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Artículo de revisión

Solving the Puzzle of Thrombopoietin

Lawrence A. Solberg Jr[1]

RESUMEN

"Thrombopoietin - at last" fue el título de un comentario que Donald Metcalf hizo en la sección de "News and Views" del número del 16 de junio de 1994 de la revista *Nature*. En ese escrito, Metcalf señaló que: "Durante décadas se sospechó la existencia de un factor vital de crecimiento de las plaquetas pero que éste se había resistido a ser caracterizado, por lo que la solución a este enigma debe ser un motivo de celebración". El comentario editorial se refería a uno de los artículos principales aparecidos en ese número de *Nature*, cuyo título era: "Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand", del que Dan Eaton era el autor principal, con la mayoría de los demás autores de la compañía Genentech, con excepción de Karl Oles, mi técnico de laboratorio y yo. El artículo se había enviado para publicación el 30 de marzo de 1994 y fue aceptado el 10 de mayo de 1994. Como ocurre con la mayor parte de los nuevos descubrimientos, la revista *Nature* promovió que otros investigadores publicaran información relacionada con el tema que motivaron la publicación de Cartas al Editor en la misma revista en el mes de junio. Lok y colaboradores y Kauschansky y su grupo publicaron el aislamiento de c-ADN murino identificado mediante tamizaje de líneas celulares mutantes autónomas para auto estimulación del receptor del c-Mpl insertado en líneas celulares. Wendling y colaboradores no clonaron el ligando de c-Mpl pero generaron información adicional de que el ligando de c-Mpl tenía las propiedades biológicas de la tan esperada trombopoyetina. En este manuscrito se describe el papel de la Clínica Mayo, de Karl Oles y mío en el descubrimiento de la trombopoyetina. Creo que se confirman los principios básicos que deben regir las actividades de investigación y las personas involucradas en la misma y me refiero a estos principios como las piezas del rompecabezas.

Palabras clave: trombopoyetina, receptor de trombopoyetina, receptor del c-Mpl.

ABSTRACT

"Thrombopoietin - at last" was the title of the News and Views article written by Donald Metcalf in the June 16, 1994 issue of Nature.[1] He went on to say "When, for decades, a vital blood-cell growth factor has been believed to exist but has resisted all efforts to characterize it, a resolution of the conundrum is a cause for celebration." The main article of the June issue was entitled "Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand˚.[2] The lead author was Dan Eaton with most other authors from Genentech except for Karl Oles, my laboratory technician, and me. The article was submitted on March 30, 1994 and accepted on May 10, 1994.[3] As is often done with major discoveries, Nature encouraged other investigators to publish letters which were accepted in June 1994.[4] Lok et al and Kauschansky et al published isolation of murine cDNA identified by screening autonomous mutants of cell lines for auto-stimulation of a c-Mpl receptor inserted into the cell lines.[3,4] Wendling and colleagues did not clone c-Mpl ligand but provided additional evidence that the c-Mpl ligand had biological properties expected of a thrombopoietin.[5,6] This article describes the role the Mayo Clinic, Karl Oles and I played in this discovery. I believe basic principles are affirmed that may prove useful for anyone engaged in research. In this article, I have referred to these principles as pieces of a puzzle.

Key words: Thrombopoietin, Trombopoietin receptor, c-Mpl ligand.

[1] ˚Ph.D., M.D. Division of Hematology-Oncology
Mayo Clinic, Jacksonville, Florida USA

Correspondence: Lawrence A. Solberg, Jr., Ph.D., M.D.
Professor of Medicine
Division of Hematology-Oncology
Mayo Clinic
4500 San Pablo Road
Jacksonville, FL USA 32224
solberg.lawrence@mayo.edu

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The first two pieces of the puzzle: Persistence and Observation

The first piece of the puzzle I found in 1967 when I started my PhD in Physiology at the University of California, Berkeley. I asked Nello Pace, the Chair of the Department of Physiology-Anatomy, for advice. He said something like "Be prepared to be persistent and to live with discouragement". I had then no idea how important persistence would become for the thrombopoietin project. Nor did I have any idea then of the importance that basing one's hypotheses on real observations from biological systems would prove. The first such observation I made at Berkeley was that sulfhydryl reagents selectively and reversibly inhibited hydrogen ion secretion in an in vitro preparation of the bullfrog gastric mucosa.[6] This observation strongly supported an hypothesis that sulfhydryl groups played a critical role in the gastric mucosal proton pump. Over 15 years later this same phenomenon would be exploited by development of proton pump inhibitors.

