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ORIGINAL PAPER

Dual action of TGF-β induces vascular growth in vivo through recruitment of angiogenic VEGF-producing hematopoietic effector cells

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Abstract The role of Transforming growth factor β (TGF-β) as a regulator of blood vessel endothelium is complicated and controversial, and the mechanisms by which TGF-β is able to induce angiogenesis in vivo are not well understood. Here we show that TGF-β causes in vivo a massive recruitment of tissue infiltrating hematopoietic cells. Concurrently, TGF-β induces strong vascular endothelial growth factor (VEGF) production in the recruited hematopoietic cells, resulting in activated angiogenesis and vascular remodeling. TGF-β also promoted abnormalities of α-smooth muscle actin-expressing pericytes on angiogenic capillaries. TGF-β-induced angiogenic effect was inhibited by a systemic treatment with VEGF-neutralizing antibodies. When studied in isolated human hematopoietic cells, physiological concentrations of TGF-β stimulated VEGF mRNA and protein expression in a dose- and time-dependent manner. This induction was p38 and p44/p42 mitogen activated kinase dependent. p38 and p44/p42 activation was also observed in vivo in TGF-β–treated angiogenic murine tissues. Taken together, our results provide a dual action mechanism by which TGF-β promotes angiogenesis in vivo via recruitment of paracrine VEGF-expressing hematopoietic effector cells. This mechanism may activate vascular growth and remodeling during inflammatory conditions and tumor growth when TGF-β activity is upregulated.

Keywords TGF-β · VEGF · Angiogenesis · Hematopoietic · Paracrine

Introduction

Transforming growth factor β (TGF-β) is a multifunctional growth and differentiation factor that regulates many diverse biological processes [1]. TGF-β has also potent immunoregulatory properties, of both pro- and anti-inflammatory effects. During mouse embryogenesis, TGF-β1 is required for yolk sac hematopoiesis and endothelial differentiation [2, 3]. The role of TGF-β as a regulator of adult vascular endothelium is complicated and controversial. While TGF-β has been shown to have an angiogenic effect in vivo [4–7], it has also been shown that TGF-β inhibits angiogenesis and induces apoptosis of endothelial cells in vitro [8–13] The mechanisms by which TGF-β is able to induce angiogenesis in vivo are not yet well defined. In the present report, we show that TGF-β promotes angiogenesis in vivo by causing a massive recruitment of hematopoietic effector cells, and by inducing in them p38 and p44/p42 mitogen activated kinase (MAPK) dependent synthesis and secretion of vascular endothelial growth factor (VEGF).

Methods

Mouse model of TGF-β1-induced angiogenesis

Age (7–10 weeks), weight and sex-matched wt C57BL/6 J mice were injected subcutaneously (s.c.) at the same

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injection site at the ear with equal volumes of TGF-β1 (60 ng/injection/mouse; R&D Systems, Minneapolis, MN, USA), VEGF164 (60 ng/injection/mouse; R&D Systems) or buffer every other day. The mice were killed 14 days later and the ears were photographed and processed for tissue analyses. To study the mechanism of action of the proangiogenic effect of TGF-β1, C57BL/6 J mice were injected s.c. at the same injection site at the ear every second day during 2 weeks with with equal volumes of 60 ng of TGF-β1 (R&D Systems) or buffer. An immunoneutralizing anti-VEGF mouse monoclonal IgG antibody (clone AVA [14] Genentech, San Francisco, CA, USA) was given intraperitoneally (i.p.) once per week at a dose of 5 mg/kg. The control group received the same amount of mouse IgG (ChromPure, Jackson ImmunoResearch Laboratories, Inc., PA, USA). The tissues were whole mounted or processed to cryosections, analyzed by immunohistochemical means, and studied by light microscopy, florescence microscopy, or multichannel laser scanning confocal microscopy.

Syngeneic bone marrow transplantations

Chimeric mice reconstituted with enhanced green fluorescent protein (GFP)-positive bone marrow (BM) were created to study the behavior of BM cells in vivo. Briefly, BM was collected by flushing femurs of C57BL/6-TgN(ACTbEGFP)1Osb mice. Unselected BM cells (6 × 106) from GFP-transgenic mice were transplanted into C57BL/6 J wild type recipient mice via tail vein injection. The recipient mice were irradiated 1 day prior to transplantation by a lethal dosage of 9.1 Gy. The mice were subjected to TGF-β-induced in vivo angiogenesis assay 5–8 weeks after the BM transplantation. All animal experiments were approved by the Provincial State Office of Southern Finland and were performed in accordance with institutional guidelines.

