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Role of the DBP Gene in the Regulation of Circadian and Cyclic Hematopoiesis: A Case for Potential Linkages

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ABSTRACT D-site albumin promoter binding protein (DBP) is a member of the PAR leucine zipper family of transcription factors and is known to be a clock controlled gene (CCG) hardwired back to the core clock components. Genetic loss of function studies in mice with DBP have shown that DBP null mice exhibit 0.5hr shorter circadian rhythms and altered locomoter activity. However, it is not known whether DBP plays any role in the regulation of hematopoiesis. This review presents the evidences from the literature which point out a role for DBP in the regulation of hematopoiesis. Further, the evidences also suggest that DBP may be associated with the etiology of the myeloproliferative disorder polycythemia vera (PV).

INTRODUCTION

D-site albumin promoter binding protein (DBP) is a member of the PAR leucine zipper family of transcription factors, initially found to be associated with the D-site of the albumin gene promoter (Wuarin and Schibler 1990). The DBP mRNA fluctuates with a similar phase and amplitude in most tissues such as liver, lung, heart muscle, pancreas, kidney and SCN (Wuarin et al. 1992). There are two more members of the PAR family of transcription factors. The Thyroid Embryonic Factor (TEF)(Drolet et al. 1991; Fonjallaz et al. 1996), and Hepatocyte Leukemia Factor (HLF) (Hunger et al. 1992; Falvey et al. 1995) have been shown to share extensive sequence similarity with DBP within their basic leucine zipper (bZip) region and an adjacent peptide segment rich in prolines and acidic amino acids, known as the PAR domain. All of the PAR transcription factors are expressed in a rhythmic fashion in several tissues. Owing to the fact that DBP has the highest abundance and the largest circadian fluctuation of the three PAR transcription factors, it has been the focus of research in recent years. Genetic loss of function studies in mice with DBP have shown that DBP null mice exhibit 0.5hr shorter circadian rhythms. However, it has been found that normal mice show 40% more wheel-running activity or 64% more infrared beam breaking activity under normal entrained conditions compared to DBP null mice (Lopez-Molina et al. 1997), and altered liver metabolism (Damiola et al. 2000). It has also been shown that DBP null mice have altered sleep parameters (Franken et al. 2000). Since the behavior and physiology of DBP null mice is affected but not the circadian rhythm, it has been suggested that DBP is a CCG regulating clock output pathways. However, substantiative data gathered suggest that DBP is indeed hardwired to the central molecular oscillator. It has been shown that DBP itself can upregulate the expression of *mPer1*, an important constituent of the molecular oscillator, by directly binding to the *mPer1* promoter at the DBP binding site (D-box) adjacent to the E-box element (Yamaguchi et al. 2000). Given the fact that DBP null mice is rhythmic, DBP is likely a clock controlled gene (CCG) involved in the output pathways but it also has a strong link to core clock loops. In this context, it is very interesting to note that $CD34^{+}$ lin^{-} fetal liver HSCs also express DBP mRNA (Phillips et al. 2000). However, it is not known whether DBP protein is expressed or what is the fold fluctuation in its amplitude of expression or what functions DBP serves in the regulation of circadian aspects of hematopoiesis.

This review presents how trains of facts and their associations have been used to generate strings of propositions to understand the role of D-site albumin promoter binding protein (DBP) in hematopoiesis. Since this logical coverage is

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complex and the developed propositions are scattered over, the next section presents the actual hypothesis (without any reference to the facts on which it has been founded). The experimental data to support some of the ideas presented in this paper will be communicated in a future publication.

