels of Epo in mice following their exposure to hypoxia (49), thus implicating nitric oxide in oxygen sensing and Epo production. Up until that time, we had given considerable weight to the role of cyclic AMP in the regulation of kidney production of Epo based on our collaborative work with Bill George and George Rodgers[18]; we observed a relationship between hypoxia, cyclic AMP, and Epo production (50, 51). However, Munihiko Ueno[19] and Jun Nakashima[20] found that cyclic AMP would not enhance Epo production in a hepatocellular carcinoma cell line under normoxic conditions (52, 53). Cyclic AMP did significantly enhance the effects of hypoxia in elevating medium levels of Epo in hepatocellular carcinoma cells in culture (52, 53). Studies carried out by some of my former graduate students and postdoctoral fellows in my laboratory supported a role for adenylate cyclase activation and Epo production; activators of adenylate cyclase such as beta-2 adrenergic agonists and several eicosanoids stimulated Epo production. Greg Fink[21] demonstrated several years ago that beta-2 adrenergic activation increased Epo production (54, 55) and beta-2 adrenergic antagonists (56) inhibited hypoxia-induced Epo production in vivo in mice. Wolfgang Jelkmann[22], a postdoctoral fellow working in my lab, demonstrated

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[19]Munihiko Ueno, MD, (1986–1987), Department of Urology, Saitama University School of Medicine, Saitama, Japan.

[20]Jun Nakashima, MD, (1988–1991), Department of Urology, Saitama University School of Medicine, Saitama, Japan.

[21]Gregory Fink, PhD, (1971–1975), Professor of Pharmacology, Michigan State University School of Medicine, East Lansing, MI.

[22]Wolfgang Jelkmann, MD, (1978–1979), Professor and Chairman, Physiology Institute and School of Medicine, University of Luebeck, Luebeck, Germany.

that hypoxia enhanced Epo production in rabbits following beta-2 adrenergic activation (57) and that indomethacin blocked this enhancement (58). Dennis Gross[23] (59), Vujadin Mujovic[24] (60), and Peter Kim Nelson[25] (61) demonstrated that PGE_2, prostacyclin, and 6-keto PGE_1 stimulated Epo production. It was clear from the work of Luiz Paulo[26], Douglas Wilkerson[27], and Byung Lim Roh that PGE_1 was a potent stimulus of Epo production (62). We postulated from these findings that cyclic AMP probably acted at a posttranscriptional level once hypoxia had produced an increase in Epo mRNA.

Jun Nakashima and Takashi Ohigashi[28] also demonstrated that adenosine A2 receptor activation increases Epo production in Hep3B cell cultures and in vivo in mice (63, 64). Of clinical interest, working in collaboration with George Bakris, a nephrologist at the Ochsner Foundation Hospital, we found that theophylline, a nonselective adenosine A1 and A2 receptor antagonist, produced a significant decrease in Epo and red cell production in polycythemic post–kidney transplant patients (65). In addition to increasing cAMP production, in all likelihood adenosine A_3 receptor activation leads to the activation of phospholipase C, causing an increase in diacylglycerol and activating protein kinase C (PKC). We knew for several years that G kinase activation, through the release of nitric oxide, was involved in Epo production. However, we were puzzled that the selective G kinase inhibitor Rp-8 Br-cGMPS produced only $\sim$40% inhibition of the effect of hypoxia in increasing Epo titers in the media and Epo mRNA in Hep3B cells in culture (49). In additional studies, we found that H-8, a compound that inhibits both G kinase and C kinase, almost completely blocked the effects of hypoxia in generating Epo and Epo mRNA in Hep3B cells in culture. We concluded from these studies that there must be an important signal transduction pathway other than the G kinase pathway for the expression of Epo mRNA. We then focused heavily on the C kinase pathway for the regulation of Epo gene expression. Earlier reports by Faquin, Schneider & Goldberg (66) showed that down-regulation of Epo production occurred with C kinase activation. Some of our earlier work with postdoctoral fellow

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[24]Vujadin Mujovic, MD, PhD, (1973–1975), Professor and Chairman (former Dean), Department of Physiology, University of Belgrade School of Medicine, Belgrade, Serbia.

[25]Peter Kim Nelson, MD, (1981–1985), Assistant Professor, Department of Radiology, New York University School of Medicine, New York, NY.

[26]Luiz Paulo, MD, (1970–1973), Professor of Pharmacology, Faculdada de Medicina da Universidade, Federol de Rio de Janeiro, Brazil.

[27]Robert D. Wilkerson, PhD, (1971–1973), Professor, Department of Pharmacology, Medical College of Ohio, Toledo, OH.

[28]Takahashi Ohigashi, MD, (1991–1992), Department of Urology, Saitama University School of Medicine, Saitama, Japan.

