

# Thrombopoietin stimulates VEGF release from c-Mpl-expressing cell lines and haematopoietic progenitors

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**Abstract** Vascular endothelial growth factor (VEGF) production was analysed in megakaryocytic cell lines and CD34<sup>+</sup> haematopoietic progenitors following treatment with thrombopoietin (TPO). In CMK cells TPO caused a time- and dose-dependent increase in the levels of VEGF released into the medium. A similar effect was observed in UT-7/mpl cells transfected with the TPO receptor c-Mpl, but not in parental UT-7 cells. In CD34<sup>+</sup> haematopoietic progenitor cell cultures TPO stimulated VEGF mRNA expression and VEGF protein release. Production of VEGF in CD34<sup>+</sup> cultures increased with TPO-induced megakaryocytic differentiation, but not with erythroid or myelomonocytic differentiation induced respectively by erythropoietin and granulocyte-macrophage colony-stimulating factor. These results demonstrate that TPO stimulates VEGF release in c-Mpl-expressing cells and suggest that this process is an integral feature of the megakaryocytic differentiation programme.

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**Key words:** Thrombopoietin; Vascular endothelial growth factor; Megakaryocyte

## 1. Introduction

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a potent angiogenic molecule *in vivo* and an endothelial cell-specific mitogen *in vitro* (reviewed in [1]). It also promotes increased vascular permeability [2] and migration of monocytes through endothelial layers [3]. VEGF plays a major role in the development of blood vessels during embryogenesis [4], and in the neovascularisation of solid tumours [5,6]. In addition, VEGF has been implicated in wound healing [7] and in pathological conditions such as diabetic retinopathy [8], rheumatoid arthritis [9] and atherosclerosis [10].

VEGF is a homodimeric glycoprotein with structural homology to platelet-derived growth factor. Four isoforms of human VEGF, arising from alternative splicing of a single gene, have been identified [11]. All isoforms have a peptide leader sequence that promotes their active secretion. The two smaller species, VEGF<sub>121</sub> and VEGF<sub>165</sub>, are freely soluble, whereas the two larger ones, VEGF<sub>189</sub> and VEGF<sub>206</sub>, are found bound to the cell surface or the extracellular matrix.

VEGF was shown to be produced constitutively by tumour cell lines of various origins [12]. In addition, it is strongly induced by hypoxia, both in transformed [13] and in non-transformed cells [14–16]. Furthermore, in a number of cell types several growth factors and cytokines, including platelet-derived growth factor BB, basic fibroblast growth factor, transforming growth factor- $\beta$ , interleukin-1 $\beta$ , interleukin-6, tumour necrosis factor- $\alpha$  and interferon- $\beta$ , have been shown to induce VEGF expression to varying degrees [15,17–21].

Recently, Mohle and his colleagues have reported that VEGF is produced constitutively by human mature megakaryocytes (MKs) [22]. MKs are generated in the bone marrow by a complex differentiation process that is controlled primarily by the hormone thrombopoietin (TPO) (reviewed in [23]). In this study, we investigated the possibility that TPO might also stimulate VEGF production. We demonstrate that TPO causes a marked increase of VEGF release in cell lines that express the TPO receptor c-Mpl. Furthermore, we show that *in vitro*, production of VEGF by haematopoietic progenitor cells is specifically associated with TPO-induced differentiation, and not with the differentiating effects of other similarly acting cytokines.

## 2. Materials and methods

### 2.1. Materials

Recombinant human TPO, granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO) and the human VEGF enzyme-linked immunoabsorbent assay kit were purchased from R&D Systems (Abingdon, UK). Tissue culture media and additives were from Gibco Life Technologies (Paisley, UK). Other reagents were from standard suppliers or as listed in the text.