CYCLIC NEUTROPENIA AND DBP

There are several studies that have pointed to a cyclic relationship wherein the rhythm impacts metabolic activity and metabolism feeds back to impinge upon the rhythm (Roenneberg and Merrow 1999). Here, it is hypothesized that this connection between metabolism and circadian rhythms may be connected to several cyclic phenomena of hematopoiesis – namely, circadian and cyclic hematopoiesis (Smaaland et al. 1991 and Haurie et al. 1998). As a CCG, it was hypothesized DBP may play an important role in regulating hematopoiesis by controlling different genes related to metabolism and other genes. First clue to connect DBP to cyclic hematopoiesis was the finding that DBP binds to promoter of serine protease inhibitor (serpin) expressed in liver (Rossi et al. 1992). It is previously known that mutation in elastase cause cyclic neutropenia (Horwitz et al. 1999) but it is not known how this mutation brings about this disease at the molecular level and hence it is previously proposed that inadvertent interaction between serpin and elastase (Horwitz et al. 1999) or elastase with other proteins may be root cause of this disease (Aprikyan and Dale 2001). It is known that inhibitors of angiotensin converting enzyme (ACE) causes neutropenia (Cooper 1983). ACE is an enzyme that acts on the angiotensin I which in turn is a byproduct of angiotensinogen to generate peptide hormone angiotensin II (Rodgers et al. 2000). This finding that ACE inhibitors cause neutropenia along with the fact that angiotensinogen itself is a target gene of DBP (Narayanan et al. 1998) was sufficient to visualize the following hypothesis. Inhibition of ACE may lead to accumulation of angiotensinogen which may in turn off or at least reduce the level of several transcription factors involved in the expression of angiotensinogen which may cause cell death (neutropenia) provided the transcription factors (in this case DBP) are connected to survival pathway. The finding that survival of bone marrow hematopoietic progenitor cells in cyclic neutropenia is impaired (Aprikyan and Dale 2001) further strengthened this hypothesis. Based on these results, it is hypothesized that inadvertent interaction between DBP and elastase may cause the disease cyclic neutropenia. Other side effects of ACE inhibitor, captopril on the hematopoietic system are also known like granulo-cytopenia, aplastic anemia, and pancytopenia but the mechanisms are not known (Chisi et al. 1999).

POLYCYTHEMIA VERA AND DBP

In order to gain more clues, studies related to polycythemia vera (PV), a cyclic hematopoietic disease were looked at. PV is one of four diseases termed as myeloproliferative disorders (MPDs). Besides PV, this group includes essential thrombocythemia (ET), idiopathic myelofibrosis (IMF), and chronic myelogenous leukemia (CML) (Tefferi 1999). All MPDs result from the clonal expansion of a mutant pluripotent hematopoietic stem cell (Pahl 2000). PV is characterized by an increased proliferation of all 3 myeloid lineages, which results in an excess production of mature red cells, granulocytes, and platelets (Ellis and Peterson 1979). Although the molecular etiology of PV remains unknown, progress has been made in characterizing the malignant cells. The PV cells are hypersensitive to several hematopoietic growth factors (Temerinac et al. 2000) including interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), and thrombopoietin (TPO). However, regarding erythropoietin (EPO) there has been long debate as to whether PV cells are hypersensitive to or in fact independent of this growth factor. Correa et al. (1994) have recently shown that PV erythroid progenitor cells are independent of EPO using a novel serum-free medium devoid of any burst-promoting activity.

In addition, these cells are also hypersensitive to insulin-like growth factor-1 (IGF-1). Interestingly, patients with PV also show 4-fold elevated plasma levels of insulin-like growth factor binding protein-1 (IGFBP-1), which, together with IGF-1, stimulates erythroid burst formation *in vitro* (Mirza et al. 1997). IGFBP-1 is previously known to be a target gene of DBP (Babajko and Groyer 1993) involved in the regulation of glucose metabolism and fetal growth (Lee et al. 1997). It has also been shown that DBP binding

site is present at the 3' flanking sequences of EPO (Lee-Huang et al. 1993). Hence, efforts are made to come into grips with what is known about the expression of EPO at the molecular level and to see the possibility of DBP doing its own part in the regulation of the EPO expression. EPO is involved in the control of red blood cell production through the promotion of survival, proliferation and differentiation of erythroid progenitors in the bone marrow. In the fetal stage, the liver is the major producer of EPO (Zanjani et al. 1981), however, the EPO gene also appears to be strongly expressed in kidney during early gestation (Wintour et al. 1996). Studies on mice have also shown that EPO gene transcription is stimulated by hypoxia and cobalt treatment (Beru et al. 1986).

