

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Regulation of Thrombopoietin in Bone Marrow

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in

Biomedical Sciences

by

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ACKNOWLEDGEMENTS

Chapter 2, in full, is a manuscript that has been prepared for submission. It has been reformatted for consistency. Figures have been inserted in to the text for easy of understanding. The references have been combined with all others in this dissertation in the last section titled References. The dissertation author was the primary investigator and author of this manuscript. Ken Kaushansky, the committee chair, is the only other author on the manuscript. No particular venue for publication has been decided.

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ABSTRACT OF THE DISSERTATION

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The normal elimination of a large number of circulating hematopoietic cells from the circulation each day necessitates substantial proliferation and maturation of progenitor cells in bone marrow. An impressive array of cytokines and growth factors help to manage these complicated processes in order to maintain a physiologically appropriate number of each of the various blood cell types. Thrombopoietin is one such factor that is important for the platelet lineage. In addition, thrombopoietin participates in sustaining pluripotent stem cells and early multi-lineage progenitors. In the following report, we have chosen to focus on the prior characteristic.

It has been observed that the expression of thrombopoietin message is regulated differently in different parts of the body. Although thrombopoietin is highly expressed in the liver its expression is largely unregulated; however, a significant up-regulation is observed in bone marrow. Therefore, bone marrow stromal cells as well as a suitable cell model were subjected to treatment with serum and platelet extracts. The primary effect of both the platelet derived and non-platelet derived serum components was to down-modulate thrombopoietin message levels indicating that any physiological increase in TPO expression is likely the result of a decrease of inhibitory factors rather than an increase in a stimulatory factor. Serum was also capable of depressing TPO protein secretion. A series of reported gene constructs based on the TPO gene demonstrated that the mechanism responsible for at least a portion of the serum suppression was transcriptional repression.

CHAPTER1: INTRODUCTION AND BACKGROUND

The hematopoietic system is characterized by massive cell turnover; each day the adult human produces 4×10^{11} cells, a number that can expand up to 20-fold in times of heightened demand. The continual demand for new mature cells relies on a process of robust cell proliferation and maturation, which is controlled by both internal and external factors that work on stem and progenitor cells in the bone marrow. These cells may be conveniently viewed as being situated in an organizational hierarchy such that cells that can give rise to multiple types of differentiated mature blood cell types are located above those with fewer developmental outcomes. Thus, the cell atop the hierarchy, the hematopoietic stem cell (HSC), following a number of expansion divisions can differentiate into all of the various mature blood cell types, with cells of progressively more restricted developmental potential lie further down the pathway. Multipotent committed progenitor cells provide hematopoietic decision points that can be influenced and provide a nimble response as demands fluctuate; the cellular output at each decision point depends on the interaction of signals arising from a variety of sources. Soluble factors, cell-to-cell contacts, cell-to-matrix contacts, intracellular signaling molecules and transcription factors all contribute to the process.

Factors affecting thrombopoiesis, the process of platelet production, act primarily of the megakaryocyte and its two immediate progenitors, the megakaryocyte-erythroid precursor (MEP) and the committed colony-forming cell-megakaryocyte (CFU-MK). Megakaryocytes grow very large in the bone marrow and attain a DNA content up to 128N in mice before the cytoplasm finally fragments to form one to two thousand individual platelets. Thrombopoietin (TPO), a 45-70 kDa

glycoprotein produced in the liver, kidney and marrow stroma, is the primary soluble regulator of megakaryocyte production. In addition to TPO, a large number of cytokines have been identified as promoting megakaryocyte proliferation and development. Cytokines such as interleukin-3 (IL-3), granulocyte monocyte colony stimulating factor (GM-CSF), stem cell factor (SCF), interleukin-6 (IL-6), and interleukin-11 (IL-11) all possess megakaryocyte promoting activity. (Bruno et al. 1992; Ishibashi et al. 1989; Briddell et al. 1992; Guinan et al. 1993; Geissler et al. 1992; Broudy et al. 1995) Although each of these proteins displays only modest activity alone, they can synergize with TPO to provide finer control of platelet count. (Broudy et al. 1995) Aside from TPO, IL-11 shows the most significant and specific stimulus of thrombopoiesis, at least in humans. The injection of recombinant IL-11 into mice or humans causes a rise in platelet count of about 50%. (Du et al. 1993; Tepler et al. 1996) In contrast, treatment with recombinant TPO leads to a massive stimulation of thrombopoiesis, with platelet counts increasing in a log-linear relationship to levels 10-fold or greater than normal, or with expression in transgenic mice, or infection with a virus that directs the production of TPO. (Lok et al. 1994; Chagraoui et al. 2003; Kakumitsu et al. 2005; Harker et al. 1997; Yan et al. 1996) The strongest evidence for the physiologic role of TPO comes from the genetic manipulation of TPO or its receptor, c-Mpl. When either gene is disrupted, platelet counts fall to just ~10-20% of normal. (de Sauvage et al. 1996; Gurney et al. 1994)

Another interesting aspect of TPO that was first established in the mouse, and later confirmed in the case of the human disease Congenital Amegakaryocytic Thrombocytopenia (CAMT), is its impressive effect on the HSC. In vitro, TPO helps stem cells maintain their pluripotency. (Sitnicka et al. 1996) In addition, a group of

cells in the mouse embryo in the region where the earliest hematopoietic stem cells first arise, the aorto-gonado-mesonephros (AGM), c-Mpl expression can be observed histologically. (Petit-Cocault et al. 2007)

In *c-Mpl*^{-/-} mice, the number of progenitor cells of all types is dramatically reduced, and the repopulating ability of knockout stem cells after bone marrow transplantation is diminished nearly 10-fold. (Kimura et al. 1998) It appears that in human biology the stem cell activity of TPO is even more crucial, as mutation of its receptor in children with CAMT leads to complete bone marrow failure during the first few years of life. (Ballmaier et al. 2001; Tonelli et al. 2000; Ihara et al. 1999; van den Oudenrijn et al. 2000) Interestingly, TPO seems to be such an important factor in stem cells that in a highly enriched population of hematopoietic stem cells, all of the repopulating ability is found in the sub-fraction of cells that expresses c-Mpl. (Solar et al. 1998)

The protein structure of TPO comprises two domains; the N-terminal domain of ~160 amino acids is closely related to the functionally related hormone erythropoietin (EPO) and binds to the c-Mpl receptor. The second domain of ~180 amino acids has no known structural homologue, is highly glycosylated, and appears to function to increase the stability of TPO in the circulation (Kuter 1997) as well as to increase the efficiency of its secretion (Linden and Kaushansky 2002). Although the cDNA sequence of TPO predicts a molecular weight of about 35 kDa, the mature protein, because of the glycosylation, actually migrates from 45-70 kDa when subjected to SDS-PAGE, depending on cell of production and species of origin.

The receptor for TPO, c-Mpl, is a member of the type I hematopoietic growth factor receptor family characterized by two prototypical cytokine receptor domains in

the extracellular portion of the molecule, a single pass transmembrane domain, and a 121 amino acid intracellular domain. (reviewed in (Ihle 1995)) Signals emanating from the receptor are mediated principally by JAK2, PI3K, STAT3, and MAPK. (reviewed in (Kaushansky 2005)) Several tissues express TPO at a very low level, but expression is highest in the liver and kidneys. (Lok et al. 1994; de Sauvage et al. 1994) Hepatic production of TPO is responsible for about half of the normal baseline platelet production, as judged by reciprocal liver transplant experiments in normal and *Tpo*^{-/-} mice. (Qian et al. 1998) The regulation of TPO production has been studied in murine models. In general, TPO levels are inversely related to platelet count; levels are low in thrombocytosis and high in thrombocytopenic conditions. (Kuter and Rosenberg 1995) To address the mechanism(s) responsible for TPO production, several investigators have provoked thrombocytopenia and monitored the TPO response. For example, when mice were challenged with chemotherapy, radiation, or antiserum directed against platelets, TPO-specific mRNA levels remained constant except for in the bone marrow. (McCarty et al. 1995; Stoffel et al. 1996) From these studies and others, two, non-mutually exclusive models of TPO regulation emerged. In the first, based on the static levels of hepatic and renal TPO mRNA in the face of severe thrombocytopenia, and the capacity of platelets to adsorb and remove TPO from solution, platelet and megakaryocyte removal of TPO from the blood by surface c-Mpl was posited to serve as a TPO sink; high numbers of platelets would consume a large amount of the hormone, leaving a low concentration of TPO in the plasma, and a low number of platelet would remove less TPO, allowing the concentration to rise. (Fielder et al. 1996) With the realization that bone marrow TPO mRNA is modulated by thrombocytopenia, a second model of TPO regulation was born. The

molecular mechanisms responsible for its modulation were not defined by the currently available reports. *The regulation of TPO mRNA expression in the marrow is the subject of this doctoral thesis.*

The TPO gene contains 5 exons and is situated between the chordin and RNA polymerase II genes, with only ~4 kb separating them one from another. Transcription of the TATA-less gene TPO has been studied in liver-derived cell lines and shown to be dependent on the ubiquitously expressed Ets family transcription factor GABP α/β . (Kamura et al. 1997) Unfortunately, little else is known of the regulation of TPO expression.

CHAPTER 2: TRANSCRIPTIONAL REGULATION OF BONE MARROW TPO BY PLATELET PROTEINS

Abstract

Platelet production is regulated primarily by the cytokine thrombopoietin (TPO). Although TPO is expressed in several different tissues, only in the bone marrow has the level been reported to increase in response to reduced numbers of platelets. In this chapter it is shown that platelet proteins are able to provoke the transcriptional repression of TPO in OP9 cells as well as primary bone marrow stromal cell cultures. In addition to TPO RNA, protein secretion was also found to be suppressed by serum treatment. Reporter gene constructs suggest that DNA elements located in an approximately 1.9 kb region between 250bp upstream of the transcriptional initiation site and the middle of the second intron are able to mediate the serum repression.