Masamichi Hagiwara[29] and Kazuhiko Nagakura[30] had shown that inhibitors of C kinase prevent Epo production. Moreover, there was a temporal difference depending on time (67); for example, early stimulation of C kinase was followed after prolonged activation of C kinase by down-regulation of C kinase activity. Wolfgang Jelkmann and his colleagues also showed an inhibition of hypoxic stimulation of Epo production with C kinase inhibitors (68). Takashi Ohigashi continued these studies and showed that Calphostin C, a specific C kinase inhibitor, significantly inhibited the increase in Epo and Epo mRNA production in Hep3B cells in response to hypoxia (T Ohigashi, unpublished observation). In addition, working with Barbara Beckman[31], Conrad Mallia[32], and Eric McGary[33], we were able to demonstrate that hypoxia selectively increases the PKCα isoform in Hep3B cells in culture (T Ohigashi, E McGary, C Mallia, I Rondon, JW Fisher & BS Beckman, unpublished observations). An antisense probe for PKCα prepared by Eric McGary showed significant inhibition of Epo production in Hep3B cells in culture (T Ohigashi, E McGary, C Mallia, I Rondon, JW Fisher & BS Beckman, unpublished observations). Therefore, it would appear that PKCα is most important in the regulation of Epo production. Kunihiko Yoshioka[34] demonstrated that G kinase plays a more selective role in the oxygen-sensing process in generating nitric oxide.

I have continued, almost uninterrupted, my research at Tulane on oxygen sensing and the regulation of Epo gene expression during my tenure as Professor, Chairman, and then Regents Professor of the Department of Pharmacology. I had to reduce my research activities during several periods, such as when I was President of the Association of Medical School Pharmacology (AMSP) (1990–1992) and Chairman of the AMSP Knowledge Objectives Committee in Pharmacology, which required developing the principles in medical school pharmacology that should be taught to medical students in all US and Canadian medical schools (1984–1994). I also served as Academic Consultant for the US Information Service to the University of Zambia School of Medicine in 1989, as well as consultant for the Pan American Health organization on four different occasions (1966, 1967, 1969, and 1971), and I assisted the Universidad

[29]Masamichi Hagiwara, MD, (1982–1984), Chief of Urology, National Tochigi Hospital, Utsunomiya Tochigi-ken, Japan.

[30]Kazuhiko Nagakura, MD, (1985–1986), National Defense Medical School, Takorozama, Japan.

[31]Barbara Beckman, PhD, (1978–1980), Professor of Pharmacology, Department of Pharmacology, Tulane University School of Medicine, New Orleans, LA.

[32]Conrad Mallia, PhD, (1991–1996), National Institutes of Health, Bethesda, MD.

[33]Eric McGary, MD, PhD, (1991–1997), Scripps Institute, La Jolla, CA.

[34]Kunihiko Yoshioka, MD, (1993–1995), Staff Urologist, Saseijai Central Hospital, Tokyo, Japan.

Nacional del Nordeste, Corrientes, Argentina, in developing their pharmacology teaching program and their research program on Epo. This Argentine program was designed to provide postdoctoral training at Tulane in pharmacology for Argentine fellows from Corrientes, in collaboration with Luis Malgor at Universidad Nacional del Nordeste. Abe Gutnisky, a physiologist in Corrientes, had applied to the NIH for a grant to provide human urinary Epo from hookworm anemia patients to the NIH under a grant from the NIH for the Epo distribution program. I was fortunate to be able to serve on the NIH Epo committee for nine years (1970–1979). The human urinary Epo from hookworm anemia patients provided under this NIH grant (managed for many years by James Stengel) through this Argentine Epo collection program was one of the few sources of Epo at that time for experimental work. This Epo was distributed at no cost to investigators all over the world for their research.

Concluding Remarks

I have thoroughly reviewed my work in pursuing the elusive hormone Epo. I retired as Chairman of the Department of Pharmacology at Tulane on July 1, 1996, and am continuing part-time in my research work on Epo. I have mentioned in my review several mentors who have had a significant impact on my career in pharmacology: LJ Klotz at Lloyd Brothers, Peter Knoefel at the University of Louisville, Robert Woodbury at the University of Tennessee in Memphis, and Lazlo Lajtha at Oxford and Manchester Universities, England. My time with each of these men changed the focus of my scientific research. I am very grateful to Tulane University for allowing me to continue my Regents endowed professorship, which includes some funds to provide a little salary and to continue to support my research. Even though I have been both pleased and honored to receive several awards for our research on Epo, notably the ASPET award in Experimental Therapeutics and the Purkinje Medal from the Czech Medical Society, I have gained most pleasure in working with my research fellows in carrying out experiments in the laboratory. I still enjoy developing hypotheses, designing experiments, generating new data, analyzing them, and deciding on future experiments to resolve unanswered questions. I rely very heavily on my two technicians, Jesse Brookins and Eugene Maulet, who have been with me for over 27 years, to assist me in the laboratory experiments in our day-to-day work. I have immensely enjoyed having a total of 62 fellows (predoctoral and postdoctoral) from all over the world (England, Germany, Argentina, Japan, Kenya, Turkey, Norway, Brazil, Korea, Poland, and of course mostly from the United States) working with me over the years on our Epo research. I am truly proud of the achievements of my research fellows, many of whom have risen to become deans, chairmen of basic and clinical science departments, faculty members in medical schools, and directors of research in the drug industry.

I still maintain contact with many of my research fellows and was pleased to receive letters from several former fellows at a retirement party given in my honor by members of my department in June 1996. I hope I can continue my research for several years yet and will do so until my health prevents me from doing otherwise.

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