Interestingly, EPO has been shown to be expressed in macrophages (Vogt et al. 1989) and also in commited CD34 lineage HSCs (Stopka et al. 1998). Control of EPO gene expression needless to say, involves complex interactions between regulatory elements surrounding both upstream and downstream of EPO gene and several transcription factors that together confer developmental, cell type or tissue specificities and other physiological specificities such as hypoxia and anemia. For example, during hypoxia adult liver contribution to EPO synthesis increases substantially whereas renal EPO mRNA increases by 150-fold. However, EPO mRNA in lung did not increase, but it became detectable in spleen (Tan et al. 1991). The 3' enhancer of EPO contains different segments to which different transcription factors bind (Semenza and Wang 1992). A 50bp DNA element responsible for hypoxia inducibility of EPO expression has been defined approximately 120bp 3' to the polyA site to which hypoxia inducible factor-1 (HIF-1) binds. Molecular cloning of HIF-1 by Wang et al (1995) showed that DNA binding complex was composed of two basic-loop-helix Per-ARNT-Sim (PAS) domain containing proteins called HIF-1α and HIF-1β. HIF-1β had previously been identified as the aryl hydro-carbon nuclear receptor translocator (ARNT), a molecule involved in the xenobiotic response (Hoffman et al. 1991). Insufficient but detailed mechanisms by which HIF-1 is involved in the regulation of EPO mRNA and other hypoxia- inducible genes like vascular endothelial growth factor (VEGF), transferrin, heme oxygenase, glucose transporter-1 (GLUT-1), inducible nitric oxide synthase (iNOS) and aldolase are known (Wenger and Gassmann 1997; Wenger 2002). Several components of the mammalian circadian clock contain PAS proteins PER1 (Sun et al. 1997; Tei et al. 1997), PER2 (Sakamoto et al. 1998), and PER3 (Zylka et al. 1998). Two other PAS proteins, CLOCK (King et al. 1997) and BMAL1 (also known as MOP3 or ARNT3) (Honma et al. 1998) were cloned and characterized as positive transcription factors that bind to the E-box elements present in the promoter of the mouse *PER1* gene (Gekakis et al. 1998; Hogenesch et al. 1998). Concurrent with this homology among genes involved in hypoxia and circadian rhythm, it has been shown that hypoxia affects expression of circadian genes *PER1* and *CLOCK* in mouse brain and has also been shown that HIF-1α indeed coimmunoprecipitates with PER1 indicating strong ties between hypoxia and circadian rhythm (Chilov et al. 2001). Getting back to 3' enhancer of EPO, adjacent to the HIF-1 binding site in the mouse is the sequence TGACCTCTTGACCC, known as a direct repeat 2 (DR2) element to which hepatocyte nuclear factor- 4 (HNF-4) binds (Galson et al. 1995; Bunn et al. 1998). It has been proposed that EPO expression with respect to DR2 element is regulated during the E9.5—E11.5 phase of fetal liver erythropoiesis by RXR alpha (retinoic acid receptor) and retinoic acid, and that expression then becomes dominated by HNF4 activity from E11.5 onward (Makita et al. 2001).

However, these results do not explain completely how the EPO is expressed in a circadian fashion or expression in hematopoietic tissues such as macrophages or commited HSCs. The presence of DBP binding site at the 3' end of EPO gene, overexpression of IGFBP-1 in PV, the connection between hypoxia and several circadian factors all have strongly suggested an important role for DBP in EPO expression. It is also interesting to note that another important feature of PV cells to give rise to all myeloid lineage cells could easily be vindicated provided the earlier proposition was true (wherein DBP is considered to be involved in survival pathways). But then there are other threads of information nicely tying with for a role of DBP in hypoxia and EPO expression. This particular clue comes again from a cyclic hematopoietic disease known as chronic granulomatous disease (CGD) (Haurie et al. 1998). CGD is an inherited immunodefi-

ciency characterized by the failure of phagocytic cells to produce superoxide upon the ingestion of microorganisms due to a lesion in a membrane-associated NADPH-oxidase (Dinauer et al. 1987; Geiszt et al. 2001).

NADPH oxidase is a multicomponent enzyme that is dormant in resting cells but becomes highly active during the phagocytosis of invading pathogens. Once activated, the enzyme transfers one electron from NADPH to molecular oxygen, resulting in the formation of superoxide anion (O_2^-). The enzyme is present in all professional phagocytes like neutrophils, eosinophils, macrophages. The activated NADPH oxidase is composed of five components: the membrane-bound flavocytochrome b_{558}; the cytosolic factors $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$; and the small GTPase Rac2. Cytochrome b_{558} itself is a complex of two subunits, $gp91^{phox}$ and $p22^{phox}$ (Babior 1999). Mutations in different subunits may cause this disease and in fact there is a database of these mutations to help researchers working with this disease (Roos 1996). Recent works also show that there are several variants of NADPH oxidase especially the catalytic subunit $gp91^{phox}$ known as Nox1, Nox2, Nox3, Nox4 and (Cheng et al. 2001) which are expressed in different cells like B-cells (Kobayashi et al. 1990), kidney (Geiszt et al. 2000), and cancer cells (Cheng et al. 2001). NADPH oxidase has been subsequently proposed to play an important role as a universal oxygen sensor (Jones et al. 2000) and has been shown to play a role in oxygen sensing (Haenze et al. 2002). The finding that superoxide release is much higher in the neutrophils from PV patients compared to normal neutrophils (Iki et al. 1997) indicated that DBP may be involved in this process (Personal communication with Prof. Schibler). With the emerging role of NADPH oxidase in oxygen sensing, it is now conceivable that DBP has its own role in the regulation of EPO expression and hypoxia given the more compelling evidences presented so far.