### 2.2. Cell culture

The human megakaryocytic cell line CMK was maintained in RPMI supplemented with 10% fetal calf serum (FCS), 0.1 mg/ml streptomycin, 100 U/ml penicillin and 0.25  $\mu$ g/ml amphotericin B. The growth factor-dependent pluripotent haematopoietic cell line UT-7 was maintained in  $\alpha$ -minimum essential medium without deoxyribonucleosides, supplemented with 2 mM L-glutamine, 10% FCS, 2.5 ng/ml GM-CSF and the above antibiotics. UT-7 cells transfected with a human *c-mpl* cDNA (UT-7/mpl) [24] were kindly supplied by Prof. W. Vainchenker (Institut Gustave Roussy, Villejuif, France) and maintained under the same conditions as parental UT-7 cells except that the medium contained 0.5 mg/ml G418. Cultures were kept in exponential growth ( $2\text{--}7 \times 10^5$  cells/ml) at 37°C in a humidified incubator under 5% CO<sub>2</sub>/95% air. For experiments, prior to stimulation, cells were rendered quiescent by incubation for 18 h in their respective media containing either 0.5% FCS (CMK), or lacking GM-CSF (UT-7 and UT-7/mpl), as previously described [25]. After washing, cells were resuspended in their respective fresh quiescence media at  $5\text{--}6 \times 10^5$  cells/ml. To induce VEGF release, quiescent cells were incubated for 24 h (unless otherwise indicated) with haematopoietic growth factors as described in the respective legends to figures. At the end of the incubation cell count and viability were determined by

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the trypan blue dye exclusion method. Cell viability remained above 85% at all culture conditions. Supernatants for measuring VEGF concentration were recovered after centrifugation at  $1000\times g$  for 10 min at  $20^{\circ}\text{C}$ .

CD34<sup>+</sup> haematopoietic progenitor cells were isolated from umbilical cord blood by magnetic immunoselection, using the anti-CD34 monoclonal antibody (mAb) QBEND/10 and super-paramagnetic MicroBeads (Miltenyi Biotec Ltd, Camberley, UK) as previously described [26]. Following purification, CD34<sup>+</sup> cells (95–98% purity) were cultured in Iscove's modified Dulbecco's medium supplemented with 0.1 mM minimum essential amino acids, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 2 mg/ml L-asparagine, 0.2% bovine serum albumin (BSA), and 10% human cord blood platelet-poor plasma, in the presence or absence of different haematopoietic growth factors as indicated in the respective legends to figures. Cells were grown in 24-well tissue culture plates (Falcon) at  $37^{\circ}\text{C}$  in a humidified incubator under 5%  $\text{CO}_2/95\%$  air, for various lengths of time. At the end of the culture period cell count and viability were determined as described above. In all the cultures containing haematopoietic growth factors viability remained above 95%. Supernatants for measuring VEGF concentration were recovered after centrifugation at  $400\times g$  for 5 min at  $4^{\circ}\text{C}$ . Cell pellets were saved for flow cytometric analysis or for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

### 2.3. Determination of VEGF release

The VEGF concentration in cell supernatants was measured using an enzyme-linked immunoabsorbent assay (Quantikine, R&D Systems) that recognises human VEGF<sub>121</sub> and VEGF<sub>165</sub>. The lower limit of VEGF detection in this assay was 3.0 pg/ml. Assays were performed in duplicate and the results averaged.

### 2.4. Flow cytometric analysis of antigen expression

Phenotypic differentiation of CD34<sup>+</sup> progenitor cell cultures was monitored by flow cytometry using the following fluorescein isothiocyanate (FITC)-conjugated mAb: Y2/51 (Dako, UK), which detects the megakaryocytic lineage-specific antigen CD61 (glycoprotein IIIa); CLB-143 (CLB, Amsterdam, The Netherlands), which recognises the erythroid differentiation marker glycophorin A; and Leu-M3 (Becton Dickinson, UK), which detects the antigen CD14 on myelomonocytic cells. Cells ( $1\text{--}2\times 10^6$ ) were stained with saturating amounts of FITC-mAb in 0.1 ml Dulbecco's phosphate-buffered saline (PBS)/1% BSA for 30 min at  $4^{\circ}\text{C}$ . Subsequently, the cells were washed once with PBS/BSA, resuspended in 0.5 ml, and finally analysed by flow cytometry using a FACScan instrument and Lysis II software (Becton Dickinson, Mountain View, CA, USA). Light-scatter parameters were used to exclude dead cells. The boundary between antigen-positive and -negative cells was determined according to the fluorescence distribution of cells stained with an FITC-conjugated isotype-matched control antibody.