Tissue immunohistochemistry

For the whole-mount staining, the ears were collected and the cartilage was removed. Tissues were fixed in 4 % paraformaldehyde (PFA), blocked with 3 % normal serum in 0.3 % Triton-X/PBS and incubated with primary antibodies overnight at 4 °C. The ears were then washed and incubated with fluorescent-conjugated secondary antibodies (Alexa594 anti-rat, Alexa594 anti-rabbit, Alexa633 anti-rat, Alexa633 anti-rabbit, Molecular Probes, Eugene, OR, USA) overnight at 4 °C. Finally, the ears were flattened and mounted with antifading medium (DABCO, Sigma-Aldrich Chemie CmbH, Steinheim, Germany). For immunohistochemistry of cryosections the ears were embedded in O.C.T. compound (Tissue-Tek, Sakura Finetek, Europe B.V., The Netherlands) and frozen at −70 °C. Tissues from the chimeric mouse with GFP-positive BM were fixed in 2 % PFA for 1 h and incubated in 20 % sucrose/PBS overnight prior to embedding and freezing. Sections (10 μm) were immunostained with the primary antibodies overnight at 4 °C and subsequently treated according to manufacturers instructions using Vectastain Elite ABC anti-rabbit or anti-rat kits (Vector Laboratories, Inc., Burlingame, CA, USA) for primary antibody detection. Primary antibodies used were; rat anti-mouse CD31/Pecam-1, rat anti-mouse CD45 (BD Pharmingen, Palo Alto, CA, USA), rat anti-mouse alpha-smooth muscle actin/α-SMA (Cy3-conjugated, Sigma-Aldrich), rabbit anti-mouse/human von Willebrand Factor/vWF (DAKO, Glostrup, Denmark), polyclonal rabbit anti-VEGF Ab-1 (NeoMarkers, Fremont, CA, USA), and polyclonal rabbit anti-mouse antibodies against phospho-p38 MAP kinase (Thr180/Thr182) and phospho-p44/p42 MAP kinase (Thr202/Thr204) (Cell Signaling Technology, Inc., Beverly, MA, USA). The samples were analyzed and photographed at room temperature with a digital camera, Leica MZFLIII stereomicroscope with 12.5:1 zoom, Leica DFC 480 camera and FireCam software (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany), Leica DMLB light microscope using 5× (0.12 numerical aperture), 20× (0.4 numerical aperture) and 40× (0.65 numerical aperture) N PLAN objectives (Leica Microsystems), Olympus DP 50 camera, and Studio Lite software (Olympus, Hamburg, Germany), and a Zeiss Axioplan 2 immunofluorescence microscope using 10× (0.3 numerical aperture) and 40× (0.75 numerical aperture) Plan-Neofluar objectives, AxioCam Hrc camera, and Axiovision 3.1 software (Carl Zeiss, Göttingen, Germany). Additionally, the samples were analyzed with a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss) using multichannel (sequential) scanning in frame mode. A 40× (1.3 numerical aperture) Plan Neofluar oil immersion objective and LSM 5 software version 3.2 were used (Carl Zeiss). Single XY-scans had an optical slice thickness of 0.9 μm or less. The and CD31+ vessel densities were determined essentially as described by Folkman and coworkers [15]. The scoring was performed blind without information on the identity of the treatment group. CD45+ or CD31+ vessel hotspots were identified by scanning the sections at low magnification using a 10× (NA = 0.3) plan-Neofluar objective. Micrographs were taken by an Axioplan2 epifluorescence microscope (Carl Zeiss) using 20× (NA = 0.5) plan-Neofluar objectives and Axiovision 4.3 software. The CD45+ cells or CD31+ vessels within each micrograph area (0.379 mm^2) were counted manually, and mean values ± standard error (SE) per tumor were calculated. In scoring of the vascularity, only CD31-positive structures that morphologically appeared as vascular were taken into

account. The thickness of TGF-β-treated ears in different groups was measured by scanning each sample for the thickest area of the ear. Subsequently, a micrograph was taken using a 5× objective and the total ear thickness (skin to skin) was measured using Studio Lite software. The groups were compared using the *t* test.

Results

TGF-β induces angiogenesis in vivo

We studied the in vivo effect of TGF-β on angiogenesis in healthy wild type C57BL/6 J mice injected s.c. with TGF-β, VEGF, or buffer every other day for 2 weeks. Macroscopic evaluation of the skin from the injection site revealed only few blood vessels in the control mice injected with buffer (Fig. 1a). In contrast, a highly elevated amount of blood vessels of varying sizes could be seen in the mice injected with TGF-β or VEGF. Markedly, the ears of the mice treated with TGF-β were several fold thicker than the ears of the animals injected with buffer or with VEGF (Fig. 1a, b). The elevated number of subcutaneous blood vessels in the TGF-β- or VEGF-treated mice was evident also when the endothelial cells were stained for the endothelial-specific marker CD31 (Fig. 1b, d). The mice injected with TGF-b (n = 6) had over two times higher vascular density than the control mice (n = 6) injected subcutaneously with PBS (mean 32, SD ± 5 vs. 12, SD ± 3 vascular elements per field, respectively; $P < 0.01$; Fig. 1c). Also the mice injected with VEGF (n = 6) had significantly higher vascular density when compared to the PBS controls ($P < 0.01$; Fig. 1c).