Introduction

Hematopoiesis, the development of bone marrow progenitor cells into mature blood cells, is regulated by a large number of cytokines and growth factors, each with a limited and defined set of target cells. Physiologically appropriate numbers of each cell type are maintained by continual adjustments in the levels of hematopoietic growth factors. For the platelet lineage, thrombopoietin (TPO) is the primary regulator of thrombopoiesis (de Sauvage et al. 1994; Lok et al. 1994; Bartley et al. 1994; Kaushansky et al. 1994). Disruption of the *TPO* gene, or that of its receptor, *c-Mpl*, results in a dramatic reduction in the number of mature platelets produced by marrow progenitors in both mouse and man (Gurney et al. 1994; de Sauvage et al. 1996;

Ballmaier et al. 2001). As the primary regulator of thrombopoiesis, blood levels of TPO are inversely related to platelet count (Kuter and Rosenberg 1995). One mechanism that accounts for this phenomenon is the binding of TPO to its receptor, present on mature platelets (Fielder et al. 1996; Stoffel et al. 1996). Thus, when platelet levels are high, a larger quantity of TPO is removed from the blood, leading to a lower concentration of the hormone. Conversely, when platelet levels are reduced, little TPO is removed and blood levels of the hormone rises, resulting in increased platelet production. A tenet of this model of regulation is that TPO expression in the liver, the primary site of TPO production (Lok et al. 1994) is unchanged (McCarty et al. 1995). However, several reports have indicated that TPO mRNA expression in the bone marrow can increase in response to pathologic reductions in platelet levels and experimental manipulation (Sungaran et al. 1997; McCarty et al. 1995). It is well established that such paracrine production of a cytokine such as TPO would have a far greater effect on blood cell development than would its endocrine production.

In order to better understand what regulates TPO mRNA expression in the bone marrow, and the responsible molecular mechanism(s) , we used quantitative-polymerase chain reaction (Q-PCR) to measure changes in TPO mRNA expression provoked by treatment with serum and platelet proteins in model cell lines, cultured primary cells, and in vivo. Additionally, we used a modified TPO-specific Q-PCR assay to test for expression of mRNAs derived from TPO genomic DNA after its introduction into a bone marrow stromal cell line. We found that TPO is subject to transcriptional regulation in marrow stromal cells in response to released platelet proteins.

Materials and Methods

Cell culture

The murine marrow stromal cell line OP9 (Nakano et al. 1994) (kindly provided by Dr. Sanford Shattil) was cultured at 37°C in 5% CO₂ in alpha-modification of Eagle's medium (alpha-MEM), freshly prepared from powder (HyClone, South Logan, UT) every 7-10 days and containing 20% preselected bovine calf serum, penicillin, streptomycin, and supplemental glutamine. The murine hepatoma cell line Hepa1c1c7 (Bernhard et al. 1973) (American Type culture Company, Manassas, VA) was cultured at 37°C in 5% CO₂ in Dulbecco's modified essential medium (DMEM) containing penicillin, streptomycin, and supplemental glutamine. Primary bone marrow stromal cells were derived from normal murine bone marrow that had been flushed from femurs and placed directly into Iscove's modified Dulbecco's medium (IMDM) supplemented with 12.5% bovine calf serum, 12.5% horse serum, penicillin, streptomycin, pyruvate, and 100nM dexamethasone. The cells were maintained at 37°C in 5%CO₂ with weekly half media changes until only an adherent layer was present. The adherent cells were then used for up to 4 passages once confluent.

Quantitative RT-PCR

Cell cultures were treated as indicated and total RNA was purified with Trizol™ (Invitrogen, Carlsbad, CA) except for transfected cells where the RNeasy Plus mini kit (Qiagen, Valencia, CA) was used instead. To further reduce DNA contamination, RNA samples were subjected to DNase (Roche, Indianapolis, IN) digestion and ethanol precipitation. When collecting bone marrow RNA, femurs first flushed lightly with several milliliters of low serum medium applied through a 25 ½ G needle. Two femurs from each mouse were then immersed together in one tube in

750µl Trizol. To complete the stromal cell solubilization, the Trizol™ was passed through the empty bone cavities with a 27 ½ G needle, and the marrow cavity scraped with the tip of the needle. A real-time-reverse transcriptase (RT)-PCR was then conducted on an iCycler (BioRad, Hercules, CA) thermal cycler using Taqman™ assay chemistry. The RT reaction (20µl) was performed with MMLV RT (50U) at 37°C for 1 hr with random hexamer primers after an initial 20 min room temperature pre-incubation. The PCR reaction (40µl) contained 15mM Tris [pH 8.0], 50mM KCl, 0.3mM dNTPs, 1 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), and 250µM of each primer. Magnesium and FAM-TAMRA labeled probes were added as indicated in Table1. The PCR protocol was: 95' for 10 min followed by 50 cycles of 95°C for 15 sec and 60°C for 1min.

Table 1.1

Gene/Acc#	Primers/Probe	MgCl₂ (mM)
Thrombopoietin (TPO) NM_009379.3	L: cccatacaggagccactt R: gacagagttagtcttgccactgcaa Prb: actgattgctcctggcggccat Mutant prb: tggagctgactgatttactactagcagcaatgc	3.0
Cyclophilin (CYC) NM_008907	R: gtctccttcgagctgtttgc L: tggcacatgaatcctggaata Prb: ttcgagctctgagcactggagaga	4.0
Bone sialoprotein (BSP) L20232.1	L: ggcgatagttccgaagagg R: cccgagagtgtgaaagtgt Prb: aggcggaggcagagaacgcc	2.0
Firefly luciferase (from pGL3, Promega)	F: ggttgtggatctggat accg R: cgcttcgattgtttacat Prb: aggcgaactgtgtgagaggctccta	3.0
18S NR_003278.1	L: cgcggttctatttgttgg R: caaatgcttctgctctggtc Prb: ggcattcgattgcgccgct	2.0

Platelet-rich and platelet-poor plasma-derived serum

TPO receptor (c-Mpl) null mice were backcrossed onto a C57/Bl6 background for greater than 10 generations (kindly provided by Dr. Warren Alexander, Melbourne, Australia). Whole blood was collected into acid-citrate-dextrose (ACD) by cardiac puncture of *c-Mpl*^{-/-} and C57/Bl6 control mice. Samples without evident clotting were pooled and centrifuged at 800xg for 15 min to generate platelet-rich plasma. Half of each sample was then centrifuged at 1500xg for 10 min to generate platelet-poor plasma. Plasma-derived serum was obtained by adding CaCl₂ to 100mM, HEPES buffer pH7.5 to 100mM, and 0.5 U/ml thrombin (Sigma-Aldrich, St. Louis, MO) and then incubating at 37°C for 30 min. Fibrin clots were disrupted by passage through a 27 ½ G needle and removed by centrifuging at 15,000g for 5 min. The samples were dialyzed against phosphate buffered saline (PBS) overnight, and the final protein concentrations adjusted to 20 mg/ml with PBS.

Metabolic labeling

OP9 cells were grown to confluency. The medium was then replaced with methionine- and cysteine-free medium containing 20% dialyzed bovine calf serum and incubated for one hr. The medium was then replaced with fresh methionine- and cysteine-free medium (MP Biomedicals, Solon, OH) containing 20% or 0.5% bovine calf serum and incubated for an additional hr, at which time 1.5mCi of a mixture of ³⁵S-labeled methionine and cysteine (MP Biomedicals, Solon, OH) was added to each T-75 culture flask (BD Bioscience, Bedford, MA) containing 7ml of medium. After 8 hr further incubation in a container packed with a large volume of activated charcoal, the culture supernatants were collected. Serum or PBS was added to standardize the

medium composition, and trichloroacetic acid (TCA) precipitable counts were measured by liquid scintillation counting. An equal quantity of cpm was combined with the primary biotinylated precipitating reagents (anti-TPO antibodies or soluble c-Mpl receptor, R&D Systems, Minneapolis, MN) and incubated at 4°C overnight with rocking. Streptavidin-agarose beads (Cedarlane Laboratories, Burlington, Canada) were added and incubated for 2 hr. The agarose beads were collected by centrifugation and washed several times in PBS. The bound protein was eluted with 1X sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer and separated under standard PAGE conditions. Before drying, the gel was soaked in ENLIGHTNING™ autoradiography enhancer (Fischer Scientific, Pittsburgh, PA) for one hr. Finally, the dried gel was exposed to film.

Subcloning of murine TPO genomic DNA by homologous recombination

Subcloning was achieved with the RED™/ET™ BAC Subcloning kit (Gene Bridges, Dresden, Germany). The protocol consisted of first transforming a bacterial artificial chromosome (BAC) clone known to contain TPO genomic region (RP23-187G15) with the pSC101-BAD-gbaA plasmid, which contained the recombination enzymes, and selecting for transformants at 30°C. The recombination enzymes were induced with arabinose for 1 hr. The cells were then electroporated with the subcloning vector pColE1-Amp that had been previously prepared by PCR to incorporate ends 50bp of sequence identical to the TPO gene (specifically, at the 5' end of TPO: gattgctcaatgatcctcttctagtctctaccactatacatgtgacaagag, and at the 3' end of TPO: gaatctcagtgctcagtttctatgtgacagagaccttcatcttctaattct). The subclones were selected on ampicillin containing plates at 37°C and screened by restriction digest.

DNA reported constructs

The BAC subclone containing 7.7 kb of TPO genomic DNA served as the basis of two series of constructs, pmutTPO and pT+L. The pmutTPO series of constructs was created by using Quikchange™ (Stratagene, La Jolla, CA) mutagenesis to introduce 5 single bases changes that preserve the reading frame and splicing signals. The pmutTPO-250pro construct was the result of restriction digest at the immediate 5' end of the cloned genomic fragment with SpeI and a XbaI site 1 kb downstream, followed by blunting and re-circularization. The pmutTPO-3'del construct was produced in the same manner with a XhoI at the 3' end of the TPO gene and a AgeI site incorporated at the immediate 3' end of the subcloned fragment. The 4th intron was removed cutting with AatII in exon 4 and SbfI in exon 5 and replaced with the corresponding cDNA fragment to produce the pmutTPO-din4 construct. The firefly luciferase cDNA followed by an SV40 polyadenylation sequence was removed from pGL3-basic (Promega, Madison, WI) at the HindIII and BamHI sites, blunted, and inserted into the EcoRV site in the 1st intron of the TPO gene. The pT+L-dBam and pT+L-dPst were subsequently produced by digestion the BamHI site (2860bp) or the PstI site (5100bp) and a AgeI site at the immediate 3' end of the subcloned fragment, followed by blunting and re-circularization.