### 2.5. Purification of CD61<sup>+</sup> cells

CD61<sup>+</sup> cells were purified by magnetic immunoselection from CD34<sup>+</sup> cultures grown for 9 days in the presence of TPO, using FITC-Y2/51 and an anti-FITC mAb coupled to MicroBeads (Miltenyi Biotec Ltd). Briefly, following addition of 1 ng/ml prostaglandin I<sub>2</sub>,  $2.5\text{--}3.0\times 10^6$  cells were harvested by centrifugation at  $300\times g$  for 10 min, stained with FITC-Y2/51 (1:20 dilution) as described above, and washed once with PBS containing 0.5% BSA and 2 mM EDTA (PBE). Cells were subsequently incubated with anti-FITC MicroBeads (1:10 dilution) in 0.1 ml PBE for 15 min at  $4^{\circ}\text{C}$ , washed once, resuspended in 0.5 ml PBE, and finally loaded onto a Large Cells Separation Column Type MS (Miltenyi Biotec Ltd). The column was washed with PBE as described by the manufacturer and CD61<sup>+</sup> cells were eluted in 1 ml culture medium with a purity of 98%.

### 2.6. Reverse transcriptase-polymerase chain reaction analysis of RNA

Cell pellets harvested from CD34<sup>+</sup> cultures were washed twice with ice-cold PBS and total cellular RNA extracted according to the acid guanidinium thiocyanate/phenol/chloroform method [27]. RT-PCR analysis was performed as described by Stavri et al. [15] using specific VEGF and  $\beta$ -actin primers. Aliquots of the PCR reactions were separated on 1.5% agarose gels. VEGF PCR products were identified by Southern blot analysis using a murine <sup>32</sup>P-labelled VEGF<sub>164</sub> cDNA probe [28].

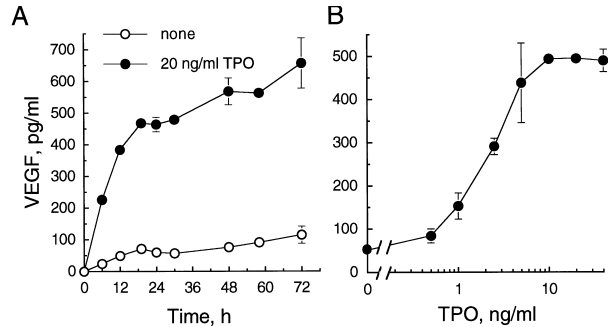


Fig. 1. Stimulation of VEGF release by TPO in CMK cells. Quiescent CMK cells were incubated (A) for various lengths of time in the presence or absence of 20 ng/ml TPO or (B) for 24 h with different concentrations of TPO. The amount of VEGF released into the medium was measured as described in Section 2. Cell numbers did not change significantly during the course of the incubation periods. Values represent the mean  $\pm$  S.D. from replicate determinations.

### 2.7. Statistical analysis

Experiments were performed at least twice and unless otherwise stated results from one representative experiment are shown. Where indicated, levels of statistical significance for VEGF release data were determined using the Student's *t*-test.

## 3. Results

### 3.1. TPO enhances VEGF release in CMK cells

The effect of TPO on VEGF production was initially examined in CMK cells (Fig. 1). This megakaryocytic cell line was chosen because it had been previously reported to display a functional response to TPO [29]. Quiescent cells released constitutively low amounts of VEGF (Fig. 1A, open circles). Addition of 20 ng/ml TPO resulted in a marked time-dependent increase in the level of VEGF accumulated in the medium (Fig. 1A, closed circles). This level increased sharply for approximately 12 h and thereafter continued to rise at a slower rate. The TPO-stimulated release of VEGF was also dose-dependent, with a maximal effect (9-fold increase) observed at 10 ng/ml TPO (Fig. 1B).

