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Numerous Growth Factors Can Influence *in Vitro* Megakaryocytopoiesis

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At least two classes of human megakaryocyte progenitor cells have been identified: the burst-forming unit megakaryocyte (BFU-MK) and the colony-forming unit megakaryocyte (CFU-MK). The BFU-MK is the most primitive progenitor cell committed to the megakaryocytic lineage. The CFU-MK appears to be a more differentiated megakaryocyte progenitor cell and is thought to be ultimately a descendant of the BFU-MK. A number of recombinant cytokines have recently been shown to be able to promote megakaryocyte colony formation *in vitro*. Recombinant GM-CSF and IL-3, in particular, have the ability to promote both CFU-MK- and BFU-MK-derived colony stimulatory formation. The activities of these two cytokines on *in vitro* megakaryocytopoiesis are also additive. Recent results of clinical trials in both primates and humans, in which these glycoproteins were administered *in vivo*, suggest that these cytokines, both alone and in combination, can enhance *in vivo* thrombopoiesis and therefore may be potentially useful in the treatment of thrombocytopenic disorders.

BACKGROUND

While the factors that control mammalian platelet production have now been studied for several decades, the mechanisms that control this process remain poorly defined. Although a number of investigators have clearly shown that the plasma of thrombocytopenic animals is capable of stimulating thrombopoiesis, the exact identity of the growth factors responsible for this activity has eluded the pursuit of a large number of research groups [1–3]. During the past decade, advances in protein purification and gene cloning have been applied to the isolation and characterization of hematopoietic growth factors. Recently, these new tools have been applied to the study of megakaryocytopoiesis, resulting in important new insights [1–4].

These investigations have been facilitated by the development of a variety of semisolid assay systems, utilized to detect megakaryocyte progenitor cells [5–15]. *In vitro,* these progenitor cells possess the ability to form colonies composed exclusively of megakaryocytic elements. The presence of a hierarchy of megakaryocyte progenitor cells is well established. This hierarchy has been defined by both the time of appearance and the cellular composition of colonies derived from such progenitors [16–19]. At

Abbreviations: Anti-GM-CSF Antisera: anti-granulocyte-macrophage colony-stimulating factor antisera Anti-IL-1α Antisera: anti-interleukin-1α antisera Anti-IL-6 Antisera: anti-interleukin 6 antisera BFU-MK: burst forming unit—megakaryocyte CFU-MK: colony-forming unit—megakaryocyte G-CSF: granulocyte colony-stimulating factor GM-CSF: granulocyte-macrophage colony-stimulating factor IL-1α: interleukin 1α IL-3: interleukin 3 IL-6: interleukin 6 MK-CSA: megakaryocyte colony-stimulating activity MK-CSF: megakaryocyte colony-stimulating factor NALDT$^-$: non-adherent low density T-cell depleted

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TABLE 1
Characteristics of Human Megakaryocyte Progenitor Cells

	BFU-MK	CFU-MK
Time required for colonies to appear *in vitro*	21 days	12 days
Cells/colony	108.6 ± 4.4	11.6 ± 1.2
Foci/colony	2.3 ± 0.4	1.2 ± 0.1
Elutriation profile	12–14 ml/minute	18–20 ml/minute
Phenotype	$CD34^+DR^-$	$CD34^+DR^+$
Sensitivity to 5 Fluorouracil	Resistant	Sensitive

present, at least two classes of megakaryocyte progenitor cells have been identified: the burst-forming unit megakaryocyte (BFU-MK) and the colony-forming unit megakaryocyte (CFU-MK)[16,18,19]. The BFU-MK is the most primitive progenitor cell committed to the megakaryocyte lineage [16,18,19]. The CFU-MK appears to be a more differentiated megakaryocyte progenitor cell and is thought to be ultimately a descendant of the BFU-MK. The assays developed to detect these progenitor cells are dependent upon their ability to form megakaryocyte colonies *in vitro* in response to the addition of cytokines to semisolid media. The characteristics that allow one to differentiate between the CFU-MK and BFU-MK present in normal human marrow are listed in Table 1 [18,19].