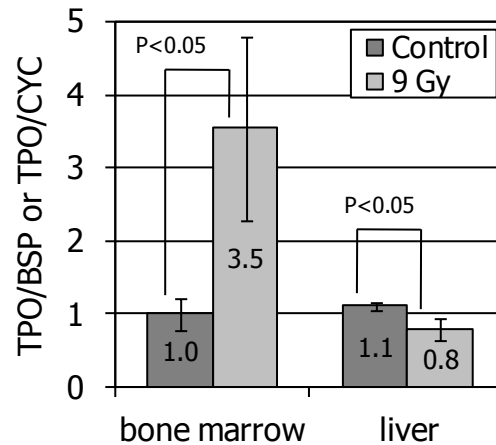
Results

Bone marrow Tpo mRNA levels are regulated by thrombocytopenia

In order to confirm earlier reports of the selective up-regulation of bone marrow TPO mRNA levels in response to myelosuppression, a Q-PCR assay was

developed. The results (Graph 2.1, left) demonstrate that an increase in TPO-specific mRNA can be observed 9 d after having received 9 Gy of γ -radiation, a point at which thrombocytopenia is severe. In contrast, the TPO mRNA levels in the liver decreased slightly (Graph 2.1, right). These results are consistent with those reported previously (McCarty et al. 1995).

Graph 2.1: TPO expression in murine bone marrow and liver. Mice were irradiated with 9 Gy of γ -radiation. Nine days later, Q-PCR was used to determine expression of TPO-specific mRNA in bone marrow stromal cells as well as in the liver. TPO transcript levels were normalized to bone sialoprotein (BSP) expression in marrow cells and to cyclophilin (CYC) in the liver. The results are expressed as the mean value of 4 independent experiments \pm SEM.



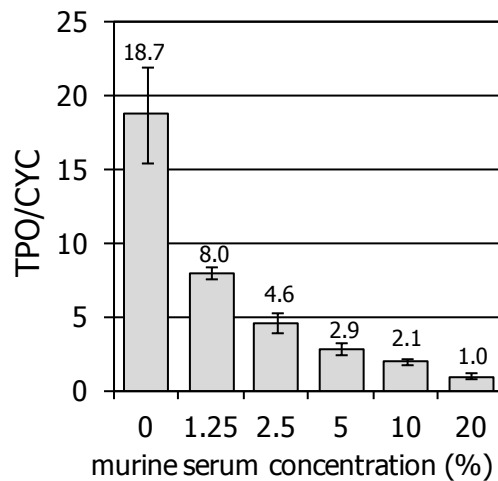
Platelet products regulate Tpo-specific mRNA expression in marrow stromal cells

To investigate the role that soluble products of platelet release or blood coagulation might play in the regulation of TPO expression, cultured primary murine bone marrow stromal cells and model stromal cell lines were treated with varying concentrations of normal serum. OP9 cells were selected as a model of bone marrow stromal cells based on their ability to support the *in vitro* differentiation of ES cells toward hematopoietic lineages (Feugier et al. 2005). The murine hepatoma derived cell line, Hepa1c1c7, was selected to represent liver cells. Interestingly, the main effect of normal murine serum on the OP9 cells was found to be suppression of TPO-specific transcripts (Graph 2.2a), whereas in Hepa1c1c7 cells, TPO expression was stimulated by serum treatment (Graph 2.2b). The suppressive ability of normal

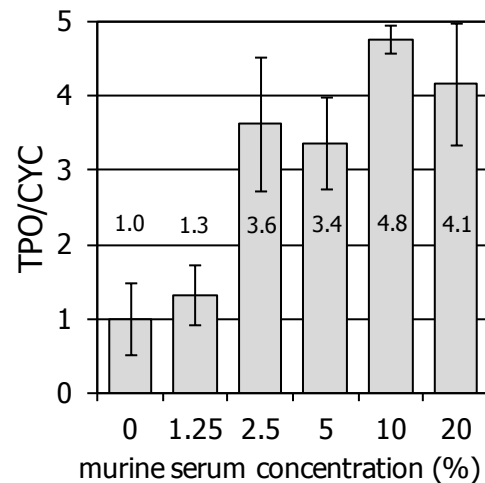
murine serum was likewise matched by bovine calf serum in OP9 cells (Graph 2.2c) and primary bone marrow stromal cells (Graph 2.2d).

Graph 2.2: TPO expression in cultured primary cells and cell lines in response to serum. Two cell lines, OP9 (A) and Hepa1c1c7 (B) were treated with increasing doses of murine serum for 10 hr. OP9 cells (C) and primary murine bone marrow stromal cells (D), were treated with bovine calf serum for 10 hr. The results are expressed as the mean value of 4 independent experiments +/- SEM.

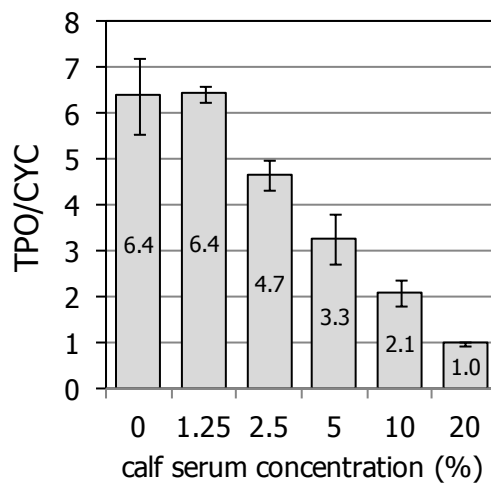
A. OP9



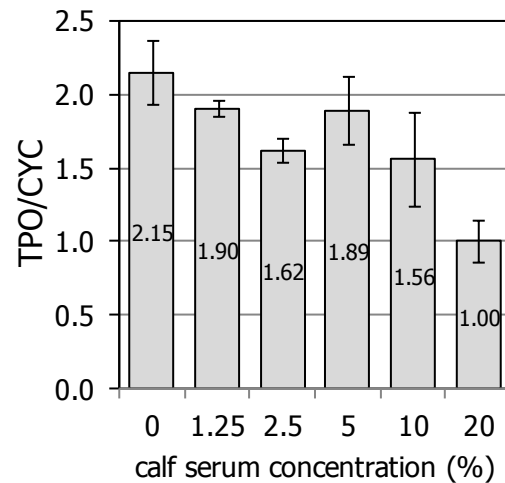
B. Hepa1c1c7



C. OP9



D. Bone marrow stromal cells



Having demonstrated the effect of serum on TPO mRNA levels, it was important to also demonstrate equivalent effects on TPO protein production. In previous reports of bone marrow TPO expression, no attempt was made to link

mRNA to protein levels. Unfortunately, OP9 cells do not secrete sufficient TPO in the culture supernatant to be determined by the available ELISA assays, nor could the culture supernatant be concentrated sufficiently owing to the 20% serum already present in the culture medium. Simple immunoprecipitation was also precluded by the cross-reactivity of bovine TPO and IgG in the calf serum. Metabolic labeling of proteins in OP9 cells was undertaken to establish the concordance of RNA and protein production. Since it was serum deprivation that led to an increase in TPO production, the time to observe the increase in protein was found to be limited to just 8 to 10 hours. Both an anti-murine TPO antibody (Figure 2.1, left) and a soluble form of the TPO receptor (Figure 2.1, middle) were able to pull down a ^{35}S labeled protein of the appropriate M^r . Metabolic labeling of BHK cells that over-express recombinant murine TPO provided a suitable positive control immunoprecipitation (Figure 2.1, right).

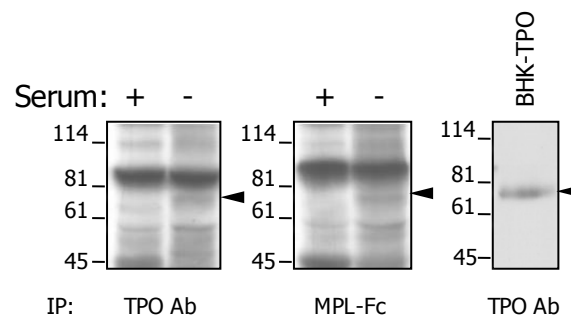
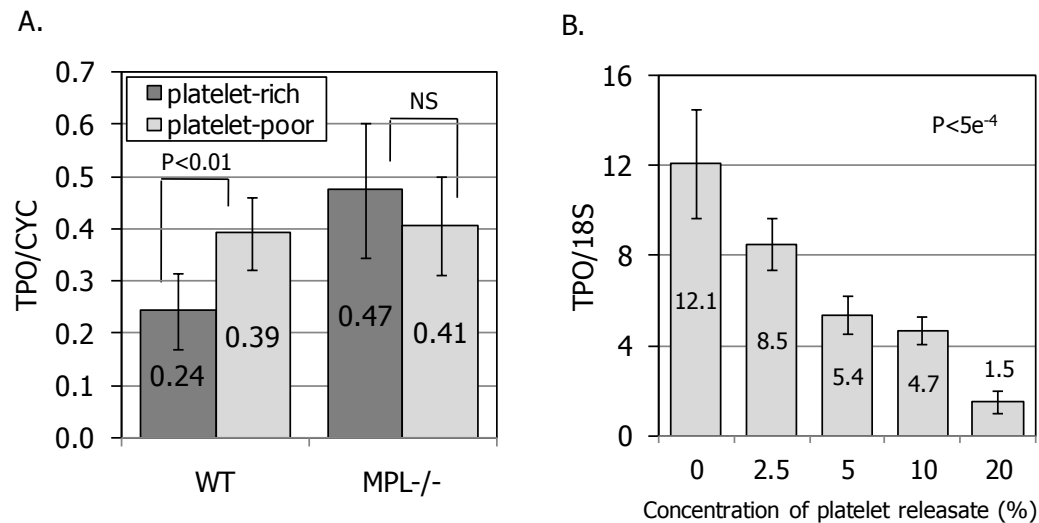


Figure 2.1: Metabolic labeling of proteins in OP9 cells. Cells were labeled with a mixture of ^{35}S -cysteine and ^{35}S -methionine for 8 hr in the presence of 20% (+) or 0.5% (-) bovine calf serum. Labeled proteins were precipitated from culture supernatants using either an anti-TPO antibody (left) or a soluble TPO receptor (middle). As a control, labeled TPO was precipitated from the supernatant of BHK cells over-expressing recombinant murine TPO (right).

Serum is a complex milieu of proteins, lipids, and other small molecules. The simplest explanation of the serum suppression would be that TPO in serum suppresses bone marrow production through feedback inhibition. However, recombinant TPO was unable to suppress TPO mRNA levels in OP9 or marrow stromal cells (data not shown). In addition, bone marrow TPO mRNA is elevated in settings where endogenous levels of TPO are known to be high, such as following myelosuppressive therapies or in other thrombocytopenic conditions (Kuter and Rosenberg 1995). To further explore the component(s) of serum that suppresses TPO production we tested serum from thrombocytopenic *c-Mpl*^{-/-} mice, which display extraordinarily high levels of TPO (Gurney et al. 1994). We found that *c-Mpl*^{-/-} mouse derived serum suppresses TPO production less well than that of control murine serum (Graph 2.3).

