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Upregulated autocrine vascular endothelial growth factor (VEGF)/VEGF receptor-2 loop prevents apoptosis in haemangioma-derived endothelial cells

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Conflicts of interest
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Summary

Background The autocrine vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR)-2 loop is required to maintain the transformed phenotype of many tumours, in part, by preventing apoptotic cell death in response to many different stimuli. However, it is unclear whether constitutive VEGF/VEGFR-2 activation in haemangioma-derived endothelial cells (HaemECs) can lead to a general suppression of apoptosis.

Objectives The objective of this study was to investigate whether the autocrine VEGF loop promotes HaemEC survival via its receptor, VEGFR-2.

Methods HaemECs and human umbilical vein endothelial cells (HUVECs) were serum-starved for 12–48 h. Cell apoptosis was measured. The potential mechanisms of VEGF/VEGFR-2-induced HaemEC survival were investigated, and the role of the autocrine VEGF/VEGFR-2 loop in preventing propranolol-induced apoptotic HaemEC death was also analysed.

Results Compared with HUVECs, HaemECs showed increased resistance to apoptosis induced by serum starvation. Upregulated VEGF/VEGFR-2 signalling in HaemECs induced an autocrine signalling loop, which resulted in Akt activation. Furthermore, this activation of Akt was necessary for VEGF/VEGFR-2-induced protection against serum deprivation-induced HaemEC apoptosis. In addition, Bcl-2, which functions as an anti-apoptotic factor and direct downstream target of PI3K/Akt, was decreased by the inhibition of VEGF/VEGFR-2, which led to an increase in caspase-3 activity, caspase-9 activity and HaemEC apoptosis. Moreover, HaemECs acquired greater resistance to propranolol treatment than HUVECs, whereas inhibition of VEGF/VEGFR-2 signalling in HaemECs sensitized these cells to propranolol-induced apoptosis.

Conclusions Our results demonstrate that upregulation of the autocrine VEGF/VEGFR-2 loop can induce general resistance to apoptotic stimuli in HaemECs.

What's already known about this topic?

- Haemangioma-derived endothelial cells (HaemECs) exhibit an X-chromosome inactivation pattern of clonality and show upregulation of vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR)-2 signalling pathways.
- Propranolol induces apoptosis in cultured vascular ECs, including HaemECs.

What does this study add?

- Compared with 'normal' ECs, HaemECs showed increased resistance to apoptosis induced by serum starvation and β-blockers.
- The upregulated autocrine VEGF/VEGFR-2 loop can induce general resistance to apoptotic stimuli in HaemECs.

Infantile haemangiomas (IHs) are the most common vascular tumour of childhood, affecting 5–10% of all infants.[1,2] One defining feature of IHs is the dramatic growth and development of a disorganized mass of blood vessels. Subsequently, a slow spontaneous involution begins at approximately 1 year of age and continues for 7–10 years.[3] Previous studies of apoptosis in proliferating and involuting haemangiomas have shown that the rate of apoptosis is substantially increased as the tumours involute,[4,5] and at least one-third of the apoptotic cells in involuting haemangiomas have been identified as endothelial cells (ECs).[6] This high level of EC apoptosis, despite ongoing cellular proliferation, may explain the decreased rate of haemangiomatous growth observed in the late proliferative and involuting phases of the tumour life cycle.

For most children with IH, the lesions are small and pose no threat or potential for complication, but in some cases, IHs grow dramatically and destroy tissue, impair function and can even threaten life.[7] Past efforts to elucidate the mechanisms underlying the angiogenesis and vasculogenesis observed within tumours have revealed that haemangioma-derived endothelial cells (HaemECs) are distinct from the ECs lining normal vessels at both the transcriptional and functional levels. These comparative studies, which focused on differences in canonical biochemical signalling pathways, revealed that HaemECs exhibit altered growth factor signalling because of the differential expression of important growth factors and their receptors.[8–10] For example, vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (KDR/Flk-1) have been a major focus of research due to their importance in numerous aspects of vascular development and pathologic angiogenesis. VEGF regulates EC proliferation, migration and differentiation via interactions with VEGFR-2.[11] Furthermore, previous studies have shown that cultured HaemECs demonstrate the phenotype of a constitutively active autocrine VEGF/VEGFR-2 loop, which renders the cells more sensitive to paracrine/external stimulation with VEGF and results in increased proliferation and migration of cells as well as tumour formation.[8,12]

In addition to stimulating EC proliferation and migration, VEGF concomitantly inhibits EC apoptosis. Of the various endothelial growth factors, VEGF seems to play a critical role in protecting ECs against apoptotic cell death, and this inhibition of EC apoptosis may improve angiogenesis and vasculogenesis in patients with ischaemia.[13,14] Furthermore, increased VEGF expression or VEGFR-2 activity has also been observed in many human tumour cell lines from various lineages and diseases and is thought to promote increased cell survival.[11,15] Previous studies also support the involvement of a VEGF-dependent autocrine loop in haematopoietic stem cell and malignant cell survival, which leads to the activation of VEGFR-2.[16–18] However, it is unknown whether the autocrine VEGF/VEGFR-2 signalling in HaemECs may exert a similar effect on cell survival.

Herein, we report on a series of studies that were performed to determine whether autocrine VEGF signalling could manipulate HaemEC survival. We focused on VEGF because of its key role as a major angiogenic factor in IHs. Our findings demonstrate that persistent activation of VEGFR-2 in HaemECs, along with the functionality of the upregulated autocrine VEGF loop, play critical roles in the survival of HaemECs.