In 1971, I decided to attend medical school and to not pursue post-doctoral work (not recommended for basic scientists in general!). Saint Louis University Medical School needed a physiologist to teach medical students so in addition to becoming a medical student I taught basic physiology to my classmates. There I had the privilege of working with Garret Hagen, M.D. studying the transport of thyroid hormones across the blood brain barrier in dogs.[7] Dr. Hagen had trained in Internal Medicine at Mayo Clinic and in Endocrinology at Massachusetts General Hospital. Stories he told me about the Mayo Clinic were ultimately to lead me to my career at the Mayo Clinic.

The third piece: Mentors

My internal medicine training and hematology fellowship (1975-1980) were at the Mayo Clinic in Rochester, MN. One day during my fellowship, I was carrying an apheresis bag full of malignant hairy cell leukemia cells and mentioned "Someone should study these!" to Robert V. Pierre, M.D., who was Head of the Section of Hematopathology. He enthusiastically encouraged me to learn how to culture hematopoietic cells. I visited the laboratory of David Golde at UCLA in 1978 to observe the study of erythroid cells in vitro. Dr. Golde suggested I consider studying megakaryocytes because they were difficult to culture. From 1980-1982 I was away from Mayo for active duty as a hematologist at Wright-Patterson AFB in Dayton Ohio. I was fortunate to spend time with Dr. Martin Murphy who had a research laboratory studying hematopoiesis in Ohio and who also had a deep interest in thrombopoietin. He stimulated me to continue my interest in hematopoiesis. In November 1981 I wrote a letter to the leadership of the Mayo Division of Hematology successfully proposing that I receive one year of additional training with Dr. Hans Messner and Nazir Jamal in Toronto, Ontario, Canada to learn how to culture human multilineage progenitors and megakaryocytic progenitors (Figure 1).[8] In my proposal I wrote "For the anticipated study of megakaryocytopoiesis, the most unique resource at our institution is the talent and expertise in the Plummer laboratory group. Their interest in the biochemistry and genetics of Factor VIII, in protein biochemistry, in the use of immunofluorescent techniques, in the generation of monoclonal antibodies, in access to the pig colony and numerous other areas – all provides an excellent environment in which to study megakaryocytopoiesis". This was to prove to be a pivotal decision on my part because there were so many talented and helpful colleagues without whom the Mayo engagement in the thrombopoietin project could not have happened. These included Walter Bowie, Bill Nichols, and Jerry Katzmann. Ken Mann became my principal mentor. Unknown to me, the NHLBI had decided to

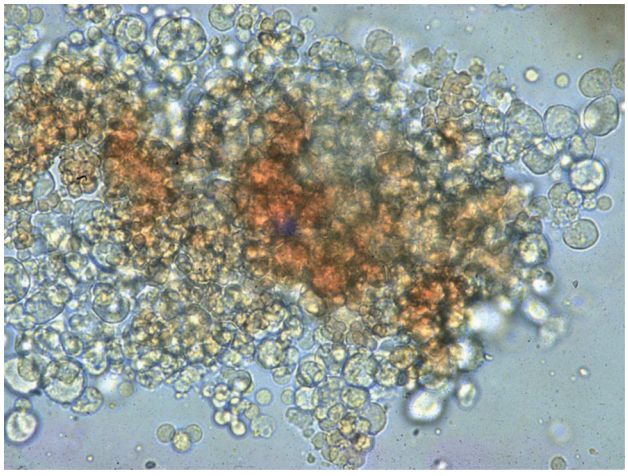

Figure 1. A colony containing erythrocytes, granulocytes, and megakaryocytes derived from a single progenitor cell and grown in methylcellulose. The megakaryocytes are the large clear cells in this colony. Photographed unstained at 250X magnification. Laboratory of LA Solberg.

stimulate more progress in isolating thrombopoietin and had generated an RFA to try and engage protein biochemists to become involved in what was largely an area of empirical, cell-culture based observations and poorly fractionated, impure solutions of colony- stimulating factors. Ken Mann had been encouraged to apply. So in 1983, Ken became the principal investigator of a grant and he involved me as a co-investigator to do human studies and Peter Quesenberrry, then at the University of Virginia, to do murine studies.