Stopka et al. (1998) showed that EPO is expressed in $CD34^+$ HSCs only after exposing the cells to the EPO or EPO mimicking peptides (Stopka et al. 1998) for 6 hours which would call for an activation of DBP preferably by kinase. It has been recently shown that mDYRK3, mouse dual-specificity Yak-related tyrosine kinase, an erythroid specific kinase related to yeast Yak1 is expressed in response to EPO (Geiger et al. 2001). It has been proposed that activation of mDYRK3 expression in rapidly expanding erythroid progenitors may act as a suppressor of colony-forming unit-erythroid (CFU-E) growth and/or development since antisense oligonucleotide to mDYRK3 significantly and specifically enhance CFU-E. This indicates that mDYRK3 may play an important role in the burst forming units-erythroid (BFU-E) stages of erythroid development that precedes CFU-E. It is also known that it is the size of BFU-E that determines the size of CFU-E which is very much dependent on EPO (Mide et al. 2001). That is a reduction in EPO leads to reduction in CFU-E whereas an increase in EPO leads to a reduction in BFU-E with concomitant increase in CFU-E. Based on these data, it is hypothesized that DBP activity may be regulated by mDYRK3, a kinase expressed in response to EPO and involved in earlier stages of erythroid differentiation apparently in coincidence with DBP.

SYNERGY WITH OTHER HEMATOPOIETIC FACTORS

It is already known that the DNA binding activity of GATA1, an erythroid transcription factor can be rescued by the addition of 30% serum when the PU.1, (a purine rich DNA binding protein involved in B-lymphoid and myeloid lineages) overexpressing murine erythroleukemia (MEL) cells are induced to differentiate with dimethylsulfoxide (DMSO) (Yamada et al. 1998). It is also known that PU.1 overexpressing cells apoptose when induced to differentiate with DMSO. Rescue of DNA binding activity of GATA1 may suggest that antagonistic interaction between GATA1 and PU.1 (Zhang et al. 2000) may be prevented by the addition of serum and hence there is an increase in GATA1 DNA binding activity. It is previously known that serum shock induces expression of DBP (Balsalobre et al. 1998). It is well known that erythrocyte or immature erythrocyte deaths display a number of features that are characteristic of apoptosis but they are not blocked, or even delayed, by peptide caspase inhibitors, and most of the cells die without activating caspases (Weil et al. 1998). It is also known that PU.1 upregulates the elastase gene expression (Srikanth and Rado 1998). It has been shown that elastase has differential ability to degrade phosphorylated proteins and unphosphorylated proteins (Nilsson Ekdahl and Nilsson 1997). It has been found that binding

sites of GATA1 and vitellogenin binding protein (VBP) are there close by in several target genes such as serpin2a and proline rich tyrosine kinase 2 (PYK2) promoters using the software MatInspector version2.2 with core and matrix similarity of 0.55 (Quandt et al. 1995) **(**Matinspector does not represent the binding sites of DBP). VBP is one of PAR leucine zipper family of transcription factors to which DBP, thyroid embryonic factor (TEF), hepatic leukemia factor (HLF) belong (Iyer et al. 1991; Haas et al. 1995). They are known to share similar binding sites with small variations in the sequence preference. In fact, TEF and DBP are known to bind the same sequence *in vitro* but the transactivations by these transcription factors are very different (Fonjallaz et al. 1996). Based on these data, it is hypothesized that GATA1 and DBP interact with each other physically and this increases the DNA binding activity of GATA1 (and DBP). This increased DNA-binding activity of GATA1 may be lost due to degradation of more susceptible phosphorylated DBP by elastase when PU.1 overexpressing MEL cells are induced to differentiate with DMSO.