Many of the important growth factors in serum that help to maintain cells in culture are platelet derived proteins released during blood clotting. The critical role that TPO plays in platelet production prompted experiments aimed at quantifying the suppressive ability of platelet proteins relative to plasma proteins. The results illustrated in figure 4a and 4b demonstrate that platelet proteins are able to suppress TPO expression. First, thrombin activated human platelet releasate is able to inhibit TPO expression (Graph 2.3b). In addition, serum produced by thrombin activation of platelet-rich plasma reduces TPO expression more than serum produced from platelet-poor plasma (Graph 2.3a), an effect that is absent in samples from *c-Mpl*^{-/-} mice, likely due to their severe thrombocytopenia. These results strongly suggest that thrombocytopenia stimulates marrow TPO expression not by increasing a stimulatory factor, but rather by reducing a negative regulatory factor.

Graph 2.3: The effect of platelet factors on the expression on TPO. A. OP9 cells were treated for 10 hr with serum derived from thrombin activated plasma that was either platelet-rich (dark bars) or platelet-poor (light bars). The results represent the mean relative TPO mRNA levels of 7 samples from 2 separately prepared serum batches and independently executed experiments +/- SEM. In order to minimize the effect of factors not released from platelets, both platelet-rich and platelet-poor serums were produced from the same batch of murine blood collected into ACD. B. OP9 cells were treated with increasing doses of thrombin-activated human platelet releasate. The platelet concentration was 400/ μ l, the same as normal human blood. The results represent the mean TPO mRNA levels in 3 independent experiments +/- SEM.



Thrombopoietin is regulation transcriptionally

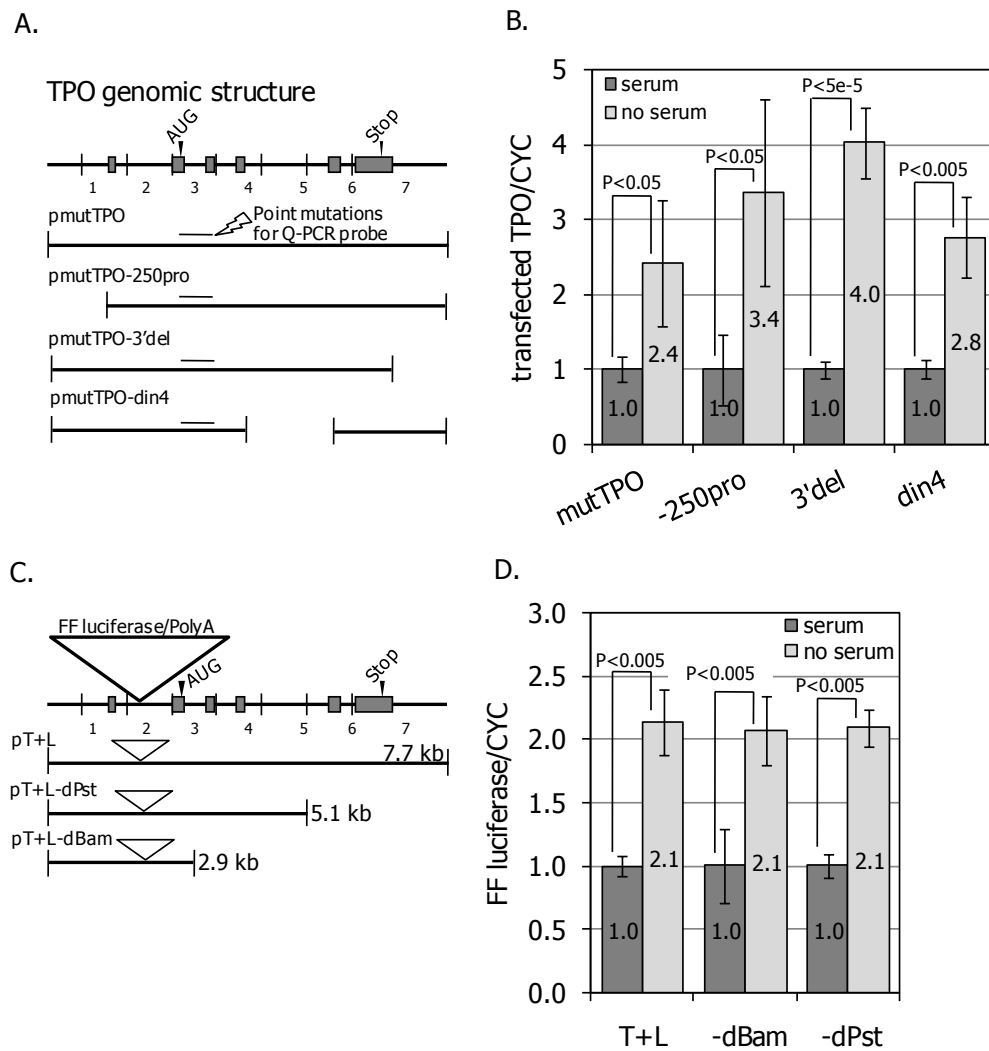
The molecular mechanism(s) that underlies the serum-induced changes in TPO expression was next investigated by introducing TPO DNA constructs into OP9 cells and evaluating the changes in transgene expression levels resulting from serum treatment by Q-PCR. The first set of constructs (Graph 2.4a) are deletion mutants of a 7.7 kb fragment of genomic DNA that contains all the exons of murine TPO. The 5' most 1 kb of DNA has been deleted in the pmutTPO-250pro construct, leaving only 250 bp upstream of the transcriptional initiation site. The last 900 base pairs have

been deleted from the 3'del construct. The 4th, and largest, intron of TPO was deleted from the pmutTPO-din4 construct. The expression of specific transcripts from this series of constructs was distinguished from endogenous TPO by the incorporation of a 5 bp change that preserves the reading frame and splicing signals, but to which a corresponding Q-PCR probe anneals. Thus, a Q-PCR signal is only detected in transfected cells, but not in non-transfected controls. As shown in Graph 2.4b, serum induced suppression of TPO expression is maintained in each of these constructs, indicating that none of the deleted regions contain required cis-acting sequence element(s) responsible for TPO regulation.

A second set of reporter gene constructs was then generated inserting the firefly luciferase cDNA and polyadenylation signal into the first intron of the TPO gene, downstream of the normal TPO transcriptional initiation site. The resulting message would be expected to terminate at the polyadenylation sequence incorporated with the luciferase gene and contain no TPO RNA sequence 3' to the insertion site in intron 1. The expression of luciferase mRNA is thus driven by TPO genomic sequences and can be followed by Q-PCR for luciferase. Based on this construct, a series of successive deletions of the 3' end of TPO were generated (Graph 2.4c). As shown in Graph 2.4d, none of the constructs was able to completely abrogate serum-induced repression. Therefore, the shortest construct (pT+L-dBam, 2860bp) contains sequence element(s) sufficient to cause serum suppression of TPO in bone marrow cells. Taking into account the first series of constructs (Graph 2.4a) in which the pmutTPO-250pro construct was able to be regulated normally, the most likely region to contain all necessary serum repressive sequence elements lies between 1000bp and 2860bp. This series of reporter gene constructs also indicates

that at least some portion of serum-mediated TPO suppression is mediated by changes in transcription, because essentially no TPO sequences on which mRNA stability mechanisms might act are incorporated into mature luciferase messages.

Graph 2.4: Expression of transcripts derived from TPO genomic DNA constructs. A. A TPO genomic construct was generated encompassing ~7.7 kb of DNA as illustrated. A Q-PCR was developed in which the probe was complimentary to 5 silent point mutations that allowed mRNA expression derived from the DNA construct to be distinguished from that of endogenous TPO-specific mRNA (shown as arrow). A series of deletion mutants was also generated. B. About 40 hr after transfection of the TPO DNA constructs, the OP9 cells were treated with or without bovine calf serum for 10 hr and cells harvested for Q-PCR. The levels of construct specific mRNA are shown from 4 independent experiments +/- SEM. C. A second series of TPO gene constructs that include a firefly luciferase reported genes and corresponding polyadenylation signal in the first intron of TPO was generated. D. The effect of serum on luciferase gene expression in the presence or absence of bovine calf serum was measured by Q-PCR. The results represent the mean luciferase mRNA levels adjusted for cyclophilin levels is shown from 4 independent experiments +/- SEM.



Discussion

Quantitative RT-PCR demonstrates that bone marrow TPO mRNA expression is increased in response to experimentally induced thrombocytopenia. Treating cultured primary stromal cells and OP9 cells with serum supports the conclusion that the elevation of TPO RNA results from the disruption of a normal negative feedback loop, rather than the action of stimulatory factors generated during the thrombocytopenia. Platelet derived proteins seem to account for a significant portion of the suppression of TPO transcripts because serum derived from platelet-rich plasma suppresses more than that of platelet-poor. In addition, the retention of suppressing activity by dialyzed serum indicates that the responsible factors are proteins of greater M_r than 10kDa. In contrast, the same stimuli seem to cause TPO expressed in the liver or a liver derived cell line to move in the opposite direction, which is most likely due to the general suppression of gene expression seen in most cultured cells deprived of the growth promoting action of serum. The enhanced TPO expression was also confirmed to occur at the level of secreted protein by metabolic labeling.