The next three pieces: a plan, an assay, and a source

Ken laid out a characteristically clear and sturdy experimental approach that was to guide the project for the next 10 years! He advised that we needed a different strategy for following growth factor activities other than tediously counting megakaryocytic colonies after 10-14 days of growth. So we developed a radioimmunoassay which measured the binding of a monoclonal antibody to human platelet GP IIb/IIIa, developed by Bill Nichols, as a surrogate for the generation and maturation of megakaryocytes.[9] Ken also stressed that if we were going to isolate anything, we needed an "inexhaustible" source of starting material. After creating a new assay and a source, the four-stage plan called for partial chemical purification of the activity from plasma followed by a last step of immunoaffinity isolation of the protein pure enough to allow N-terminal amino acid sequencing. This was an approach Dr. Mann had used successfully in isolating functional human coagulation Factor V.[10]

Finding the final source took time. Karl and I had tried identifying and establishing malignant cell lines producing thrombopoietin from patients with cancer and thrombocytosis. We made other efforts to identify a source but all such efforts had failed. It was work I was doing with Dr. E.J. Walter Bowie in the Program Project Grant in Hemostasis at Rochester that led to the most important observation that was to sustain the thrombopoietin project. Walter was studying von Willebrand disease (VWD) using a pig model. One day at Medical Grand Rounds he asked if I could transplant normal pig marrow into VWD pigs and VWD marrow into normal pigs (Figure 2). Von Willebrand factor (VWF) is synthesized by endothelial cells (plasmatic VWF) and in megakaryocytes (platelet-associated VWF), and Walter was interested in studying the phenotypic disturbance of hemostasis in pigs with only one of the

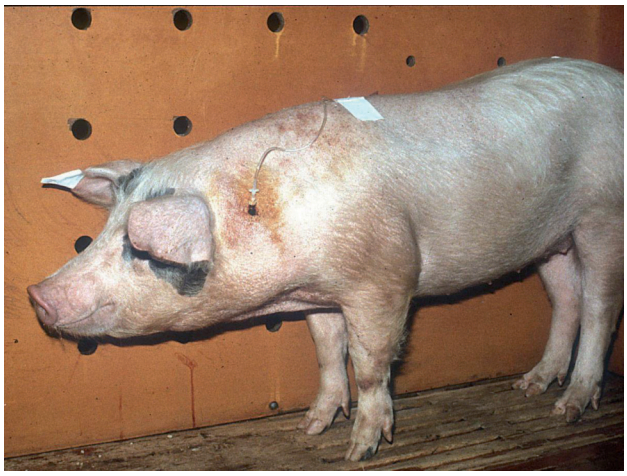

Figure 2. A normal pig receiving transplantation of marrow from a related pig with VWD. A Hickman catheter is in place. Special cages were designed to support the pigs as they received total body irradiation.

two compartments deficient. We were able to do this, but only able to transplant marrow from a normal pig into a pig with VWD.[11] As I was doing this work, I was certainly aware from my work with humans in Hans Messner's laboratory that megakaryocyte stimulating activities develop in patients receiving radiation, so I monitored the plasma from our irradiated pigs for a thrombopoietin-like activity. Bill Nichols and Jerry Katzmann helped Karl and I set up a pig radioimmunoassay that we could run in parallel to human assays, using a monoclonal antibody to porcine platelet GP IIb/IIIa.[12] What we observed and what was always reproducible and became the eventual source for the growth factor was thrombopoietin-like activity in plasma harvested from pigs 6 days after total body irradiation (Figure 3). We quickly studied all known growth factors to see if we were just observing activity from factors such as IL-3 or IL-6 but none behaved in our assay as did the pig plasma. The importance of this observation was that although we discovered this source in 1987 and were not able to successfully isolate thrombopoietin until 1993, it was the fact that we had such a source that would ultimately sustain the project. Eventually, in 1993 when the Genentech-Mayo Clinic collaboration was most active, we were irradiating over 20 pigs at Mayo Rochester and shipping several liters of plasma to South San Francisco. Mary Lou Stewart, a laboratory technician in Rochester, was essential for helping me do this from my Mayo Clinic Florida location.