DBP AND TUMOR PROMOTING PHORBOL ESTERS

The mechanism of EPO production is multifactorial and involves several signal transduction pathways. Since it is hypothesized that DBP is involved in the expression of EPO, it is worth understanding what is known about the signal transduction pathways that regulate EPO synthesis to see whether DBP could indeed fit into the previously known pathways or is it involved in new pathways that are yet to be elucidated and also to see whether there are any indications that would further reinforce a role of DBP in EPO expression. It has been proposed that regulation of expression of EPO may depend on two different oxygen sensing mechanisms. One involves the participation of a specific heme protein capable of reversible oxygenation and the other depends on the intracellular concentration of reactive oxygen species (ROS), assumed to be a function of pO_2 (Daghman et al. 1999). Studies in HeLa cell cultures treated with cAMP analog suggest that protein kinase A is involved in oxygen sensing through the transcriptional factor HIF-1 (Kvietikova et al. 1995). It has been reported that addition of phorbol 12-myristate 13-acetate (PMA, also known as TPA), an activator of protein kinase C and calcium ionophore, A23-187 to human hepatoma cell line, Hep 3B cells grown under hypoxic conditions resulted in a dose-dependent inhibition of hypoxia-induced EPO production by 95 +/- 1% and 82 +/- 4% respectively (Faquin et al. 1993). However, it has been recently shown that addition of 1-oleoyl-2-acetyl-ras-glycerol (OAG) and N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfo-namide (SC-9), two well-known protein kinase C activators, significantly increased medium levels of EPO as well as EPO mRNA levels in normoxic Hep3B cells whereas a potent protein kinase C inhibitor, chelerythrine chloride, significantly decreased hypoxia-induced increases in medium levels of EPO and mRNA levels in Hep3B cells (Yoshioka et al. 1998). Similar contrasting results with different inhibitors are also seen with another human hepatoma cell line Hep G2 (Kurtz et al. 1992). Likewise, there are reports to support and oppose the role of calcium in EPO synthesis (Fisher 1988; Metzen et al. 1999). The important message from these data is that known PKC activator, PMA is actually colliding with a molecule or bunch of molecules (or pathways) which is inhibitory to EPO expression whereas other PKC activators that does not collide with these hypothetical molecule(s) actually increase the EPO expression even under normoxic conditions. It is hypothesized that this hypothetical molecule is DBP and PMA hits at/near DBP in the signaling cascades.

It is very well known that PMA is involved in the activation of AP-1 family of transcription factors apart from PKC activation (Ventura and Maioli 2001) which plays an important role in variety of processes. In fact, the consensus site at which AP-1 family of transcription factors bind is called as TPA responsive element or TRE and it is used in transactivation experiments to study the AP-1 activation (Angel et al. 1987; Jonat et al. 1990). Hence, it is further hypothesized that DBP may be involved in the activation of AP-1 family of transcription factors during hypoxia and differentiation.

Numerous studies from hematopoietic cells not only support the above hypothesis that PMA may impinge on/near DBP but suggest a role for DBP in commitment. Here this hypothesis is arrived from the studies that are primarily done with HL-60 and K562 cells. HL-60 cells give rise to either granulocytic or monocytic lineages

whereas the K562 cells give rise to erythroid and megakaryocytic lineages. The differentiation of these hematopoietic cells could be divided into a commitment step and a phenotypic expression step. Effects of agonists and antagonists of protein kinase A (PKA) and calcium or phospholipid-dependent protein kinase C (PKC) modulators at each steps for these two different cells are well documented. These studies indicate that PKA acts as a positive regulatory signal at both steps whereas PKC has a dual role in the process of HL-60 cell differentiation, that is, as a positive regulatory signal in the commitment step and as a negative one in the phenotypic expression step. But in K562 cell differentiation into erythroid lineage, PKA may serve as a negative regulatory signal in both steps; however, PKC may act dually, namely as a negative regulatory signal in the commitment step and as a positive one in the phenotypic expression step (Okuda et al. 1991). The fact that PKA has a negative role in both steps in erythroid differentiation may suggest that HIF-1 may not be involved in the expression of EPO during the differentiation. Interestingly, PMA, the PKC agonist used in this study is well known to induce K562 cells (and variety of cells like MEL cells) to differentiate into megakaryocytic lineage (Rosson and O' Brien 1995). It is previously known that DBP is involved in the expression of several clotting factors such as factor VIII and factor IX (McGlynn et al. 1996 and Begbie et al. 1999). This suggests DBP may play a role at a particular stage of megakaryocytic differentiation.

DBP AND ITS ROLE IN OTHER MYELOID LINEAGES

Since DBP has already been hypothesized to play an important role during the early stages of erythroid differentiation, it is conceivable that if not all the activities, at least the erythroid-specific activities of DBP may need to be suppressed at the initial stages of the megakaryocytic development. Since PMA is known to inhibit EPO expression, it may promote megakaryocytic differentiation by suppressing the erythroid specific activities of DBP. This also suggests that DBP may act as a switch regulating the commitment between erythroid and megakaryocytic lineage. The fact that DBP is involved in the regulation of alpha (2-6)-sialyltransferase (Svensson et al. 1990, 1992), and possibly EPO and NADPH oxidase which are expressed in several granulocytic/monocytic or neutrophilic lineages at particular stage may call for temporal regulation of the activity of DBP during differentiation. The role of DBP in the commitment towards erythroid/megakaryocytic lineages may suggest that it may play an important role in the commitment of HSCs since HSCs are known to give rise to mixed colonies during differentiation. For example, when FDCP-Mix are differentiated towards erythroid lineage by supplying EPO and hemin, at the end of the differentiation, there will be lots of mature/immature erythrocytes along with significant numbers of granulocytes/macrophages but never 100% erythrocytes (Heyworth et al. 1995). At present, it is not known how DBP may be involved in these processes. Though the suppression of DBP activities by PMA is very apparent from the above studies, it is not clear how it is achieved at the molecular level. Further studies are done to gain better understanding that may shed light on the possible functions and regulations of DBP.