### 3.2. The stimulation of VEGF release by TPO is mediated by *c-mpl*

The receptor for TPO, a member of the cytokine receptor superfamily, is encoded by the proto-oncogene *c-mpl* [30]. To confirm that this receptor mediates the TPO-stimulated release of VEGF, we compared the haematopoietic cell line UT-7/*mpl*, which expresses a transfected *c-mpl* [25], with its parental counterpart UT-7; the latter does not express the endogenous gene in sufficient amounts to elicit a functional response to TPO [25]. As shown in Fig. 2, TPO was unable to stimulate the release of VEGF in parental UT-7 cells. In contrast, in UT-7/*mpl* cells a dose-dependent effect was observed.

UT-7 cells express functional receptors for EPO and GM-CSF [31], which also belong to the cytokine receptor superfamily. Therefore, the ability of these haematopoietic growth factors to stimulate the release of VEGF was examined for comparison (Fig. 2). EPO had no significant effect in either parental or *c-mpl*-transfected UT-7 cells. In contrast, GM-CSF stimulated a significant release of VEGF in both cell lines. In this case the magnitude of the response was similar to that observed in UT-7/*mpl* cells following treatment with

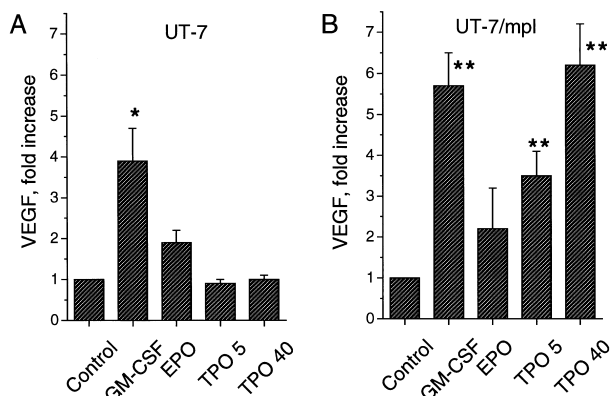


Fig. 2. Stimulation of VEGF release by haematopoietic growth factors in UT-7 cells. (A) Parental and (B) c-Mpl-expressing quiescent UT-7 cells were incubated for 24 h with 5 or 40 ng/ml TPO, 2 ng/ml GM-CSF or 2 U/ml EPO, as indicated. The level of VEGF released into the medium was determined as described in Section 2 and is expressed relative to the level in non-stimulated cells (control) incubated in parallel. Values represent the mean  $\pm$  S.E. of three (UT-7) or eight (UT-7/mpl) experiments. In the case of UT-7/mpl, results obtained with two different clones (four experiments per clone) were pooled and averaged. \* $P < 0.05$ ; \*\* $P < 0.01$ .

TPO (Fig. 2). These results confirmed that it is the absence of functional c-Mpl receptors that was responsible for the failure of TPO to stimulate the release of VEGF in parental UT-7 cells.

### 3.3. TPO induces VEGF mRNA expression and VEGF protein release in liquid cultures of CD34<sup>+</sup> cells

Since VEGF secretion is a common feature of transformed cells, it was important to establish that TPO can stimulate VEGF production also in non-transformed cells. For this purpose we isolated CD34<sup>+</sup> normal human haematopoietic progenitors from umbilical cord blood. Semi-quantitative PCR analysis of VEGF mRNA showed that treatment of these cells with 20 ng/ml TPO for 24 h resulted in a marked increase of two PCR products of 563 bp and 431 bp, which correspond respectively to the sizes expected for VEGF<sub>165</sub> and VEGF<sub>121</sub> (Fig. 3). These VEGF isoforms have been shown to be readily secreted by producer cells [11]. VEGF transcripts encoding the larger cell-associated isoforms were not detected.