TGF-β-induced angiogenesis is accompanied by irregular vasculature and abnormalities of α-smooth muscle actin-expressing pericytes

A disorganized, irregular vascular pattern was observed in whole mounted tissues of the mice treated with TGF-β (Fig. 2). Typically, areas of dense microcapillary networks could be seen, and the blood vessel showed irregular variation in vascular diameter. In mice injected with buffer, α-smooth muscle actin α-SMA)-immunoreactive pericytes were abundant and had a tight contact with endothelial cells in vessels (Fig. 2). The distribution of α-SMA + cells in TGF-β-treated mice was different from that in PBS-treated mice since some of the smallest capillaries had attached α-SMA + pericytes. Unlike the tight association of pericytes and endothelial cells in normal vessels, the tissues from TGF-β-treated mice also contained single periendothelial cells that were immunoreactive for α-SMA but were only loosely attached to the blood vessels, or had no apparent association with the vessels (Fig. 2).

TGF-β causes massive recruitment of VEGF-expressing tissue-infiltrating hematopoietic cells

Very high numbers of tissue-infiltrating hematopoietic cells were observed at the injection site in the mice treated with TGF-β (Fig. 3a, b). Significantly, the recruited hematopoietic cells were strong producers of VEGF (Fig. 3b, c) In contrast, only a few infiltrating hematopoietic cells or cells expressing VEGF could be seen in the control mice injected with buffer. As a consequence of the hematopoietic cell infiltrate and increased vascularity, the thickness of the ears of the TGF-β-treated mice was several fold higher than that of the mice treated with buffer only (Fig. 3b). Many of the infiltrating perivascular BM-derived cells were in a very close contact with the blood vessel endothelium in the TGF-β-treated tissues (Fig. 3d).

TGF-β-induced angiogenesis is mediated via VEGF

To study the mechanism of action of the proangiogenic effect of TGF-β, we injected mice subcutaneously with TGF-β every other day for 2 weeks, and simultaneously treated the mice with VEGF immunoneutralizing mAbs or control IgG (Fig. 4; n = 6 in each group). The recruitment of tissue-infiltrating hematopoietic cells was not affected by the treatment with VEGF immunoneutralizing mAbs (Fig. 4b). Accordingly, when the TGF-β-induced thickening of the ears was studied, no significant differences were found between the thickness of ears in mice receiving systemic treatment with immunoneutralizing anti-VEGF mAbs, and in mice receiving the same amount of control IgG (mean 934 vs. 956 μm respectively; $P > 0.1$). However, TGF-β-induced blood vessel growth was significantly inhibited when the mice were treated with VEGF immunoneutralizing mAbs ($P < 0.01$; Fig. 4c).

Physiological concentrations of TGF-β induce VEGF mRNA and protein in human hematopoietic cells in a dose- and time-dependent manner

To test our findings in human hematopoietic cells, peripheral blood monocuclear cells (PBMNCs) were isolated from healthy donors. The freshly isolated cells were incubated for 14 h in serum free DMEM medium. Northern analyses demonstrated that treatment of PBMNCs with TGF-β resulted in a significant induction of the major 3.7 kb transcripts of VEGF. A 14-h incubation with TGF-β at 0.2 or 2 ng/mL enhanced VEGF mRNA levels 1.6 fold and 2.6 fold, respectively, over those in unstimulated cells