As shown in Graph 2.4, a 1.9 kb region between 250 bp upstream of the transcriptional initiation site and the middle of the second intron of the TPO gene appears to contain genetic elements sufficient to mediate serum-induced suppression of TPO gene transcription. One notable transcription factor for which 5 predicted binding sites (Akiyama; Heinemeyer et al. 1998) are found within the region between 1000bp and 2860bp is δ -crystallin enhancer factor-1 (δ EF-1). This transcription factor is widely expressed and has been shown to translocate to the nucleus in response to serum (Franklin et al. 1994). δ EF-1 generally acts to down-modulate gene

expression (Jethanandani and Kramer 2005; Shirakihara et al. 2007; van Grunsven et al. 2003; Postigo 2003; Ponticos et al. 2004; Postigo et al. 2003; van Grunsven et al. 2001; Sekido et al. 1994; Murray et al. 2000; Sekido et al. 1997) Of particular interest, previous reports indicate the importance of δ EF-1 in contributing to the suppression of osteoblastic genes such as collagen Ia and osteocalcin (Terraz et al. 2001; Sooy and Demay 2002). The association of δ EF-1 with osteoblasts is interesting because evidence suggests that TPO expression in the bone marrow occurs primarily in osteoblasts. (Dr. Toshio Suda personal communication) In fact, during the course of the current investigation a variety of methods for the detection TPO in bone marrow by Q-PCR were attempted, and only after flushing away the hematopoietic marrow components and extracting the RNA from cells lining the endosteal surface of the bone was TPO able to be detected (data not shown). The expression of TPO by bone marrow osteoblasts is relevant considering the recent attention that endosteal osteoblasts have garnered as the stem cell niche (Calvi et al. 2003; Zhang et al. 2003) and the well-known role of TPO in supporting stem cells (Ballmaier et al. 2001; Solar et al. 1998; Sitnicka et al. 1996).

When considering osteoblasts as the location of TPO expression in the bone marrow, a body of literature concerning the interconnection of osteoblast, osteoclast, and megakaryocyte development may be enlightening. Osteoblasts and osteoclasts participate in a well-developed interrelated regulatory network of cytokine and growth factors, each inhibiting the development of the other and stimulating their own (for a review see(Boyce and Xing 2007)). Interestingly, megakaryocytes have been recognized as a component of this interplay (reviewed by (Kacena et al. 2006)). A large number of reports indicate that megakaryocytes stimulate osteoblastogenesis

and inhibit osteoclastogenesis. (Chagraoui et al. 2003; Bord et al. 2000; Bord et al. 2004; Bord et al. 2005; Miao et al.; Kacena et al. 2006; Kacena et al. 2004; Thiede et al. 1994) Thus, one might postulate that osteoclasts, which while acting to inhibit osteoblast development, might also act indirectly to inhibit megakaryocyte growth by secreting factors that suppress TPO expression and inhibit megakaryocyte development.

With regard to potential platelet derived serum factor that may suppress TPO, one report demonstrated that transforming growth factor- β 1 (TGF- β 1) and platelet factor-4 (PF-4) were able to suppress TPO expression in human bone marrow cultures (Sungaran et al. 2000). In addition, insulin and insulin-like growth factor (IGF-I) have been shown to induce δ EF-1 expression (Alemany et al. 1989). In actuality, the possibilities are substantial considering that platelets are known to absorb a variety of non-platelet proteins from the circulation, which they are then able to release in abundance when activated.

We conclude that when attempting to understand mechanisms that might explain aberrant platelet production the regulation of TPO in the bone marrow must be considered.

ACKNOWLEDGEMENTS

Chapter 2, in full, is a manuscript that has been prepared for submission. It has been reformatted for consistency. Figures have been inserted in to the text for easy of understanding. The references have been combined with all others in this dissertation in the last section titled References. The dissertation author was the primary investigator and author of this manuscript. Ken Kaushansky, the committee chair, is the only other author on the manuscript. No particular venue for publication has been decided.

CHAPTER 3: THE EFFECT OF PLASMA ON BONE MARROW TPO EXPRESSION

Introduction

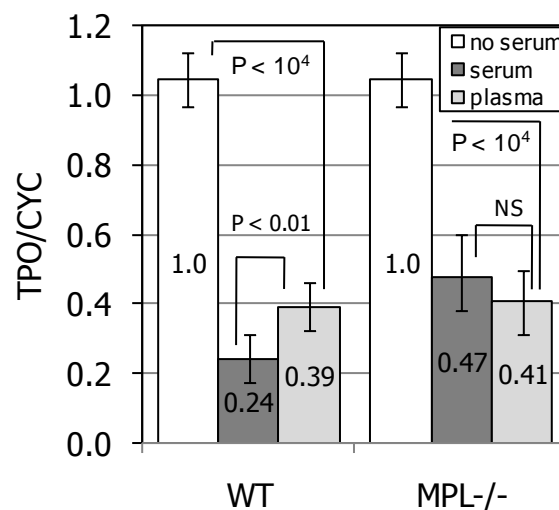
In assessing the serum-mediated suppression of TPO mRNA it was important to first determine which major fraction of serum contained the responsible factor(s), the platelet derived fraction or the plasma fraction. The approach was to compare the effect of serum and plasma on TPO expression in OP9 cells, as they differ in the presence or absence of platelet derived proteins, respectively. Because plasma would clot if placed in calcium-containing medium, it first needed to be incubated with thrombin to cleave and clot the fibrinogen to allow its removal as insoluble fibrin. Another key to understanding any difference between plasma and serum was to pool carefully collected blood samples and to only separate the fractions when absolutely necessary, so that platelet activation was the sole variable. Therefore, platelet-rich plasma was first generated by a low speed centrifugation and then divided into two equal parts, one of which was centrifuged a second time at higher speed to remove platelets. The platelet-rich and platelet-poor plasmas were then activated with thrombin and centrifuged to remove the fibrin. Acid citrate dextrose (ACD) was chosen as the anticoagulant because its effect could be reversed by the addition of neutral buffer and calcium before the thrombin activation.

Results and discussion

The quantitative RT-PCR results shown in Graph 3.1 demonstrate that both the platelet factors as well as plasma factors are able to suppress TPO expression from stromal cells relative to no serum. Samples from *c-Mpl* knockout mice were included because these mice have severe thrombocytopenia, and the contribution

made by platelets should be minimal. The difference between platelet-rich plasma-derived serum and platelet-poor plasma-derived serum is abrogated in *c-Mpl*^{-/-} mice, thus confirming that the difference seen in wild-type samples truly represent the effect of platelet-derived substances.

Graph 3.1: The effect of serum versus plasma on TPO expression. Blood was collected into acid citrate dextrose (ACD) from 5-6 WT mice and 5-6 *MPL*^{-/-} mice. Samples were pooled. After low speed centrifugation, the platelet rich fraction was divided into two parts. The platelets were removed from one half before thrombin activation (light bars; plasma). The platelet rich half was activated with thrombin as well (dark bars; serum). Results represent mean of 7 different RNA samples from 2 separately prepared blood samples and 2 independently preformed experiments. Statistics by student's t-test.



Methods

Refer to chapter 2. No new methods were used in this chapter.

CHAPTER 4: ALTERNATE HYPOTHESES OF TPO REGULATION

Introduction

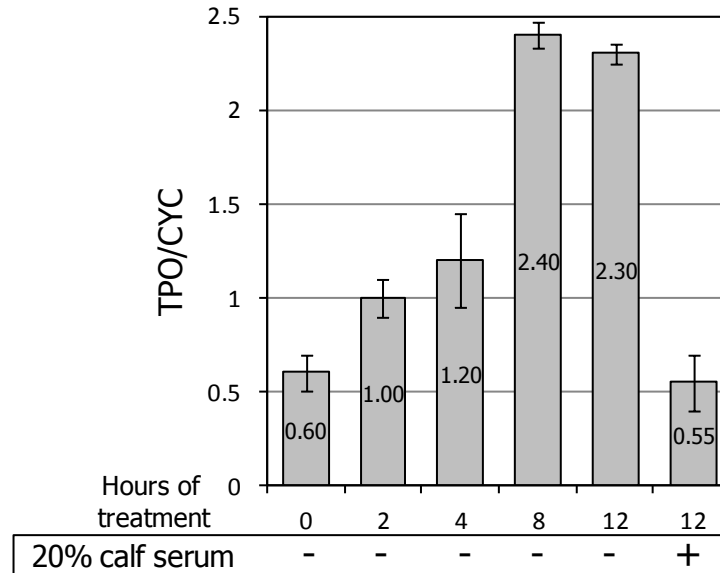
Two additional tests of what might regulate TPO production were conducted during the tenure of my graduate studies. It was formally possible that a simple feedback loop of TPO protein inhibiting TPO mRNA production existed. The effects of heparin interacting with the serum factors that suppress TPO production were tested because this polyamine has been shown to affect the activity of a large range of biologically active proteins, and the demonstration of an effect would aid in identifying a specific mediator of TPO production.

Results and Discussion

The TPO gene is not regulated by its product

The simple hypothesis that TPO protein feeds back to shut off its own expression in the bone marrow was one of the first tested. Starved OP9 cells were incubated in the presence of recombinant TPO and assayed for TPO mRNA by Q-RT-PCR. The result shows that TPO does not feedback to inhibit its own expression in the marrow. (Graph 4.1) This result was not surprising, as in the presence of thrombocytopenia, TPO levels are high, and if blood TPO concentration was to inhibit marrow TPO production, the thrombopoietic response would be blunted.

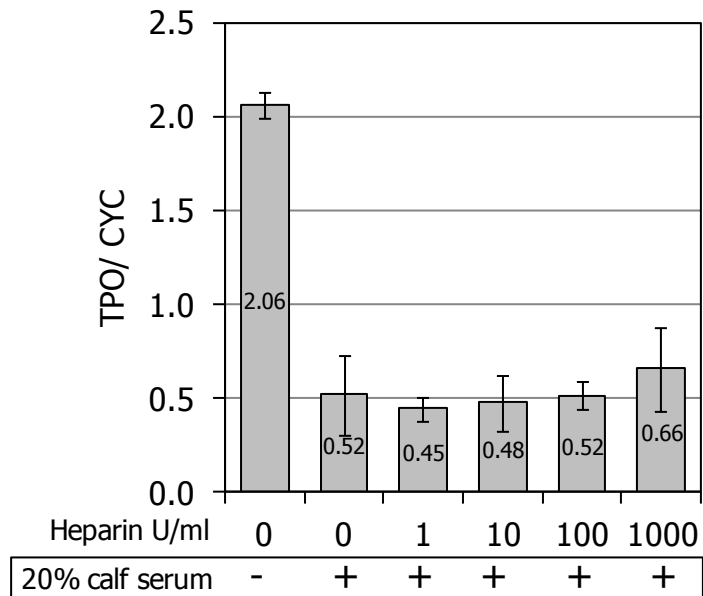
Graph 4.1: OP9 cells treated with recombinant TPO. Cells were incubated for 8hrs with 50ng/ml recombinant human TPO was used. Results represent 3 independent experiments. Mean +/- SEM.