In agreement with previous studies [32], Fig. 4 shows that treatment of CD34<sup>+</sup> cells with TPO in liquid culture resulted in a time-dependent increase in the number of cells expressing the megakaryocytic differentiation marker CD61 (Fig. 4, open diamonds). The increase in the number of megakaryocytic cells was clearly evident after 4 days in culture and continued for at least 12 days. Furthermore, consonant with the induction of mRNA for the secreted forms of VEGF (Fig. 3), incubation with TPO also resulted in a time-dependent increase of VEGF protein in the culture medium (Fig. 4, open circles). Under our experimental conditions, however, a substantial rise in the level of VEGF protein was detectable only after 1 week incubation. Thereafter, VEGF levels increased at a faster rate, following closely the increase in the number of CD61<sup>+</sup> megakaryocytic cells. Addition of a second dose of TPO after 8 days resulted in a more rapid accumulation of megakaryocytic cells (Fig. 4, closed diamonds). Similarly, a faster increase in the rate of VEGF accumulation was also observed in this case (Fig. 4, closed circles). Thus, the increase in TPO-stimulated release of

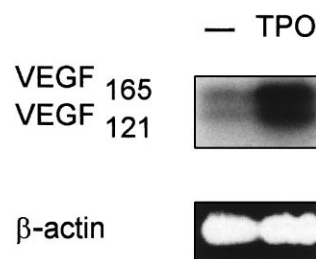


Fig. 3. Characterisation of VEGF transcripts upregulated by TPO in CD34<sup>+</sup> progenitor cells. CD34<sup>+</sup> cells were plated at  $2.4 \times 10^6$  cells/ml and incubated with or without 20 ng/ml TPO for 24 h. Total cellular RNA was prepared from  $1.2 \times 10^6$  cells and then analysed by RT-PCR. VEGF cDNA amplification products were visualised by Southern blot analysis using a <sup>32</sup>P-labelled VEGF<sub>164</sub> cDNA probe. β-Actin cDNA amplification was visualised by direct staining of the agarose gel with ethidium bromide.

VEGF appeared to correlate with the increase in the number of megakaryocytic cells.

### 3.4. VEGF production correlates with megakaryocytic differentiation

The results described above suggested that the TPO-stimulated release of VEGF was associated with TPO induction of megakaryocytic differentiation. On the other hand it could be argued that the time-dependent rise of VEGF levels in culture supernatants was unrelated to the differentiation effects of TPO, and resulted merely from the increase in cell number. To distinguish between these two possibilities we examined the effects of other haematopoietic growth factors on the stimulation of VEGF release. Fig. 5A shows that incubation of CD34<sup>+</sup> cells for 9 days with either GM-CSF, EPO or TPO caused similar increases in the number of cells. However, only TPO induced megakaryocytic differentiation (Fig. 5B). In contrast, in the presence of EPO 65% of cells became positive for the erythroid marker glycophorin A, and in the presence of GM-CSF 35% were positively stained for myelomonocytic differentiation marker CD14 (data not shown). Furthermore, as shown in Fig. 5C, only TPO was able to stimulate a significant rise in VEGF levels. Thus, while all three factors, induced similar increases in the number of cells, only TPO,

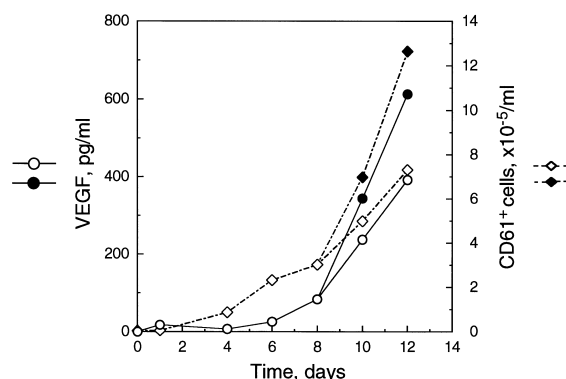


Fig. 4. Stimulation of VEGF release and megakaryocytic differentiation by TPO in CD34<sup>+</sup> progenitor cell cultures. CD34<sup>+</sup> cells were plated at  $2.5 \times 10^5$  cells/ml and cultured in the presence of 20 ng/ml TPO for 12 days (open symbols). Parallel cultures were given a second dose of TPO at day 8 (closed symbols). The number of CD61<sup>+</sup> cells and the amount of VEGF released into the medium was determined every 2 days as described in Section 2.