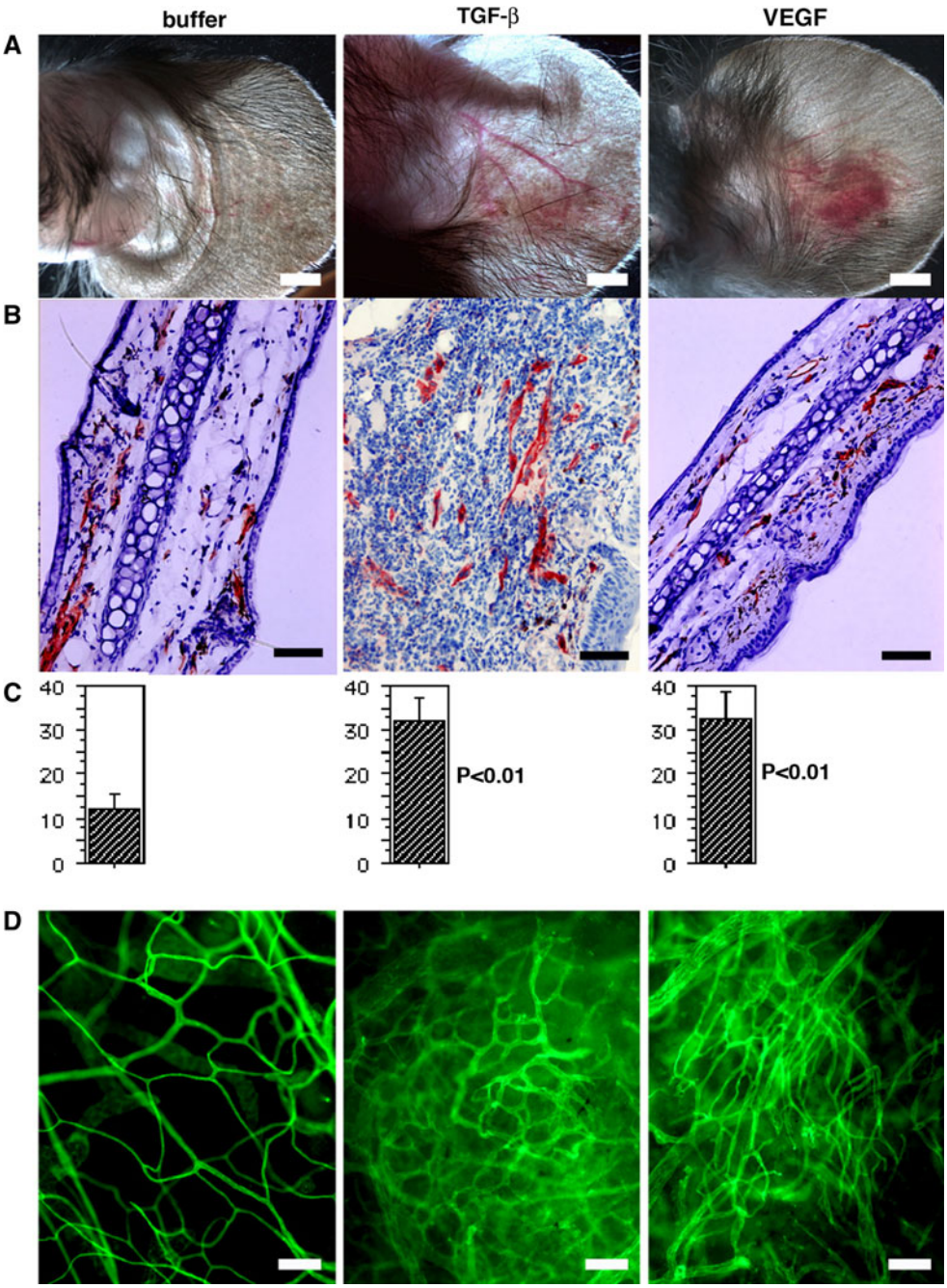

Fig. 1 TGF-β promotes angiogenesis in vivo. The in vivo effect of TGF-β on angiogenesis in wild type C57BL/6 J mice injected subcutaneously with buffer, TGF-β (60 ng/injection), or VEGF (60 ng/injection) every other day for 2 weeks. N = 6 in each treatment group. **a** The ears were photographed using a digital camera, *space bar* 2 mm. **b** Bright field microscopy of frozen sections immunostained for CD31, *space bar* 100 μm. Note the strong thickening of the ear injected with TGF-β. **c** Significantly elevated vascular density is observed in the mice with TGF-β or VEGF when compared to the control group injected with buffer. The frequency of CD31+ vascular structures is shown (mean ± SD). **d** Immunofluorescence microscopy of CD31 in whole mounted tissues also demontrates the angiogenic responce in mice injected with of TGF-β or VEGF, *space bar* 100 μm

(Fig. 5a). Four isoforms of VEGF mRNA are detected by RT-PCR analysis both in nonstimulated and TGF-β-stimulated PBMNCs. Amplification of cDNA from unstimulated PBMNCs and cells stimulated for 24 h with TGF-β (2 ng/mL) give rice to four bands of 243, 375, 447, and 498 base pairs (Fig. 5b). These are the sizes predicted for the amplification products for transcripts encoding for VEGF121, VEGF165, VEGF189, and VEGF206, based on the published cDNA sequences of the VEGF mRNA splice variants [16, 17]. The mRNAs encoding for the smaller, freely diffusible VEGF isoforms VEGF121 and VEGF165 were the major VEGF mRNAs both in untreated and TGF-β-stimulated cells. The effect of TGF-β on VEGF protein production was examined next. PBMNCs cultured for 24 h in serum free DMEM medium in the absence of TGF-β released VEGF continuously into the surrounding medium (Fig. 5c). Stimulation of the cells with TGF-β (2 ng/mL) caused a significant increase in VEGF secretion. After 24 h stimulation, the mean VEGF production of TGF-β stimulated cells (82 pg/10^6 cells) was over two-fold higher than

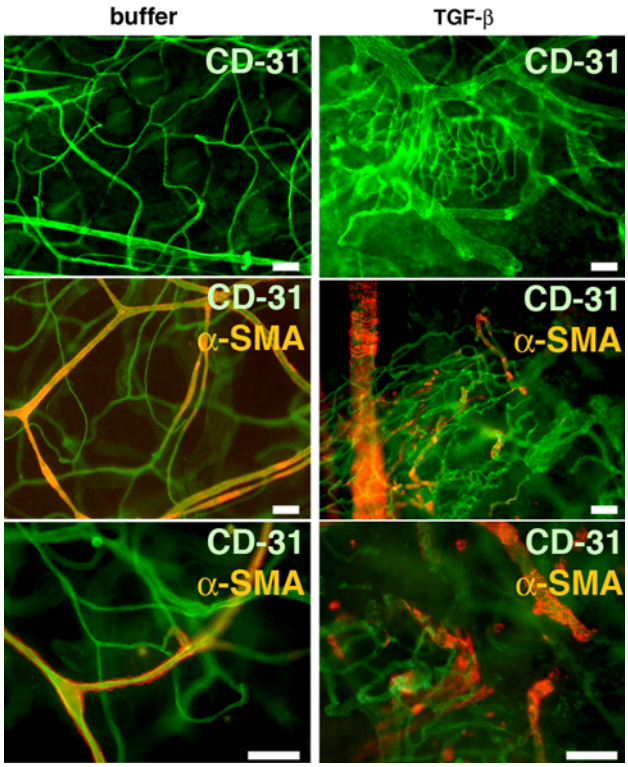

Fig. 2 TGF-β induces irregular microvasculatureand promotes abnormalities of α-smooth muscle actin-expressing pericytes. Immunofluorescence micrographs of whole mounted tissues of mice injected with buffer or TGF-β are shown. Dense microcapillary networks and irregular variation of vessel diameter can be seen in tissues injected with TGF-β. The tissues from TGF-β-treated mice also contain single periendothelial cells that are immunoreactive for α-SMA but only loosely attached to the blood vessels, or have no apparent association with the vessels. *Space bars* 50 μm