Heparin does not enhance the suppressive factors in serum

Previous observations have shown that the effect of certain growth factors can be enhanced (e.g. FGF-2) (Kan et al. 1993; Ornitz et al. 1995; Faham et al. 1996) or diminished (TGF- β 1) (Han et al. 1996) by interacting with heparin. To test if the TPO-suppressive serum factor could be enhanced by heparin OP9 cells were incubated with serum and a range of heparin concentrations. However, heparin concentrations up to 1000U/ml failed to further suppress TPO production. (Graph 4.2)

Graph 4.2: OP9 cells treated with heparin. Cells were incubated for 8 hr in the presence of serum and increasing concentrations of heparin. The results are from 3 independent experiments. Mean \pm SEM.



Methods

Refer to chapter 2. No new methods were used in this chapter. Heparin was purchased from Sigma-Aldrich (St. Louis, MO).

CHAPTER 5: CONSIDERATION OF POTENTIAL EFFECTS OF TRANSLATION ON TPO TRANSCRIPT STABILITY

Introduction

Because an increase in TPO expression could be the result of two possible mechanisms, enhanced stability of TPO-specific transcript or increased transcription initiation, it was hypothesized that transcript stability may be behind the observed change in TPO mRNA levels. Numerous attempts under a variety of conditions were made to investigate the rate of TPO-specific message decay under serum-starved and serum-replete conditions, by employing several different chemical inhibitors of transcription. However, the inhibitors resulted in significant toxicity when combined with serum starvation, causing widely varying results. No credible conclusions could be reached.

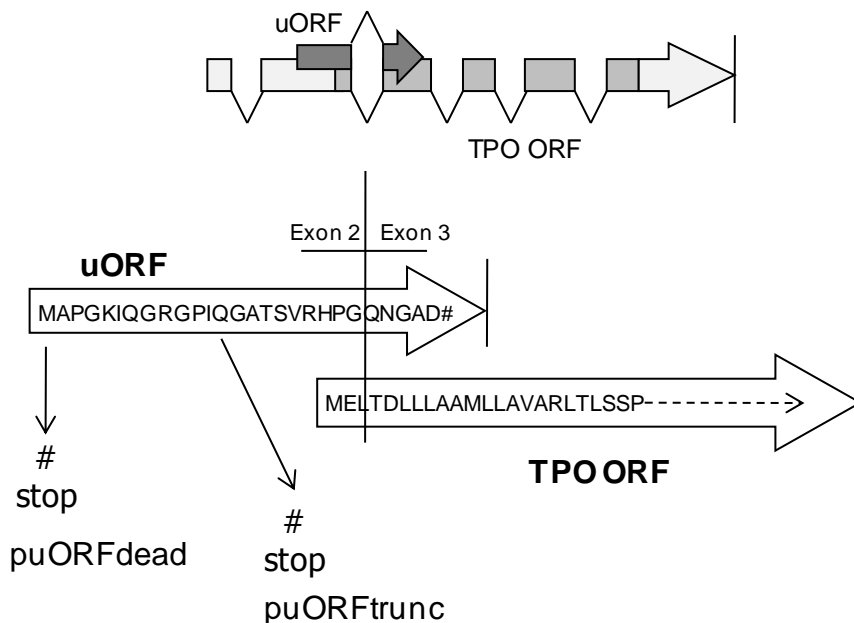
However, there is one mechanism of RNA stability that could be tested without inhibitors. There are many examples of mRNA stability being dependent on translation of the corresponding transcript. For example, the gene *c-myc* cannot be properly down-regulated during myogenesis if point mutations are introduced into its open reading frame. (Wisdom and Lee 1991; Wisdom and Lee 1990) In other cases extending the reading frames, of histone H4 and α 2-globulin, by deleting the normal stop code causes the transcripts to become less stable. (Capasso et al. 1987; Weiss and Liebhaber 1995)

An interesting aspect of TPO biology that has been learned in trying to understand the molecular mechanism of a human disease suggested that it could be regulated in this fashion. It has been reported that most cases of familial thrombocytosis can be explained by mutations in the TPO gene. The mutations, however, do not occur within the normal TPO protein, but in a short upstream reading

frame (uORF) that is out of frame and overlaps the translational start site that gives rise to full-length TPO. (Scheme 5.1,top) Although this uORF should make TPO a target for non-sense mediated decay (NMD), it does not. (Stockklauser et al. 2006) Thus, when mutation of the human gene eliminates this uORF, translation efficiency of TPO is increased greatly, leading to high TPO levels and high platelet numbers. The mutations can alter translation in a number of ways by either: 1) introducing a stop code before the correct TPO start site is reached (Kikuchi et al. 1995; Ghilardi et al. 1999), 2) shifting the uORF's reading frame such that it is in frame with TPO (Ghilardi and Skoda 1999; Kondo et al. 1998), or 3) splicing around the first coding exon allowing an alternate start site to be used. (Wiestner et al. 1998; Schlemper et al. 1994)

Results and discussion

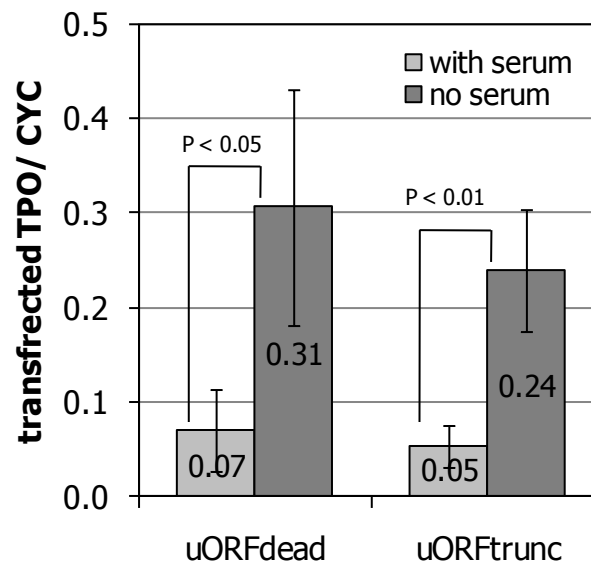
The potential connection between TPO mRNA stability and translation was tested by generating two gene constructs, one in which the start site of the uORF was mutated to a stop code, uORFdead, and another in which the uORF was truncated before reaching the normal TPO reading frame, uORFtrunc. (Scheme 5.1, bottom) Both of these constructs mimic the gene structure found in humans with familial thrombocytosis.



Scheme 5.1: Depiction of relationship between the uORF and the exon structure of TPO. The uORF (dark gray) spans the 2/3 exon boundary and terminates in the 3rd exon. The ORF responsible for full-length TPO is shown in light gray. The location of mutations in the uORF to stop codons are displayed at the bottom. In the puORFdead construct the start of the uORF is mutated to a stop. In the puORFtrunc, the second glutamine residue is mutated to a stop codon.

The constructs were introduced into OP9 cells, and 40 hr later the cells were treated with or without calf serum for 8hr. Quantitative RT-PCR indicates the both constructs display down-regulation in response to serum, consistent with unmodified constructs. (Graph 5.1) Thus, a change in the translation of TPO message is not responsible for the down-regulation of TPO transcripts that occurs upon serum treatment, as both of these constructs should enhance TPO translation, and yet neither affects TPO mRNA levels.

Graph 5.1: Analysis of TPO expressed from genomic DNA constructs that are modified to alter the small upstream open reading frame that overlaps the start site of full-length TPO. Both constructs were transfected into OP9 cells and then 40hrs later treated with (light bars) or with no serum (dark bars) for 8hrs. Transcripts derived from the transfected gene assayed by quantitative RT-PCR. Results represent the mean of 4 independent experiments. Statistics by student's t-test.



Methods

Refer to chapter 2. No new methods were used in this chapter.

CHAPTER 6: CONSIDERATION OF BIOACTIVE LIPIDS

Introduction

Lipid mediators are an important class of signaling molecules. They can act locally or circulate in the blood by binding to albumin. In fact, platelets produce large quantities of bioactive lipids during activation. It was therefore reasonable to ask if the platelet derived suppressing factors might be lipids. Two of the most highly induced and biologically active lipids released by platelets upon activation are lysophosphatidic acid (LPA) (Gerrard et al.; Eichholtz et al. 1993) and sphingosine 1-phosphate (S1P) (Yatomi et al. 2000). One other lipid was tested because of its newly discovered effect on stem cells (North et al. 2007); prostaglandin E₂ (PGE₂) has been shown to increase spleen forming units and the repopulating ability of stem cells after a short incubation with cultured marrow cells. Since TPO is an important stem cell factor, the potential of a regulatory loop existed. If TPO triggered the production of PGE₂ by stem cells as part of the mechanism by which it maintains stem cells, perhaps PGE₂ could feedback to down-modulate TPO expression.

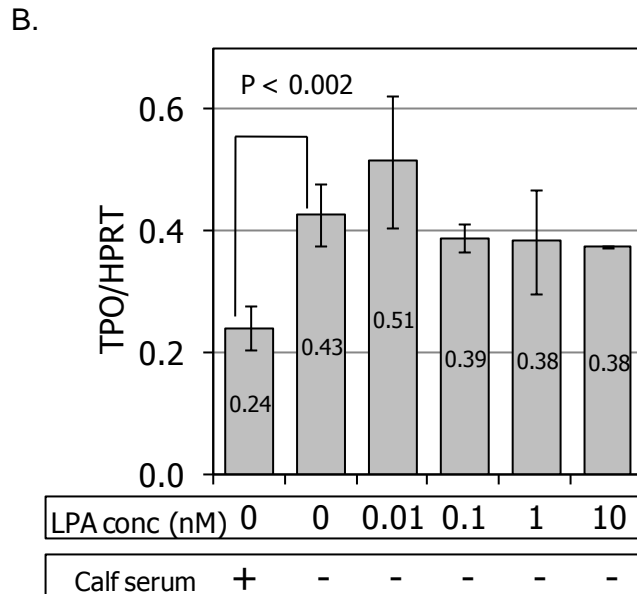
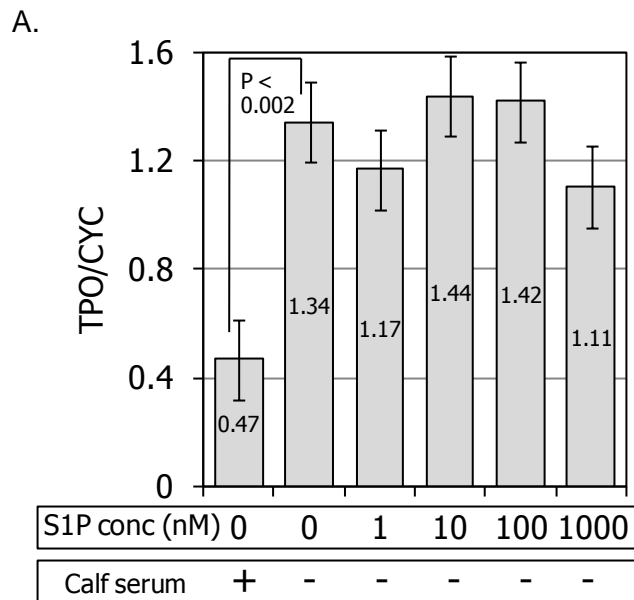
Results and discussion