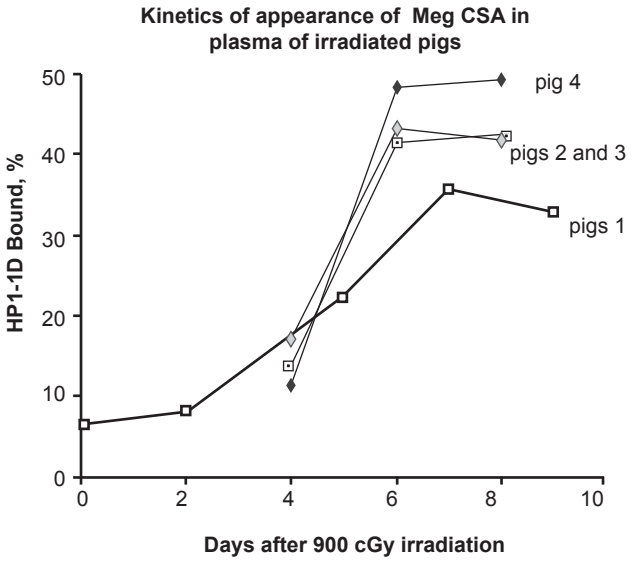

Figure 3. A graph showing the appearance in plasma from irradiated pigs of an activity (MEG-CSA) that stimulated growth of porcine and human megakaryocytic colonies in vitro. HP1-1D is a monoclonal antibody that binds to GP IIb/IIIa. The extent of this binding was measured after 10 days in culture and reflects the total number of megakaryocytes generated in vitro during that time.

The final piece: Relationships

Science is a social enterprise and relationships matter. In 1987, in response to a request from Dr. Howard Jaffe and Bharat Aggarwal, Ph.D., who were working at Genentech, Bill Nichols and I had helped establish our radioimmunoassay for human megakaryocytopoiesis in a laboratory of Marc A. Shuman, M.D. of the University of California in San Francisco. The Genentech group was working on trying to purify thrombopoietin from conditioned medium from rat kidney cells. Relationships with Marc Shuman and Dan Eaton of Genentech were to become essential as part of the final assembly of the puzzle.

Karl and I were working unsuccessfully on the next two steps of the plan: partial purification and immunoaffinity isolation. In July 1989, I asked staff at Genetics Institute to help in these final two steps but my request was not accepted. Karl and I had done pre-clinical studies on recombinant human erythropoietin in pigs for Genetics Institute. We had made the intriguing observation that when infused into pigs, human recombinant erythropoietin (EPO) stimulated thrombocytosis -and also in vitro human recombinant human EPO markedly stimulated porcine megakaryocytopoiesis. We suspected there might be some homology between thrombopoietin and erythropoietin and I had tried to entice a post-doctoral fellow at Mayo to help "clone" from the pig a gene with DNA sequence related to EPO. This was a failure.

In 1990 we made an observation that was to be a crucial in engaging the interest of Dan Eaton, Ph.D., of Genentech in our work. We had been screening plasma from patients with thrombocytopenia for years hoping to identify a patient with an antibody that might bind to thrombopoietin or to the thrombopoietin receptor. We had plasma from a patient with aplastic anemia who had developed pancytopenia after a marrow transplant and we found that his plasma contained an antibody that did not directly bind the thrombopoietin activity in our irradiated pig plasma, but which would abrogate the effect of that plasma on megakaryocyte generation while having no blocking effect on stimulation by IL-3 or other growth factors (Figure 4). We hypothesized this antibody might be against a growth factor receptor on megakaryocytes or their progenitors that was binding our thrombopoietic activity. Karl and I had been working on trying to identify and isolate this cell surface molecule -again unsuccessfully.

Then I was approached by Adair Hotchkiss, Ph.D. from Genentech at an American Society of Hematology meeting in Denver in 1990 and was invited to present my research

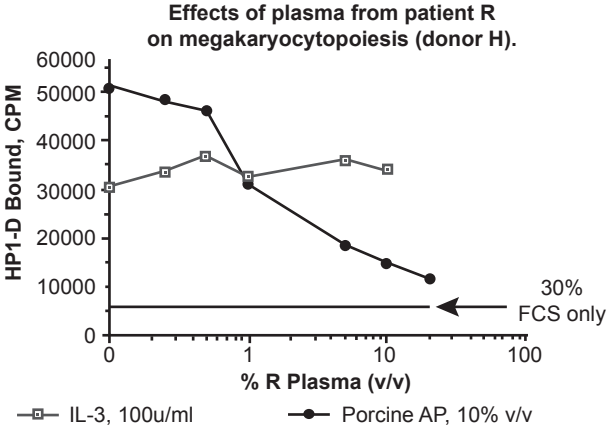

Figure 4. This graph shows the effect of adding increasing percentages of plasma from patient R (volume to volume) on the generation of GP IIb/IIIa in vitro as reflected by the binding of HP1-1D after 10 days in culture. Megakaryocytopoiesis stimulated by IL-3 was not inhibited – however, the antibody in patient R plasma clearly inhibited stimulation of megakaryocytopoiesis by the activity in the irradiated pig plasma (Porcine AP was used interchangeably with MEG-CSA in our laboratory notebooks).