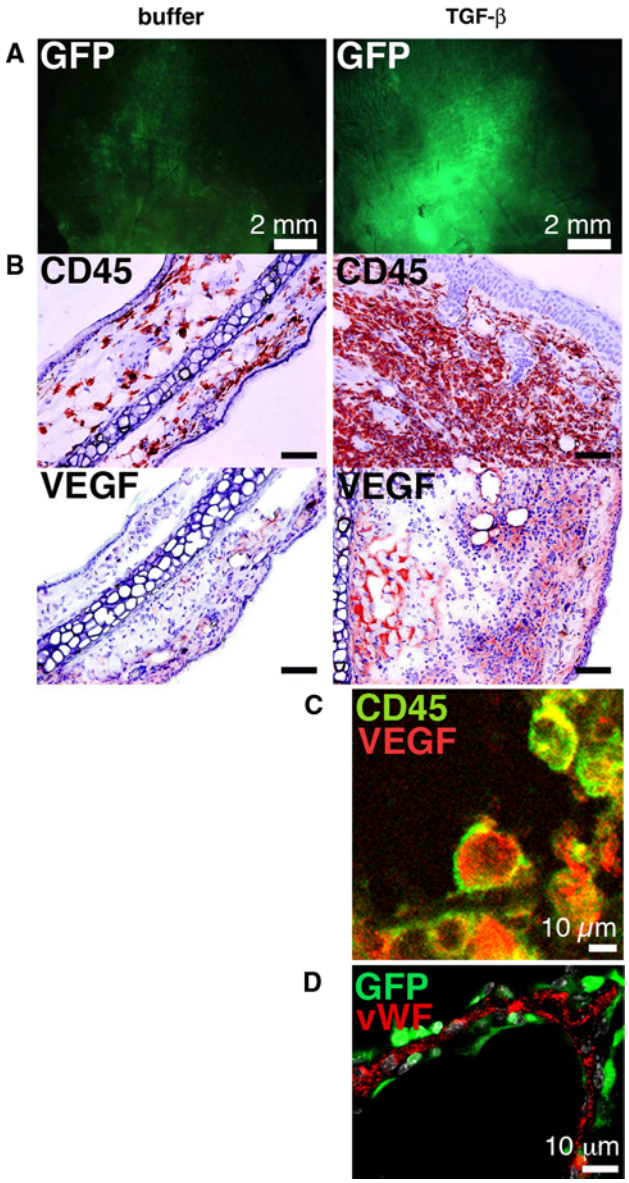

Fig. 3 TGF-β causes massive recruitment of tissue-infiltrating hematopoietic VEGF-expressing cells. Mice with genetically GFP-tagged hematopoietic system or detection of the pan-hematopoietic marker CD45 were used to identify the tissue-infiltrating hematopoietic cells. **a** Immunofluorescence photographs of whole ears demonstrate the massive recruitment of GFP-tagged hematopoietic cells in tissues treated with TGF-β. **b** Bright field micrographs of frozen sections stained for CD45 or VEGF demonstrate the massive infiltrate of VEGF-expressing hematopoietic cells in mice injected with TGF-β. Note also the strong thickening of the TGF-β-treated tissues. *Space bars* 100 μm. **c** Confocal scanning confirms the localization of VEGF in CD45+ hematopoietic cells. *Space bar* 10 μm. **d** Confocal section shows the close contact of GFP-tagged perivascular BM-derived cells with the underlying vascular ECs. *Space bar* 10 μm

that of nonstimulated cells (37 pg/10^6 cells; Fig. 5c). In cultures both with and without TGF-β, 1 mmol/L cycloheximide completely blocked VEGF release into the media (Fig. 5c and data not shown). The concentration dependence by TGF-β induction of VEGF protein synthesis/release was next determined. The PBMNCs were incubated for 24 h in serum free DMEM medium with various concentrations of TGF-β. At a concentration of TGF-β as low as 0.02 ng/mL an increase in VEGF secretion was detectable. Higher concentrations of TGF-β further increased VEGF secretion (Fig. 5d).