A number of complex biological processes occur as the megakaryocyte progenitor cell differentiates and proceeds toward the ultimate production of mature platelets (Table 2). The megakaryocyte, a large but relatively rare cell present in human marrow, is unique because of its ability to undergo nuclear endoreplication while acquiring multiples of the normal amount of nuclear DNA. In this report we will discuss exclusively the regulatory factors that control megakaryocyte progenitor cell proliferation. It should be emphasized that while the processes of megakaryocyte maturation and endoreduplication are also probably controlled by a variety of cytokines, this subject is beyond the scope of this discussion [1–3]. A number of groups have shown that the process of platelet production is regulated by one or more factors that influence different cellular steps along the schema of megakaryocyte development [20–22]. Williams et al. first suggested a two-factor requirement for megakaryocytopoiesis, whereby a megakaryocyte colony-stimulating factor (MK-CSF) selectively promotes megakaryocyte colony formation while an additional factor, megakaryocyte potentiator factor, influences megakaryocyte maturation [22]. MK-CSF is defined as a unique lineage-specific cytokine which selectively promotes megakaryocyte colony formation *in vitro*. The hypothesis that megakaryocytopoiesis is regulated by distinct factors acting at different levels of cellular development has provided a framework for investigation of the cytokine regulation of megakaryocytopoiesis.

TABLE 2
Events Occurring During Megakaryocytopoiesis That Are Possibly Regulated by Humoral Factors

1. Progenitor cell proliferation (BFU-MK, CFU-MK)
2. Megakaryocyte polyploidization
3. Cytoplasmic maturation (membrane and organelle development) of megakaryocytes
4. Platelet release from megakaryocytes

MEGAKARYOCYTE COLONY-STIMULATING FACTORS IN PLASMA

Urine, serum, and plasma obtained from patients with hypomegakaryocytic thrombocytopenia have been shown to be capable of promoting the formation of megakaryocytic colonies *in vitro* [23–29]. This megakaryocyte colony-stimulating activity (MK-CSA) is defined as the ability of conditioned media, plasma, serum, and purified or recombinant cytokines to promote megakaryocyte colony formation in semisolid media. It is unknown whether this MK-CSA is due to the presence of a single unique MK-CSF or a combination of several cytokines with MK-CSA. Data have been provided by several laboratories suggesting that the elaboration of MK-CSA in human plasma or serum is not directly related to platelet numbers but rather inversely related to megakaryocyte mass [23,24]. The elaboration of MK-CSA has been observed in a variety of clinical disorders, including selective amegakaryocytic thrombocytopenia, aplastic anemia, and marrow hypocellularity following cytotoxic chemotherapy or chemoradiotherapy in preparation for allogeneic marrow transplantation [23–29]. Miura et al. have developed a small animal model that provides an opportunity to define the relationship between platelet numbers and detectability of MK-CSA [24]. These investigators compared assayable MK-CSA in plasma of severely thrombocytopenic rats following irradiation to that detected in similarly irradiated animals who received platelet transfusions in order to maintain normal platelet numbers [24]. Assayable MK-CSA was equally elevated in each of these experimental groups as compared with animals not irradiated [24]. This work is in agreement with the hypothesis that MK-CSA elaboration occurs independently of platelet numbers.

The cytokines responsible for plasma MK-CSA in hypomegakaryocytic individuals must be identified in order to understand the contribution of various growth factors to CFU-MK regulation *in vivo*. The biochemical purification of each of these cytokines has recently been pursued by a number of different laboratories with limited success [27,30,31]. A number of sources of MK-CSA, including aplastic anemia urine, selective amegakaryocytic thrombocytopenia plasma, or thrombocytopenic plasma obtained following the administration of sublethal irradiation to dogs have each been used as starting materials for these purification procedures [23–29].