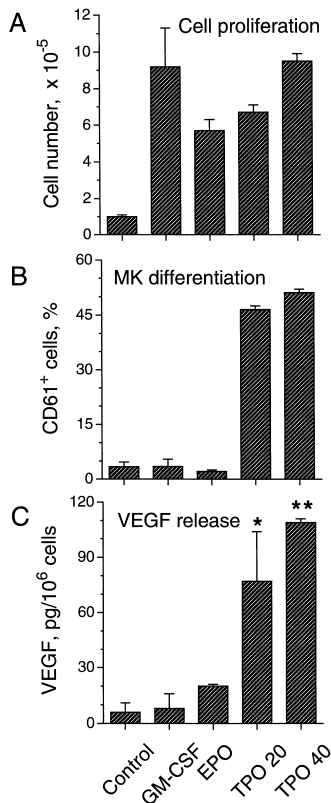


Fig. 5. Comparison of the effect of TPO, EPO and GM-CSF on the stimulation of VEGF release in CD34<sup>+</sup> progenitor cell cultures. CD34<sup>+</sup> cells were plated at  $1.5 \times 10^5$  cells/ml and cultured for 9 days with 20 or 40 ng/ml TPO, 2 ng/ml GM-CSF or 2 U/ml EPO, as indicated. Cell number, percentage of CD61<sup>+</sup> cells and the amount of VEGF released into the medium were determined as described in Section 2. Results represent the mean  $\pm$  S.E. of two experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

which induced megakaryocytic differentiation, stimulated the release of VEGF in these cultures.

### 3.5. VEGF is released from megakaryocytic cells

In order to confirm that the VEGF released into the culture medium originated directly from megakaryocytic cells, CD61<sup>+</sup> cells were purified by magnetic immunoselection from CD34<sup>+</sup> cultures grown for 9 days in the presence of TPO. Fig. 6 shows that when purified CD61<sup>+</sup> cells were subcultured for a further 3 days, measurable levels of VEGF were detected in the medium. Furthermore, in the presence of TPO the levels of VEGF increased by 3-fold (Fig. 6). In contrast, VEGF was not detected in cultures of CD61<sup>-</sup> cells incubated in parallel (data not shown). Thus, TPO stimulated the release of VEGF straight from cells of the megakaryocytic lineage.

## 4. Discussion

In vivo, TPO is the major regulator of megakaryocytopoiesis and platelet production [23,33]. In vitro, TPO can stimulate the proliferation of MK progenitor cells and promote MK terminal differentiation [29,32]. The present study demonstrates a novel action of TPO, the stimulation of VEGF production in cells that express the TPO receptor c-Mpl.

Recently, Mohle et al. reported that the megakaryocytic cell line DAMI as well as ex vivo generated mature MK produce

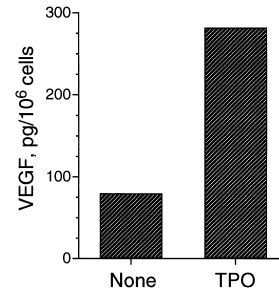


Fig. 6. Stimulation of VEGF release by TPO in CD61<sup>+</sup> cells. CD61<sup>+</sup> cells were purified by magnetic immunoselection and plated at  $6 \times 10^5$  cells/ml. The cells were incubated in absence (none) or presence of 20 ng/ml TPO for 3 days. The amount of VEGF released into the medium was determined as described in Section 2.