Induction hematopoietic cell VEGF production by TGF-β1 is p38 MAPK and p44/p42 MAPK (Erk1/Erk2) dependent

We wanted to study the major regulatory pathways involved in TGF-β-stimulated VEGF synthesis in hematopoietic cells (Fig. 6). Human PBMNCs were incubated for 12 h in serum free conditions with or without TGF-β (at 2 ng/mL). The cells were pretreated with the blocking agents for 60 min prior to adding TGF-β or buffer. Blocking of the p38 MAPK pathway by a specific inhibitor

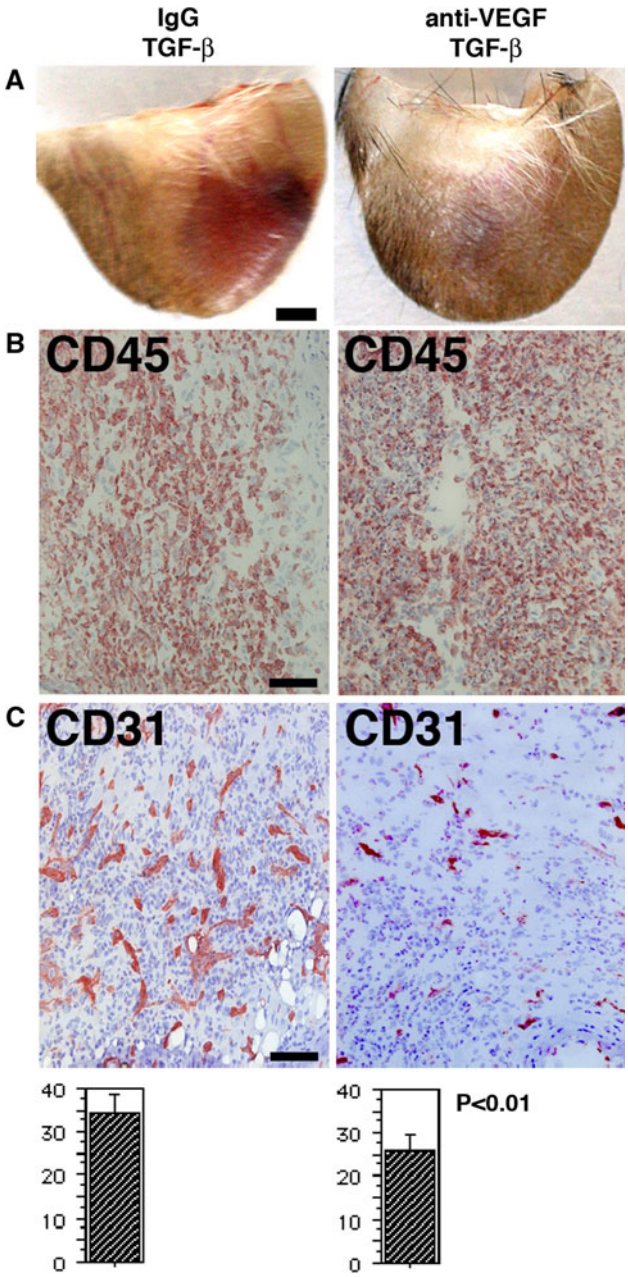

Fig. 4 Treatment with VEGF immunoneutralizing mAbs blocks TGF-β-induced blood vessel growth. Mice were injected with TGF-β (60 ng/injection) for 2 weeks and simultaneously treated with VEGF immunoneutralizing mAbs or control IgG. **a** The ears were photographed using a digital camera. *Space bar* 2 mm. **b** TGF-β-induced hematopoietic cell recruitment is not affected in mice treated with VEGF immunoneutralizting mAbs. Bright field micrographs of tissue-infiltrating CD45+ cells are shown. *Space bar* 100 µm. **c** TGF-β-induced blood vessel growth is blocked in mice treated with VEGF immunoneutralizting mAbs. Bright field micrographs of CD31 vessels are shown. *Space bar* 100 µm. The frequency of CD31+ vascular structures is also indicated (mean ± SD; n = 6 in each group)

PD169316 at 10 µmol/L inhibited strongly the nonstimulated, steady state VEGF production (median, 36 pg/10^6 untreated cells, vs. 16 pg/10^6 in PD169316 treated cells; $P < 0.001$, the t test; Fig. 6a) and also prevented completely the induction of VEGF production by TGF-β (median, 15 pg/10^6 in TGF-β and PD169316 treated cells; $P > 0.5$ when compared to cells treated with PD169316 only; Fig. 6a). Similarly, the specific p44/p42 MAPK (Erk1/Erk2) pathway inhibitor PD98059 (at 10 µmol/L) had a strong suppressing effect on the steady state VEGF production of the cells (median, 36 pg/10^6 untreated cells, vs. 24 pg/10^6 in PD98059 treated cells; $P < 0.001$; Fig. 6a). PD98059 also blocked the induction of VEGF production by TGF-β (median, 27 pg/10^6 in TGF-β and PD98059 treated cells; $P > 0.5$ when compared to cells treated with PD98059 only; Fig. 6a). To confirm our findings in vivo, we next studied the effect of TGF-β on MAPK signaling in mice by using monoclonal antibodies specifically recognizing the activated, phosphorylated forms of p38 or p44/p42 MAPKs. Active forms of p38 and p44/p42 MAPKs were observed in only sporadic cells in the ears of the mice injected with buffer (Fig. 6b). Activated p38 or p44/p42 could particularly be seen in the cells within hair follicles. In line with our in vitro findings, TGF-β caused in vivo a strong increase of active, phosphorylated forms of the MAPKs. When the mice were treated with TGF-β, activated p38 or p44/p42 could be seen in numerous tissue-infiltrating hematopoietic cells (Fig. 6b).