A polyclonal rabbit antiserum that neutralizes MK-CSA present in human plasma has been devloped [32]. This antiserum did not neutralize the MK-CSA of interleukin 3 (IL-3) or granulocyte-macrophage colony-stimulating factor (GM-CSF) but did neutralize MK-CSA in a protein fraction partially purified from human plasma [32]. In addition, Mazur et al. recently reported that IL-3 and granulocyte-macrophage (GM)-CSF neutralizing antibodies do not diminish the MK-CSA present in plasma obtained from either thrombocytopenic dogs or humans [33]. These data collectively indicate the presence of an additional cytokine(s) apart from GM-CSF or IL-3 in human plasma exhibiting MK-CSA.

RECOMBINANT CYTOKINES WITH MEGAKARYOCYTE COLONY-STIMULATING ACTIVITY

Determination of the range of activities of the purified and recombinant cytokines already available have indicated that these cytokines can affect both *in vitro* and *in vivo* megakaryocytopoiesis. Some of these cytokines actually have MK-CSA, while others act in synergy with other factors to affect CFU-MK proliferation.

Data generated in a large number of laboratories are in agreement that recombinant

TABLE 3
The Effect of Cytokine Combinations on BFU-MK-Derived Colony Formation

Cytokine(s)	BFU-MK-Derived Colonies/ 5×10^3 $CD34^+DR^-$ Cells Plated
None	0.0 ± 0.0[a]
GM-CSF 200.0 pg/ml	2.6 ± 0.4
IL-1α 500.0 pg/ml	0.0 ± 0.0
IL-3 1.0 ng/ml	5.8 ± 0.4
GM-CSF + IL-1α	2.2 ± 0.6
GM-CSF + IL-3	10.0 ± 1.8
IL-1α + IL-3	9.4 ± 0.6

[a]Each point represents the mean ± the standard error of the mean of pooled data obtained from experiments performed in duplicate on at least four separate occasions.

GM-CSF and IL-3 both individually have MK-CSA in addition to their ability to affect a number of other hematopoietic lineages [14,16,34–42]. In addition, the effects of GM-CSF and IL-3 are additive in that colony formation by a combination of these two growth factors approximates the sum of colony formation by each growth factor alone [14,35,42]. GM-CSF and IL-3 have also been shown to enhance BFU-MK-derived colony formation [19]. These actions on the BFU-MK are also additive. These cytokines not only increase colony formation, but also increase the number of cells comprising individual CFU-MK-derived colonies [14]. In addition, Briddell and Hoffman have shown that IL-1α augments the ability of IL-3 to promote BFU-MK-derived colony formation [19]. Although IL-1α by itself had no colony-stimulating activity, its synergistic effect occurred in a dose-related fashion [19]. These observations are in no way subtle in that optimal concentrations of both IL-1α and IL-3 promote similar numbers of BFU-MK-derived colonies, as do combinations of optimal concentrations of GM-CSF and IL-3 (Table 3) [19].

Recently, continuous infusions of human IL-3 in a primate, the cynomolgus macaque, have been reported to result in profound increases in mean platelet counts [43,44]. Administration of recombinant GM-CSF to primates also has resulted in some modest, albeit inconsistent, effects on platelet numbers [45]. Krumwieh and Seiler have further explored the *in vivo* action of these cytokines by administering them in sequence [43,44]. Primates were first injected with IL-3 and then subsequently with GM-CSF [43,44]. Such priming with IL-3 followed by GM-CSF administration resulted in a dose-dependent significant increase in platelet numbers, even though IL-3 and GM-CSF alone, at the doses administered, had no significant influence on platelet numbers [44]. Most exciting of all, infusions of IL-3 to patients with a variety of hematological disorders and bone marrow failure states have resulted in a sixfold increase in platelet numbers in five out of eight evaluable patients [46]. This increase in platelet numbers resulted in increased marrow cellularity and appearance of greater numbers of marrow megakaryocytes [46]. These preliminary findings suggest that IL-3 might be a clinically useful pharmacological agent for the treatment of thrombocytopenic disorders.