VEGF constitutively [22]. These workers also reported that TPO increased the secretion of VEGF in mature MKs, but in contrast to our study, they did not investigate this effect of TPO in cell lines or in immature haematopoietic cells undergoing differentiation in vitro. Furthermore, they showed that thrombin, which has a different physiological role and mode of action, also stimulated VEGF secretion. Thus, the significance of Mohle's findings with regard to the mechanism of action and biological role of TPO remained unclear.

In this work we found that TPO stimulates the release of VEGF in *c-mpl*-transfected UT-7 cells and also in CMK cells; the latter expresses a functional TPO receptor from the endogenous gene. Thus, this effect of TPO cannot be attributed to an artefact of the transfection, or to a property of one particular cell line. Furthermore, the doses of TPO required to stimulate the release of VEGF in these cells were comparable to those reported to stimulate c-Mpl-mediated signalling events and functional responses. Taken together these findings demonstrate that engagement of the c-Mpl receptor by TPO leads to the induction of VEGF release.

A major finding of the present study is that TPO induced both VEGF mRNA expression and protein release from normal human haematopoietic progenitor cells undergoing differentiation in liquid culture. In these cells the effect was selective for TPO as EPO and GM-CSF, two other haematopoietic growth factors with comparable mitogenic and differentiation capacities, did not stimulate VEGF release. A simple interpretation of these results is that the release of VEGF stimulated by TPO is an integral feature of the process of megakaryocytic differentiation which is induced by this haematopoietic factor. This interpretation would be consistent with the finding that in UT-7/mpl cells EPO was unable to induce VEGF release to a significant degree, in spite of the fact that it shares with TPO a very similar intracellular signal transduction machinery [34].

Although induction of VEGF mRNA expression in CD34<sup>+</sup> cell cultures could be detected by the sensitive PCR within 24 h of incubation with TPO (Fig. 3), VEGF protein release, as detected by ELISA, was not observed until 6–8 days of culture (Fig. 4). Since MKs are not seen in these cultures earlier than day 4 [35], it could be argued that while VEGF mRNA might be up-regulated in MK progenitors, VEGF protein might be released only from cells that have undergone further megakaryocytic maturation, or from mature MKs proper. However, since the VEGF mRNA species detected by PCR in this work correspond to those isoforms that are promptly

secreted in other cell types [11], it is worth considering whether VEGF protein may have been released earlier than we could detect it. CD34<sup>+</sup> cells constitute a heterogeneous population enriched in primitive and committed progenitors for all hematopoietic lineages. Vainchenker and his colleagues have previously shown that only a small subset of these cells (less than 2%) express c-Mpl [35]. A majority of CD34<sup>+</sup> c-Mpl<sup>+</sup> cells also expresses low levels of CD61<sup>+</sup>, indicating that they are late progenitors, already committed to the megakaryocytic lineage. In the presence of TPO, CD34<sup>+</sup> c-Mpl<sup>+</sup> cells undergo mitotic amplification and, along with this, further megakaryocytic maturation [35]. Hence, because the number of late MK progenitors initially present in these cultures is very small, the less sensitive VEGF ELISA may not detect the presence of this factor in the supernatant before this subset has expanded substantially; this expansion would then enable sufficient VEGF accumulation to occur. Taken together, this suggests that TPO stimulates the release of VEGF at least from the late MK progenitor stage onwards.

In histological sections of bone marrow MKs are often found in intimate contact with the abluminal surface of sinusoidal endothelial cells. Theories of platelet production have implicated either the passage of entire MKs through the sinusoidal endothelium or the projection (and subsequent fragmentation) of MK cytoplasmic processes (proplatelets) into the circulation [36]. Whatever the actual mechanism of platelet release, our findings raise the possibility that VEGF produced in response to TPO by differentiating MKs, could act as a paracrine factor on bone marrow microvascular endothelium to facilitate the migration and release of platelets into the circulation. This possibility would be entirely consistent with the already documented role of VEGF as a mediator of trans-endothelial migration for other cell types [3].

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