Discussion

The mechanisms by which TGF-β is able to induce angiogenesis in vivo have not been well defined. Previously, the application of TGF-β to chicken chorioallantoic membrane or in rabbit cornea has been shown to result in a dose-dependent, gross angiogenic response which is associated with an influx of a vast number of inflammatory cells [4, 7]. VEGF has earlier been shown to be induced in vitro in fibroblastic and epithelial cell lines in response to TGF-β, suggesting that the angiogenic effect of TGF-β in vivo might be mediated by a paracrine induction of VEGF [18]. It has also been shown in vitro that TGF-β may induce mouse peritoneal macrophages and dendtritic cell lines to produce VEGF [19, 20].

Perivascular tissues in various angiogenic situations are often characterized by numerous tissue-infiltrating BM-derived cells [21–25]. There exist now compelling evidence that hematopoietic cells indeed play an important role in promoting angiogenesis in a paracrine manner by expressing various factors (including VEGF) that promote the growth and expansion of de novo vessels from the pre-existing vasculature—either by stimulating ECs or by remodeling the extracellular matrix [26–33]. BM-derived proangiogenic VEGF-producing cells are heterogeneous and include myeloid and other blood cells such as

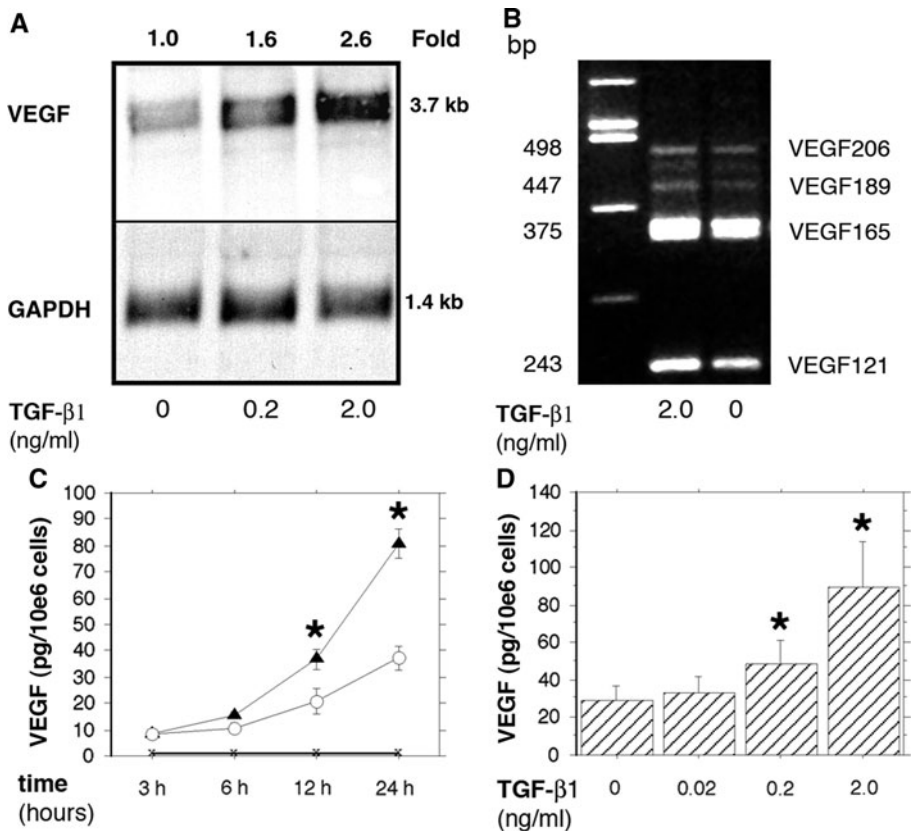

Fig. 5 TGF-β induces VEGF mRNA and protein in human hematopoietic cells in a dose- and time-dependent manner. **a** Northern analysis of the dose-dependent induction of the VEGF gene in PBMNCs after TGF-β treatment. A significant induction of the major 3.7 kb transcripts of VEGF can be seen. Relative VEGF mRNA levels represent arbitrary units normalized to GAPDH mRNA levels. VEGF mRNA expression is enhanced 1.6 fold and 2.6 fold with TGF-β1 induction at 0.2 and 2 ng/mL respectively, compared to untreated cells. **b** RT-PCR analysis of the VEGF splice variant expression pattern in PBMNCs stimulated with TGF-β1 (2 ng/mL) compared to untreated cells. The mRNAs for the freely diffusible VEGF isoforms VEGF121 and VEGF165 are dominant in the analysis. **c** Time course of VEGF protein secretion (pg/10^6 cells) by PBMNCs incubated in serum free medium (o), by PBMNCs treated with TGF-β1 at 2 ng/mL (*filled triangle*), and by PBMNCs treated with a combination of TGF-β1 (2 ng/mL) and cycloheximide at 1 mmol/L (X). The values are given as means $\pm$ 1 SE of seven independent experiments.* $P < 0.05$. **d** Dose-dependent secretion of VEGF (pg/10^6 cells) by PBMNCs stimulated with TGF-β. The values are given as means $\pm$ 1 SE of six independent experiments.* $P < 0.05$