Another recombinant cytokine, IL-6, has recently been shown to have profound effects on thrombopoiesis. IL-6 was originally characterized as a T-cell-derived factor that promoted the terminal maturation of activated B cells to immunoglobulin-

producing cells [47]. This cytokine has been shown to be identical to B_2 interferon, 26 kd protein, and hybridoma growth factor [47]. Recently Ishibashi et al. have shown that *in vivo* administration of IL-6 produces an increase in peripheral platelet numbers while promoting megakaryocyte maturation and ploidy *in vitro* [48,49]. Hill et al. have also demonstrated a dose-response relationship between the amount of IL-6 administered and the level of increased platelet numbers detected in mice [50]. In addition, this group has shown that both the number of detectable murine splenic and marrow megakaryocyte colony-forming cells were increased in mice that had received IL-6 [50]. Ziedler et al. demonstrated that IL-6 administered to seven cynomolgus monkeys resulted in a 2–3.5-fold increase in the number of platelets [51]. Interestingly, no synergistic effects were observed in the peripheral blood of these animals when IL-6 was combined simultaneously with G-CSF, GM-CSF, or IL-3 [51].

Some question has arisen concerning the mechanism by which IL-6 affects megakaryocyte colony formation. Several investigators have reported that IL-6 alone has no MK-CSA [51–55]. Koike and co-workers have further suggested that IL-6 requires a factor or factors present in serum in order to promote megakaryocyte colony formation [56]. Other groups have, however, reported that IL-6 can act independently on non-adherent low-density T-cell-depleted bone marrow subpopulations ($NALDT^-$) to promote megakaryocyte colony growth [57,58]. In addition, Lotem et al. have presented data to indicate that IL-6 plays an even more critical role in the regulation of the CFU-MK [59]. They have suggested that the induction of megakaryocyte colony formation by IL-3 is actually due to the endogenous production of IL-6 by bone marrow accessory cells [59].

Our group has shown that the addition of both human IL-1α and IL-6 to the $NALDT^-$ cell population resulted in the stimulation of CFU-MK-derived colony formation, while neither IL-1α or IL-6 alone stimulated megakaryocyte colony growth [60]. A similar synergistic effect of IL-1α and IL-6 on hematopoiesis affecting other cell lineages has been previously reported by several groups [61–64]. In addition, our studies indicate that the MK-CSA of IL-6 on low-density bone marrow cells can be significantly inhibited by the addition of anti-IL-1α polyclonal neutralizing antisera [60]. Similar additions of anti-GM-CSF antisera to low-density bone marrow cultures containing IL-6 had no effect on the MK-CSA of IL-6, indicating that the MK-CSA of IL-6 was not due to the secondary elaboration of GM-CSF [60]. Our findings therefore suggest that IL-6 cannot act alone on the CFU-MK from human bone marrow cells but rather requires the presence of IL-1α in order to promote megakaryocyte colony formation [60].

The hypothesis by Lotem et al. that IL-3 exerts its MK-CSA through the action of IL-6 is not supported by data generated in our laboratory [59]. The addition of anti-IL-6 antisera to cultures containing IL-3 had no significant effect on the MK-CSA of IL-3 [60]. Another discrepancy between our report and that of Lotem et al. concerns the appearance of megakaryocyte colonies in assays to which no exogenous cytokines are added. While Lotem et al. attributed such so-called baseline colony formation to the endogenous production of IL-6, our data failed to substantiate this conclusion [59,60]. The reasons for such discrepancies remain unknown.

The effect of the *in vivo* administration of IL-6 alone or in combination with other cytokines to animals or humans with disorders of thrombopoiesis remains unknown. The results of such studies are eagerly awaited.

CONCLUSION

Our understanding of the growth factors that control platelet production has greatly expanded over the last decade. The entry of these growth factors into clinical trial provides promise that in the future we will have to rely to a lesser degree upon platelet transfusion therapy for the treatment of patients with life-threatening thrombocytopenia. In addition, the availability of these cytokines may permit *in vitro* production of sufficient numbers of platelets that might be useful for transfusion therapy.

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