work on thrombopoietin to Dan Eaton and colleagues at Genentech. What I subsequently learned was that Genentech had been working to isolate thrombopoietin from kidney conditioned cell medium -an approach that was not successful. I understand the idea for inviting me to speak at Genentech emerged from discussions involving Marc Shuman and Dan Eaton, Ph.D., a research scientist at Genentech.

I vividly remember my trip to Genentech in February 1991 because I got on the plane in Rochester MN and noticed that I had the wrong cassette of 35 mm slides! So I got off the plane, went to my lab in the Plummer Building and drove to Minneapolis in time to catch my connecting flight! I presented all my data – including the interesting possible human antibody Karl and I had identified that might interfere with the porcine thrombopoietin activity. Fortunately, Dan Eaton became interested in helping us so he started working in September 1991 on identifying the target of this antibody. Dan and colleagues later showed this antibody did react with an epitope on the extracellular domain of c-Mpl (the thrombopoietin receptor) but as the thrombopoietin project progressed, this was not to contribute to the final completion of our project.

At the ASH annual meeting in December 1992 in Los Angeles, CA, I attended a presentation by F. Wendling, N. Methia, F. Louache and W. Vainchenker reporting that antisense oligonucleotides to Mpl proto-oncogene specifically inhibited megakaryocytic differentiation (Abstract 973, ASH Abstracts, 1992;246 a). At the same meeting, V. Mignotte, S. Chretien, I. Vignon, J.P. Cartron, S. Gisselbrecht and P.H. Romeo reported on the cloning of the human c-Mpl gene (Abstract 972, page 245, ASH Abstracts 1992). I called Dan Eaton from Los Angeles and described these observations and he set out quickly to learn about c-Mpl. In that this was a putative growth factor receptor for thrombopoietin, Dan and the Genentech group cloned c-Mpl. To circumvent having to get human marrow from Dr Shuman at UC San Francisco, they created a better assay for the putative ligand for c-Mpl by creating a Mpl-dependent cell proliferation assay in Ba/F3 cells. They also generated a human Mpl-IgG fusion protein containing the extracellular domain of Mpl. To support the Genentech effort to isolate the TPO we irradiated 20 pigs in 1993 in Rochester, MN, and shipped several liters of plasma to Genentech. It was profoundly exciting when the Genentech group found that the Mpl-IgG fusion protein bound the activity from our irradiated pig plasma! The beautiful work of purifying Mpl ligand from 5 liters of pig plasma and then subsequent purification on a Mpl- affinity column is described in detail in our June 1994 Nature paper.[2]

The puzzle solved

Only looking back do I understand now how all these pieces came together such that my colleagues at the Mayo Clinic, Karl Oles and I became part of the exciting discovery of thrombopoietin. Thrombopoietin would have been discovered without us, and the insights into how real science is done as reflected in this work are not novel – but this story of solving of the puzzle of thrombopoietin may have some value to someone starting their career.

What happened to the clinical use of recombinant thrombopoietin?

Trials with recombinant thrombopoietin produced by Genentech and subsequently licensed for clinical development to Pharmacia-Upjohn did not lead to a clinically approved therapeutic product. In July 1994, scientists at Amgen reported cloning the c-Mpl ligand from canine plasma.[13] Amgen developed a pegylated form of thrombopoietin but discontinued clinical development in 1998 because clinically significant antibodies developed in recipients.[14] Interestingly, a glycosylated recombinant human thrombopoietin (TPAIO) expressed by a Chinese Hamster Ovary cell line is produced by Shenyang Sunshine Pharmaceutical Co., Ltd .in Shenyang, China and is approved for use by the China State Food and Drug Administration for the treatment of thrombocytopenia arising from chemotherapy and immune thrombocytopenic purpura.[15] New pharmacologic approaches to creating thrombopoietin mimetics allowed both romiplostim, a 14 amino acid agonist peptide and eltrombopag, a non-peptide agonist, to be synthesized[14] and eventually both to be approved by the FDA and used in current clinical practice for immune thrombocytopenic purpura and other indications.

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