In the first experiment (Graph 6.1), high doses of LPA or S1P were added to primary bone marrow stromal cell cultures for 10 hrs in the absence of serum. However, both failed to affect TPO expression. High levels of prostaglandin E₂ also failed to block the increase in TPO RNA that occurred in the absence of serum. (Graph 6.2)

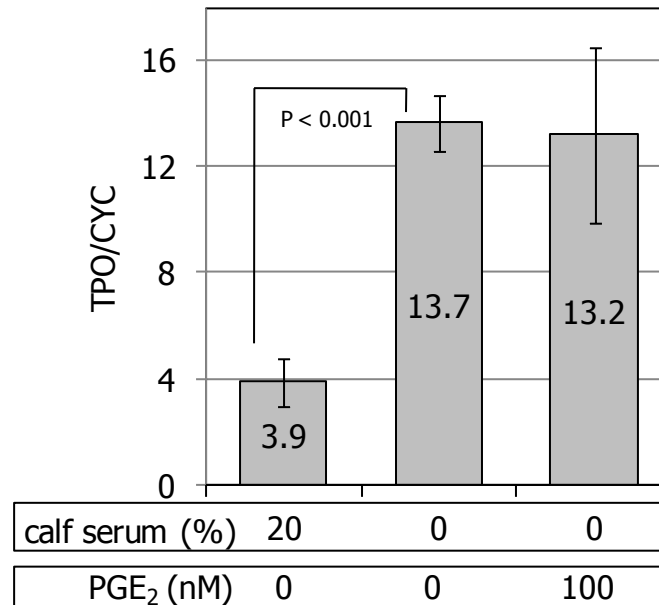
It's important to note that neither experiment contained a true positive control, which demonstrated the activity of the lipid compounds. Instead, we relied on the

supplier to furnish biologically active reagents. Thus, to further study whether lipids might be the agent in serum responsible for TPO suppression, we both dialyzed and charcoal stripped serum samples. We found that despite this treatment the serum retained its inhibitory effect on TPO production, further supporting the conclusion that the serum suppressing factor(s) is/are not lipids.

Graph 6.1: Treatment of primary bone marrow stromal cells with bioactive lipids. Primary cells were incubated for 12 hrs in presence of increasing concentrations of LPA (A) or S1P (B), but in the absence of calf serum. Results represent 3 independent experiments. Statistics by student's t-test.



Graph 6.2: PGE₂ treatment of OP9 cells. OP9 cells were incubated for 10 hrs with or without calf serum. PGE₂ was added to one of the without serum conditions at a concentration of 100nM. Results are from 3 independent experiments. Statistics by student's t-test.



Methods

All lipids were diluted more than 1000-fold from a 1:1 methanol: water stock into PBS containing 1% fatty acid free BSA to create a working stock (Sigma-Aldrich, St Louis, MO). LPA and S1P were purchased from Avanti Polar Lipids (Alabaster, AL) and PGE₂ from Sigma-Aldrich (St. Louis, MO)

The Q-RT-PCR for hypoxanthine-guanosine phosphoribosyl tranferase (HPRT) was conducted as described in chapter 2 methods at a MgCl₂ concentration of 3mM and with the following primers and probe: F:caaacttgcttccctggt
R:ctggcctgtatccaacacttc Prb:tgcaagcttgctggtgaaaagga.

Refer to chapter 2 for all other method

CHAPTER 7: CONSIDERATION OF POTENTIAL ARTIFACTS CAUSED BY SERUM STARVATION

Introduction

Because the condition I have focused on that stimulates an increase in TPO transcription is serum withdrawal, which evokes many cellular changes, alternative and less specific explanations required exploration. When serum is withdrawn from most cells, they will undergo cell cycle arrest; could this be the cause of the increased TPO expression? Likewise, serum withdrawal is often a potent cell stress. In order to eliminate these possibilities cells were treated in two ways, with agents to halt cell cycling, and with ultraviolet light and heavy metals to induce a stress response.

Results and discussion

When OP9 cells were starved of serum for 8 hrs, cell cycle analysis revealed an incomplete arrest in G1. In contrast, we found that both aphidicolin and hydroxyurea were able to cause a durable S-phase arrest. Despite this, neither agent was able to induce TPO mRNA expression in the presence of serum (Figure 7.1). Thus, blocking the cell cycle by withdrawing serum is not a significant contributor to changes in TPO expression.

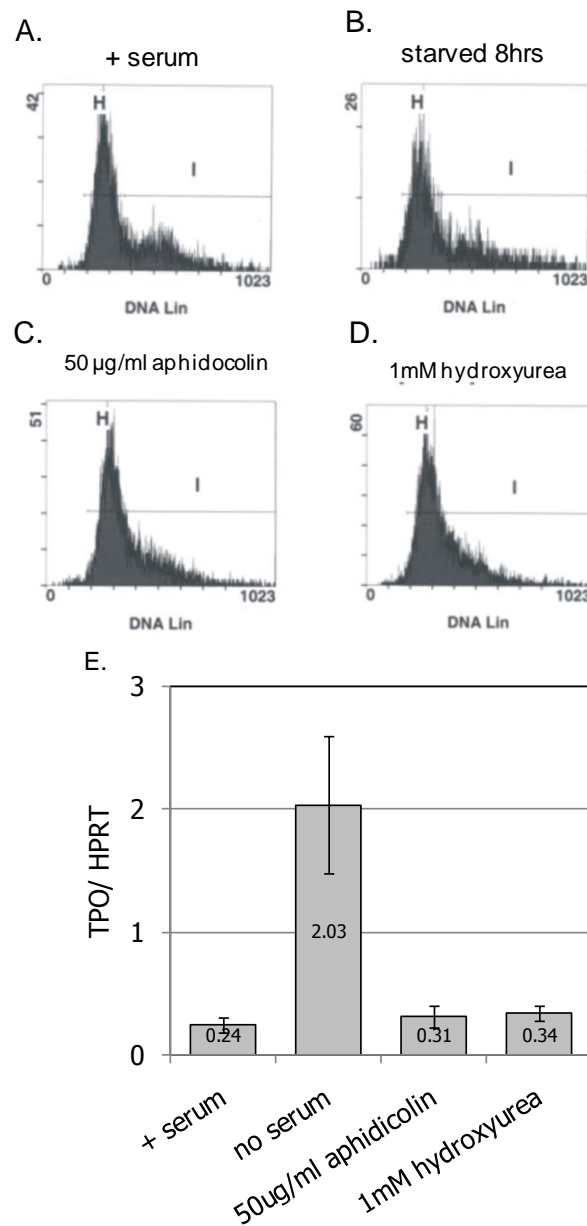
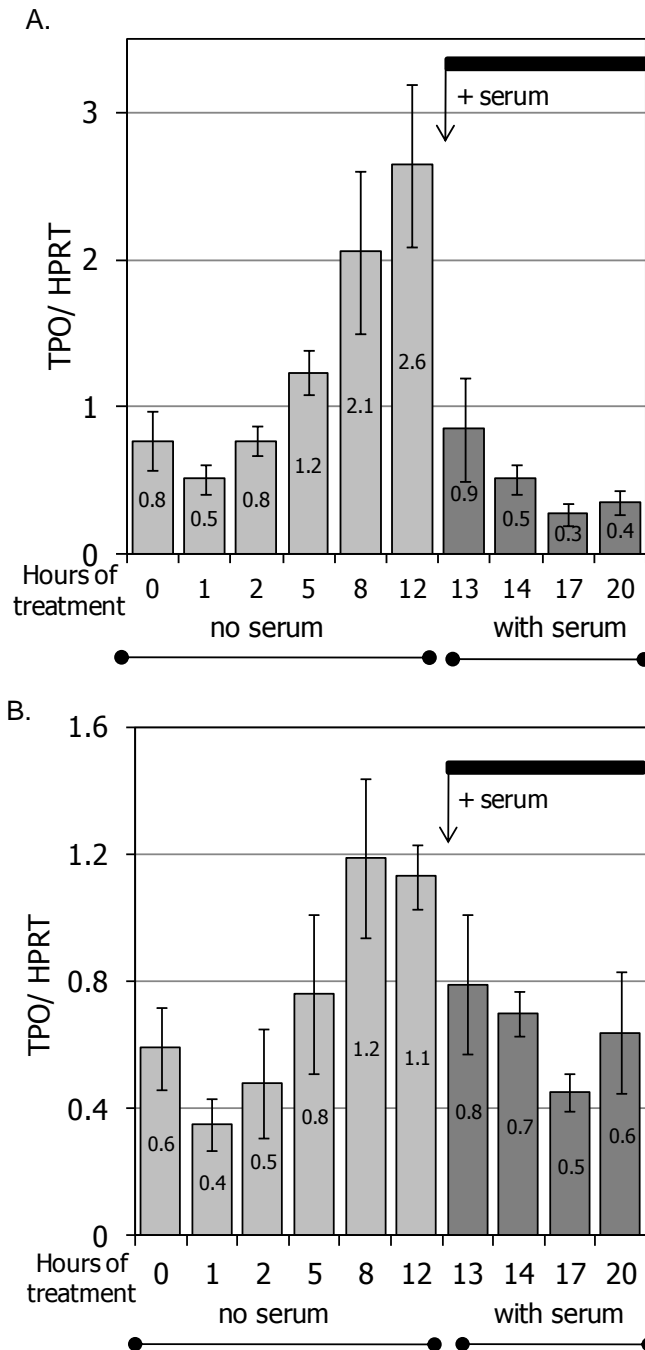


Figure 7.1: Chemically induced cell cycle arrest does not raise TPO expression. OP9 cells stained with propidium iodide and analyzed by flow cytometry for distribution of cells across the cell cycle. OP9 cells were grown in 20% calf serum (A) or starved of serum (B) for 8 hrs before analysis. OP9 cells were treated with 50 μ g/ml aphidicolin (C) and 1mM hydroxyurea (D) for 8hrs in the presence of 20% serum. Cells subjected to the same 4 conditions were also analyzed by quantitative RT-PCR for TPO expression (E).