monocytes, macrophages, neutrophils, eosinophils, mast cells, dentritic cells, T- and B-lymphocytes, and platelets [26, 27, 30, 31, 33–35]. In the present study we demonstrate that TGF-β promotes angiogenesis in vivo by causing a massive recruitment of tissue-infiltrating hematopoietic cells, and by inducing them to produce VEGF. This dual action of TGF-β then results in induction of angiogenesis and vascular remodeling involving irregular enlargement of blood vessels. Physiological TGF-β concentrations stimulated VEGF secretion in hematopoietic cells in a dose-dependent manner, representing an induction of de novo VEGF synthesis. The angiogenic effect induced by TGF-β was strongly inhibited by VEGF neutralizing antibodies. In addition to inducing angiogenesis with areas of dense microcapillary networks, treatment with TGF-β resulted in enlargement of capillaries, and the blood vessel showed irregular variation in vascular diameter. This increase of vascular diameter was similar to what has earlier been described during microvascular remodeling in chronic inflammation [36]. TGF-β also promoted multiple abnormalities on α-SMA-expressing pericytes. These pericyte abnormalities are similar to those described earlier in tumor vasculature [37]. In line, malignant cells often secrete TGF-β [38]. The effect of TGF-β on α-SMA + pericytes may not be surprising, since TGF-β is known to be a regulator of vascular smooth muscle cell differentiation, migration and organization [39, 40]. In conclusion, our present results provide a novel mechanism by which TGF-β promotes angiogenesis in vivo via recruitment of paracrine VEGF-expressing hematopoietic effector cells. This VEGF-mediated mechanism of action for TGF-β may affect the

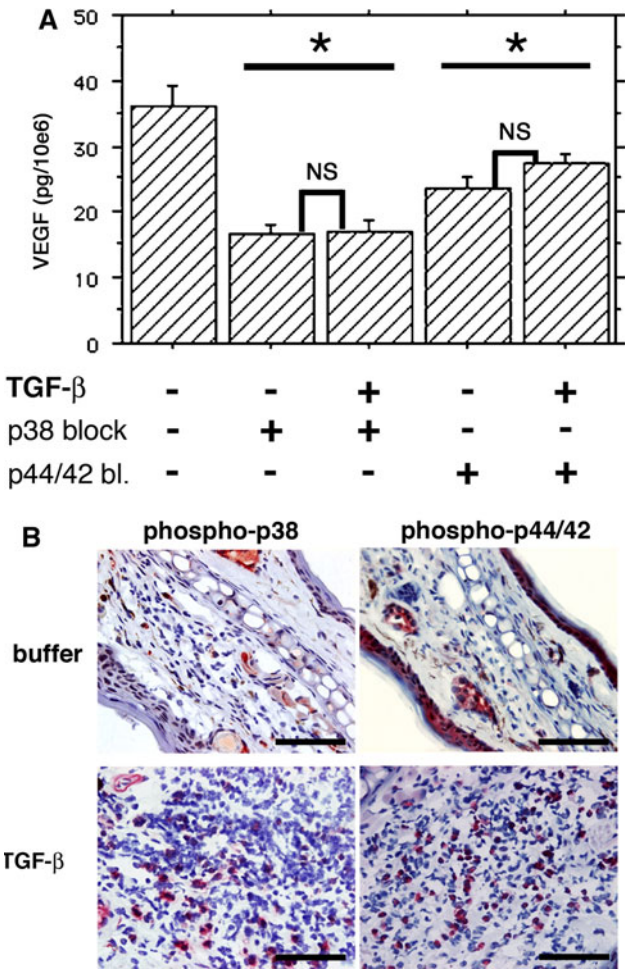

Fig. 6 Induction of hematopoietic cell VEGF production by TGF-β is p38 MAPK and p44/p42 MAPK dependent. **a** Blocking the p38 MAPK pathway with a specific inhibitor (PD169316) strongly inhibited the nonstimulated, steady state VEGF production of isolated human mononuclear cells, and also prevented completely the induction of VEGF production by TGF-β. Similarly, inhibiting the p44/p42 MAPK pathway using a specific inhibitor (PD98059) had a strong suppressing effect on the steady state VEGF production, and also blocked the induction of VEGF production by TGF-β. Secretion of VEGF (pg/10^6 cells) by mononuclear cells is shown. The values are given as means $\pm$ 1 SE of four independent experiments performed in triplicates. * $P < 0.001$, the t test. *NS* non significant. **b** In vivo in mice, the active, phosphorylated forms of p38 and p44/p42 MAPKs were observed in only sporadic cells in the ears of the mice injected with buffer (*panels* in the *upper row*). Activated p38 or p44/p42 could particularly be seen in the cells within hair follicles. Treatment with TGF-β caused a strong increase of phosphorylated forms the MAPKs in numerous infiltrating hematopoietic cells. Please note that the magnification is the same in all the *panels*, but the ears of the mice injected with TGF-β are greatly thickened. *Space bars* 100 μm

angiogenic balance during processes such as inflammatory conditions and tumor growth where TGF-β activity is upregulated.

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