Human granulocyte colony-stimulating factor (hG-CSF) is widely used to combat chemotherapy induced neutropenia, cyclic neutropenia, severe congenital neutropenia (also caused due to mutations in elastase) (Levine and Boxer 2002; Aprikyan and Dale 2001) and also for mobilization of HSCs from bone marrow to peripheral blood. It is previously known that hG-CSF potentiates *in vivo* superoxide production by polymorphonuclear cells (PMNs) stimulated with receptor-mediated agonists via G-protein (for example, synthetic chemotactic peptide N-formylmethionine leucyl-phenylalanine, known as fMLP), but not by those stimulated with agonists that bypass receptors via protein kinase C (for example, PMA; Iacobini et al. 1995). This fMLP-mediated superoxide generation coupled to human tumor necrosis factor alpha (TNF-alpha) or hG-CSF priming is inhibited by tyro-sine kinase inhibitors like genistein, alpha-cy-ano-3-ethoxy-4-hydroxy-5-phenylthiomethyl cinnamamid (ST638) whereas PMA induced ROS generation that is not coupled to TNF-alpha or G-CSF-priming is inhibited by PKC inhibitors (Utsumi et al. 1992; Kusunoki et al. 1992). The important idea from these data is that PMA does not act via tyrosine kinase whereas the hG-CSF potentiation of superoxide generation is via tyrosine phosphorylation of bunch of molecule(s).

DBP Calcium and Sialyation

Studies from severe congenital neutropenia (SN) patients suggest that there are two populations of neutrophils based on the response to fMLP. One subpopulation does not respond to fMLP with the normal increase in intracellular calcium concentration ([Ca2+]i) whereas the other subpopoulation requires 10^{-6} M fMLP to respond homogeneously with an increase in [Ca2+]i compared to the normal neutrophils that respond at 10^{-9} M fMLP. In contrast, G-CSF induced neutrophils from patients with cyclic neutropenia as well as chemotherapy-induced neutropenia showed a normal increase in [Ca2+]i after stimulation. The [Ca2+]i-dependent superoxide anion (O_2-) generation in response to fMLP is significantly diminished in neutrophils from SN patients compared to normal neutrophils. However, O_2- generation elicited by PMA, which directly activates PKC, is not affected in SN neutrophils (Elsner et al. 1993). It is previously known that altered sialylation caused by sialyltransferases in chronic myelogenous leukemia (CML) granulocytes lead to reduction in cytosolic calcium rise upon binding of fMLP and also showed reduced sensitivity to fMLP (Cyopick et al. 1993). After sialidase treatment, a significant augmentation in fMLP-induced increases in [Ca2+]i has been noted in CML granulocytes, indicating that the decreased signalling may be a consequence of aberrant sialylation. Interestingly, aberrant glycosylation of CML granulocytes have been shown to reduce the binding of hematopoietic growth factors (GM-CSF), which in turn may be responsible for the immature phenotype of CML granulocytes (Cyopick et al. 1993). So, simple sialylations of membrane proteins may play an important role in regulating how the cells perceive and respond to the signals. As mentioned earlier, DBP is involved in the transcriptional control of alpha (2-6)-sialyltransferase (EC 2.4.99.1), an enzyme regulating glycosylation. The above results together with the connection between NADPH oxidase and DBP suggest that DBP may be involved in the pathophysiology of the neutrophils from these diseases.

DBP AND PROLINE-RICH TYROSINE KINASE-2

DBP may be involved in the potentiation of O_2- generation is further supported by the finding that O_2- release is much higher in the neutrophils from several MPDs diseases including PV compared to normal neutrophils but the fold priming by hG-CSF is much reduced suggesting that the patient neutrophils are already primed *in vivo* (Iki et al. 1997). A number of important conclusions can be drawn from the above data. After hG-CSF treatment, the neutrophils from cyclic neutropenia patients have been responding normally meaning that there are no lesions surrounding DBP. This may actually imply that the inadvertent interaction (proteolytic cleavage) between DBP and elastase initially hypothesized to cause this disease may have been restored to be normal at least temporarily. This may occur possibly through G-protein coupled calcium dependent proline-rich tyrosine kinase, PYK2 (Avraham et al. 2000) which may phosphorylate DBP to make it resistant to the proteolytic degradation by elastase. The reasons for visualizing that tyrosine phosphorylated DBP is resistant to elastase are given below.