It could also be argued that withdrawing serum causes a stress response, and the change in TPO expression is the result of that stress. However, after serum starvation, the re-introduction of serum brings TPO expression back down to a normal level. (Graph 7.1) This result suggests that TPO production by serum-starved stromal cells is not a stress response.

Graph 7.1: Starvation and re-feeding. OP9 cells (A) and primary bone marrow stromal cells (B) were incubated without serum for 12 hrs before adding back serum (dark bars and arrow). The results represent the mean of 3 independent experiment +/- SEM.



To more directly test whether serum withdrawal induced cell stress is responsible for TPO production, we intentionally induced a stress response in OP9 cells using exposure to heavy metals or UV irradiation. As shown in Figures 7.2 and 7.3, despite the strong stress response to both treatments, as judged by cell morphology (not shown) or induction of c-Jun N-terminal kinase (JNK) or heat shock protein 70 (HSP70), TPO expression did not increase. Simple cell stress does not appear to be the source of the changes in TPO mRNA observed upon removal of serum.

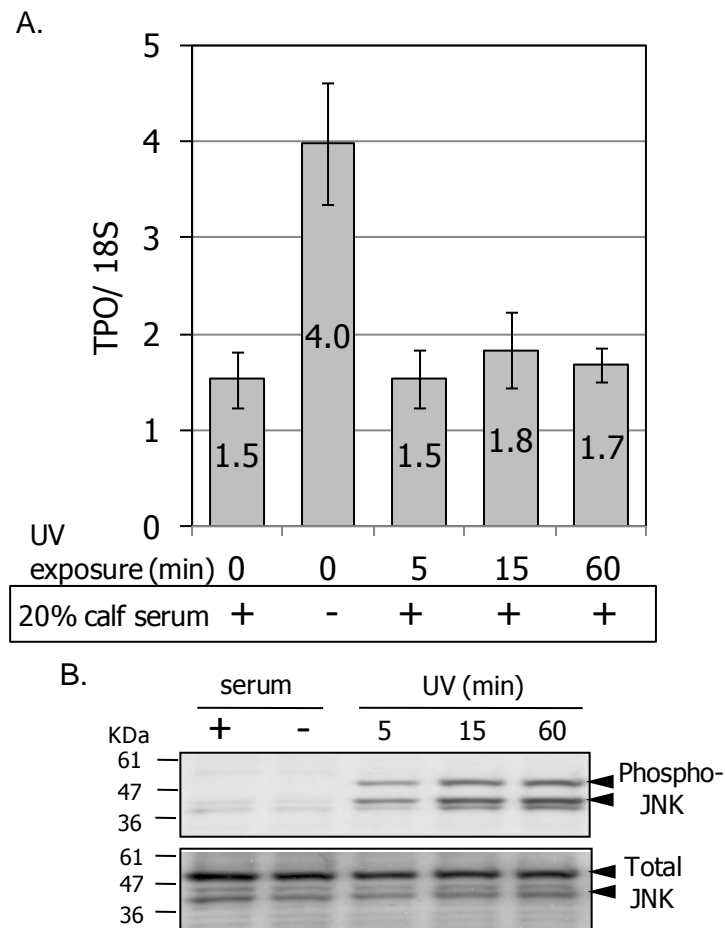


Figure 7.2: Ultraviolet (UV) radiation activates a stress response but fails to increase TPO expression. OP9 cells were treated for the indicated amount of minutes by placing under a UV light. A. Samples were then collected for RNA extraction and subsequent quantitative RT-PCR. B. Additional samples were analyzed by western blot for the activation of the stress regulated c-jun n-terminal kinase (JNK) (indicated by arrowheads).

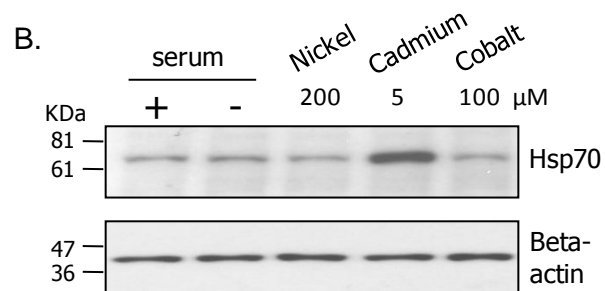
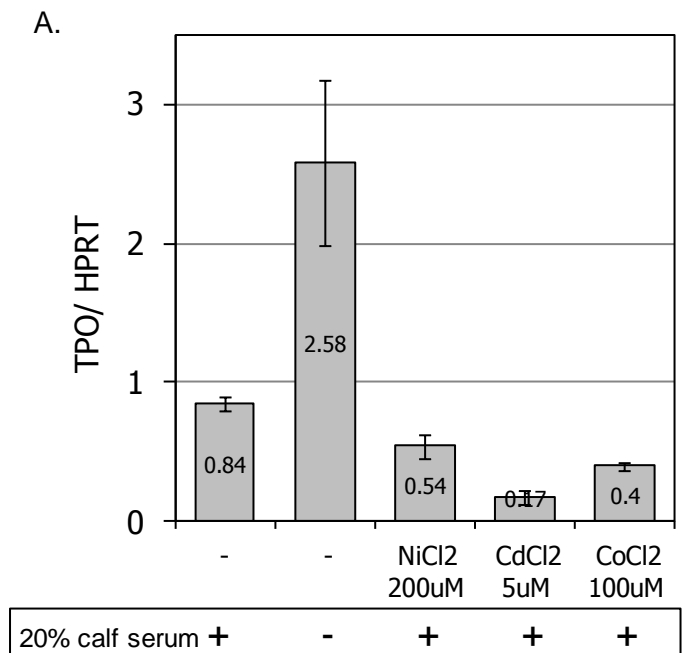


Figure 7.3: Cadmium induced stress, but not TPO expression. OP9 cells were treated with various heavy metals in the presence of 20% calf serum. A. The expression of TPO was analysis by quantitative RT-PCR and normalized to HPRT. B. activation of a stress response was assessed by probing a western blot of heavy metal treated cells with heat shock protein 70 (Hsp70). Beta-actin was used as a loading control.

Methods*Western blotting*

OP9 cells were lysed in buffer 50 mM Tris, pH7.5, 150mM NaCl, 1mM NaF, 5mM MgCl₂, 1mM sodium orthovanadate, and 1:100 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and 1% Triton X-100 and cleared by centrifugation at 15,000x g for 15 minutes. 20 µg was loaded per lane. Blots were developed with 1:1000 dilution of JNK, phosphor-JNK, and HSP70 antibodies (Cell Signaling Technology, Boston, MA).

CHAPTER 8: POSSIBLE FURTHER INVESTIGATION

A number of experiments may be proposed to follow up on the ideas discussed at the end of chapter two. The purpose of these experiments would be to investigate the possible roles of both the transcription factor δ EF-1 and osteoclasts in the regulation of TPO expression in bone marrow. Based on the hypothesis that δ EF-1 is the transcription factor that mediates the serum suppression of TPO transcription, one would predict that if δ EF-1 is expressed in OP9 cells and levels of the transcription factor would follow serum starvation and repletion. Once Q-RT-PCR and western blotting were carried out to verify this prediction, I would turn to gain-of-function and loss-of-function strategies with dominant negative and constitutively active forms of δ EF-1 to establish its role in the regulation of TPO transcription. Similar experiments would be designed in murine marrow stromal cell cultures to extend the conclusions to primary cells.

A first experiment aimed at linking osteoclast-derived factor(s) to TPO expression would be to incubate OP9 cells with osteoclast conditioned medium. If the conditioned medium was found to suppress TPO more than control medium, the connection with osteoclasts, osteoblasts, and a megakaryocyte regulatory network would further elucidate the complexity of bone metabolism.

In order to determine exactly which serum factor are exerting the greatest influence on TPO expression, a simpler screening assay than quantitative RT-PCR of a myriad of candidate platelet proteins would need to be developed. Typically, one would start with biochemical fractionation of platelet lysates, and assay for TPO suppression in OP9 cells. A high throughput assay could be developed that links a TPO reporter gene to a chromogenic reporter gene, and assay for changes in the

chromophore. Once chromatography fractions were assayed in this way, and purification techniques identified, a number of candidate proteins would likely emerge. Successful identification of individual factors could open the door to many regulatory hypotheses.

A long-standing debate in the field of thrombopoiesis is: what is the relative importance of liver- and marrow-derived TPO. There is little doubt that hepatic TPO is important; patients with liver failure have moderate thrombocytopenia, and while several mechanisms are responsible, there is little doubt that reduced expression of hepatic TPO is one of them. However, the absence of alteration of hepatic TPO mRNA, in spite of wide fluctuations in TPO-driven thrombopoiesis in disease, argues there is an additional and important source of the hormone. Ultimately, a bone marrow specific knock out of TPO expression would be needed to characterize the physiologic importance of TPO expressed in the marrow. Because of the buffering capacity of liver TPO production, and our and others' finding that basal production of marrow TPO is quite low, such a mouse is unlikely to present any lower platelet count at steady state, but would very likely display a reduced response to thrombocytopenic stimuli. For instance, perhaps the rate of recovery to normal blood cell counts following myelosuppression would not be as swift. It would also be interesting to explore if any disease states that depend on TPO and megakaryocyte biology such as myelofibrosis would be altered. Depending on the identity of the serum factors, understanding any diseases associated with them might be advanced by exploiting a bone marrow specific knockout of TPO.

Another unexplained phenomenon might also be investigated with this type of knockout model. When mice are myelosuppressed with 5-fluorouracil (5-FU), the

platelet number “overshoots,” or rises above the normal count, at the end of the recovery phase. The “overshoot” is a feature that is unique to treatment with 5-FU; radiation and other chemotherapy agent produce recovery phases without an “overshoot.” An interesting hypothesis that arises directly from the current research is that 5-FU has a disproportionate toxicity towards osteoclasts such that the recovery phase following 5-FU treatment is marked by a relative abundance of TPO secreting cells, osteoblasts, compared to TPO suppressing cells, osteoclasts. It may be that the lag in the return of the correct number of osteoclasts to balance osteoblasts that produces the “overshoot”. Understanding the dynamics of bone marrow stromal cell populations may yield important clinical implications as there appears to have been little research into just how the stromal cells behave during a myelosuppressive regime as seen during routine chemotherapy or stem cell transplantation.

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