DBP AND ITS DEGRADATION BY ELASTASE

It is known in a number of cell systems including hepatic cells and PMNs (Berna et al. 2001; Smith et al. 2001; Brecht et al. 1993; Sethi et al. 1999) that calcium level increases in response to hypoxia. It has been shown that NADH and NADPH (which increase during hypoxia since electron transport is reduced in mitochondria) accelerate the *in vitro* proteolytic activity of proteases including elastase (Knecht and Roche 1986). It is also known that increases in calcium perturbs the serpin-protease complexes *in vitro* (Calugaru et al. 2001). If DBP is to serve any function in hypoxia, DBP protein has to survive this increased proteolysis in response to hypoxic stress. Since elastase has differential proteolytic activity to phosphorylated/unphospho-rylated proteins (Nilsson Ekdahl and Nilsson 1997), it is hypothesized that PYK2 phosphorylated DBP is resistant to elastase. It is further hypothesized that DYRK3 phosphorylated DBP is more susceptible to elastase (since PU.1 causes cell death during erythroid differentiation (Yamada et al. 1998) possibly via elastase).

In case of SN neutrophils, the pathomechanisms may be more complicated but there are no defects in PMA induced O_2- generation. This may indicate that PMA may be acting downstream of

DBP. This activation at the downstream may suppress/inactivate physiological functions of DBP in normal neutrophils and hence there is a potentiation of O_2- generation when cytokines such as platelet activating factor (PAF), TNF or G-CSF or OAG (a PKC activator, Ohtsuka et al. 1990) are used but there is no potentiation when PMA is used. However, it is still not clear how the actions of PMA at the downstream may adversely affect DBP at the molecular level. Further, complications are introduced when it was found that PMA itself at low concentrations can potentiate O_2- generation by fMLP (Tyagi et al. 1988) but at high concentrations it does not potentiate O_2- generation. The fact that PMA activates PYK2 in megakaryocytes (Hiregowdara et al. 1997) also suggested that at low concentrations it may tyrosine phosphorylate DBP, while at high concentrations it may activate the PKCs which could interfere with the functions of DBP.

Since it is hypothesized that hG-CSF (tyrosine) phosphorylates DBP via PYK2, it is further hypothesized that this same modification represent a commitment decision to granulocytes. If this is indeed true, then DBP should inhibit the activation of c-Jun mediated by PYK2 via c-Jun amino terminal kinase (JNK) (Tokiwa et al. 1996). c-Jun is an AP-1 family of transcription factor known to play an important role in the early stages of monocytic differentiation (Rangatia et al. 2002). It has been known that PYK2 is a kinase that gets activated under hypertonic conditions (Tokiwa et al. 1996; Rizoli et al. 1999), which suggested that PYK2 causes a reduction in the concentration of DBP at the protein level. When the cells shrink in their size under hypertonic condition, there must be a reduction in DBP at the protein level since DBP protein has been correlated to cell size (Schmidt and Schibler 1995). There are two contradicting statements here: DBP phosphorylated by PYK2 is resistant to degradation by elastase, while PYK2 causes a substantial portion of DBP to be degraded. Although the mechanism by which these two contrasting outcomes can be accomplished was not known before doing the experiments, the data presented above suggest the presence of these two outcomes. The presence of VBP binding sites on PYK2 promoter, involvement of PYK2 in memory (Huang et al. 2001) and the circadian aspects of memory (Wright et al. 2002) together suggested that DBP may upregulate PYK2 transcriptionally. When the level of PYK2 reaches a threshold, PYK2 likely phosphorylates a portion of DBP, while the rest of the DBP protein is likely destined to degradation by PYK2 via an unknown pathway.

DBP AND OTHER SIGNALING KINASES

It is further hypothesized that serum glucocorticoid regulated kinase (SGK1) (Lang and Cohen 2001), known to be activated by glucocorticoid, a hormone through which the SCN may influence the peripheral tissues (Balsalobre et al. 2000), may also be involved in the regulation of the activities of DBP by phosphorylation. It is known that SGK1 is upregulated by GM-CSF (Cowling and Birnboim 2000) and it is also known to be expressed in the $CD34^+$ lin^- fetal liver HSCs (Phillips et al. 2000) at the mRNA level. The fact that SGK1 inactivates glycogen synthase kinase-3 (GSK3; Kobayashi and Cohen 1999) suggested that it can indirectly activate c-Jun (Boyle et al. 1991), an AP-1 family of transcription factor known to be needed for the monocytic commitment and differentiation.

The presence of the GSK3 consensus in DBP (find out using pattern search at Phosphobase, Blom et al. 1999) and the finding that DYRK kinases (DYRK2 and 1A) may prime the protein substrate for subsequent phosphorylation by GSK3 (Woods et al. 2001) suggested that GSK3 may also regulate the DBP by phosphorylation. However, the functional consequence of this phosphorylation of DBP by GSK3 was not known at this time. It is already known that retinoblastoma (pRb) control element binding proteins bind to the DBP promoter during differentiation (Leggett and Mueller 1994). This suggested that DBP may have tumor suppressive effects and indicated that DBP may inhibit the NF-κB activity mediated by serine/threonine kinase, protein kinase B (PKB) or also known as AKT (a cellular homolog of an oncogene isolated from the retrovirus AKT8; Staal and Hartley 1988) pathway (Dhawan et al. 2002). In Jan 2008, Dr. Scott Devine (University of Maryland) has reported that there is a large deletion in the DBP gene in humans to the single nucleotide polymorphism (SNP) database maintained at the NCBI. However, it is not known the significance of this mutations. The above data may suggest

that it is likely involved in the hematological maligancies such as PV and could be a potential target for gene therapy.

CONCLUSION

It should be mentioned here that the whole hypothesis on how DBP may function to influence different aspects of hematopoiesis has been developed over a period of an year and half, largely independent of the results obtained through the work done during this project. This may appear as a deviation but taking note of the following words of Peyton Rous (Nobel laureate 1966; Weiss 1968),

"*Until now, in terms of human time, most of the facts brought to light by purposeful finders had already existed unbeknownst for 'ever so long'. Searching and finding these facts has been archeology at its best, the new facts coming to life, as one might say, and often adding largely to life's betterment. Their discoverers, if gifted, have eagerly pressed on: in most instances no great harm has come from their further revelations*."

it was decided to garner as many clues as possible from the literature to guide oneself to a better understanding of the process of hematopoiesis. There were indeed unexpected results and also a number of results that have solely born out of the results obtained while carrying out the work, which will be communicated soon. However, the initial work on DBP and their results (transactivation experiments particularly with Serpin2a) reinforced the notion that DBP may play a role in hematopoiesis allowing oneself to dwell upon developing the hypothesis further.

In the previous section, it has been shown how each and every proposition of the hypothesis has been derived from the known facts from the literature. The evidences on which this hypothesis is based may be classified into either primary evidences which are strong and direct ones or the secondary evidences which are not direct but circumstantial or incidental evidences. It is also possible to view the above arguments as too discursive, intuitive or even sheer imagination depending on the particular proposition derived. However, it has not passed through the rigors of science such as quantitation and reproducibility. It may also be possible to assemble sets of arguments from the literature to counter an important role for DBP in hematopoiesis, although no such information has been come crossed during the exploration of the vast literature in the field of hematopoiesis and other fields.

Since the prime attribute of any hypothesis whether true or false lies in its testability, here all the relevant propositions of the hypothesis on how DBP may control hematopoiesis is presented without any reference to the matter of fact on which it is founded to make the hypothesis legible and to proceed further towards proving or disproving the propositions. In a nutshell, the whole hypothesis can be summarized as a simple statement: DBP is involved in the regulation of circadian and cyclic hematopoiesis. Given the mantra of the reductionist biology "break it down", this statement is broken down into small constituents that are amenable to the simple tools of the modern biology in order to prove or disprove the hypothesis.

DBP may be involved in the regulation of the expression of serpin2a and thymidine kinase. Further, it is likely involved in the activation and inhibition of NF-κB and AP-1 family of transcription factors. It may function synergistically with GATA1 while PU.1 may function as an inhibitor of DBP's activity through elastase pathway. It may be involved in the regulation of EPO expression and HIF-1 activity. The activity of DBP may be regulated through phosphorylation by proline-rich tyrosine kinase (PYK2), dual-specificity Yak1-related tyrosine kinase 3 (DYRK3), protein kinase C (PKC), serum-glucocorticoid regulated kinase 1 (SGK1) and glycogen synthase kinase (GSK3). DBP may control the self-renewal, survival, proliferation, apoptosis, lineage commitment and differentiation of hematopoietic progenitors.

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