Materials and methods

Reagents and antibodies

Endothelial basal medium (EBM-2) and SingleQuot, which contains human epidermal growth factor, VEGF, human basic fibroblast growth factor, insulin-like growth factor, hydrocortisone, heparin, ascorbic acid and gentamicin/amphotericin B, were obtained from Lonza (Walkersville, MD, U.S.A.). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, U.S.A.). Propranolol, ICI118551, metoprolol, bovine serum albumin (BSA), Hoechst 33342 and the PI3K inhibitor LY294002 were purchased from Sigma (St Louis, MO, U.S.A.). The VEGFR-2 inhibitor PTK787 was obtained from Novartis Pharmaceuticals (Basel, Switzerland). The human VEGF ELISA (enzyme-linked immunosorbent assay) kit, human recombinant VEGF-A and VEGF-A neutralizing antibody (VEGF Ab) were purchased from R&D Systems (Minneapolis, MN, U.S.A.). The fluorescein–isothiocyanate (FITC)-labelled annexin-V/propidium iodide (PI) apoptosis detection kit and the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay kit were obtained from Calbiochem (Darmstadt, Germany) and Promega (Madison, WI, U.S.A.), respectively. The caspase-3 and -9 colorimetric assay kits were purchased from Biovision (Mountain, CA, U.S.A.). The primary polyclonal antibodies recognizing the VEGF-A and Bcl-2 proteins were purchased from Santa Cruz Biotechnology (Delaware, CA, U.S.A.). The antibodies for VEGFR-2, phospho-VEGFR-2 (Tyr1175), Akt and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Boston, MA, U.S.A.).

Preparation of haemangioma specimens

This study was approved by the Ethics Committee of the Children's Hospital of Fudan University. Proliferating IH tissues were surgically removed from a female patient who was referred to our department due to a rapidly growing scalp mass. Written informed consent was obtained from the patient's parents for all tissues obtained for the study. The clinical diagnosis of the vascular neoplasm was confirmed by the Department of Pathology at the Children's Hospital of Fudan University based on staining for GLUT-1, a marker specific for haemangioma tissue. The tissues were used immediately for cell isolation.

Cell extraction, isolation and culture

HaemEC extraction and isolation were performed as previously described.[19,20] The purity of the HaemECs was > 95%, as determined by positive von Willebrand factor and CD31 expression and by negative expression of vimentin (fibroblasts) and α-actin (vascular smooth muscle cells). Primary human umbilical vein endothelial cells (HUVECs) were

obtained from the Chinese Academy of Sciences (Shanghai, China). ECs were cultured on gelatin-coated 60-mm plates in EBM-2 medium supplemented with 20% heat-inactivated FBS, SingleQuot, penicillin (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}). The cells were grown in humidified air containing 5% CO_2 at 37 °C. Cells at passages 3–6 were used for all experiments.

After a 24-h incubation period to allow for cell attachment in standard medium, the cells were washed twice with phosphate-buffered saline (PBS). Apoptosis was induced by serum deprivation using EBM supplemented with 0·1% BSA and penicillin–streptomycin in the absence of FBS. SingleQuot was excluded because this agent affects cell survival.[21]

Nuclear fragmentation and TUNEL staining

Nuclear fragmentation was detected in fixed (4% paraformaldehyde) cells either by incubating in 10 μmol L^{-1} Hoechst 33342 or by TUNEL staining with a commercially available kit using fluorescein-12-dUTP for detection. The nuclear structure of the cells was examined using an Olympus fluorescence microscope. Quantitative analysis was performed by counting the green fluorescent (i.e. apoptosis-positive) cells under 400× magnification in three randomly selected fields. The values are expressed as the percentage of apoptotic cells relative to the total number of cells per field. At least two dishes were counted in this manner for each experiment, and at least three experiments were performed for each manipulation.

Annexin-V/propidium iodide staining assay

After treatment, both floating and attached cells were harvested and combined before further processing. The apoptosis-mediated cell death of ECs was examined with a double-staining method using a FITC-labelled annexin-V/PI apoptosis detection kit according to the manufacturer's instructions. The data for $3{\cdot}0 \times 10^4$ cells were collected in the list mode.

Enzyme-linked immunosorbent assay for vascular endothelial cell growth factor

HaemECs or HUVECs were plated at $1{\cdot}0 \times 10^5$ cells per well in 24-well plates in complete growth medium. After 24 h, cells were washed in PBS and incubated in serum-free medium containing 0·1% BSA. The cells were incubated for 24 h, and the conditioned media were collected, centrifuged and stored at −80 °C. VEGF levels in conditioned media were determined using the Human VEGF ELISA kit according to the manufacturer's protocol.

Caspase colorimetric assay

Caspase activation analysis was performed using the caspase-3 and caspase-9 colorimetric assay kits. This assay is based on the spectrophotometric detection of the p-nitroaniline (pNA) chromophore after its cleavage by caspase-3 or caspase-9 from the labelled substrate, CEVD-pNA. The absorbance produced by the pNA chromophore was measured using an automatic microplate reader at 405 nm. Caspase activity was presented as a percentage of the control.

Western blot analysis

The cells were harvested using RIPA B lysis buffer containing a protease inhibitor cocktail. The protein concentration was determined using the Bradford protein assay kit. The protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with TBST (10 mmol L^{-1} Tris-HCl, pH 7·4, 150 mmol L^{-1} NaCl, 0·1% Tween 20) containing 5% (wt/vol) nonfat dry milk and incubated with primary antibody in TBST. Next, the membrane was washed three times and incubated with the appropriate secondary antibody. The protein bands were visualized using enhanced ECL-associated fluorography.

All data are expressed as the mean ± SD for all paired statistical comparisons. An analysis of variance (ANOVA) followed by Tukey's *t*-test was performed, and a P-value $< 0{\cdot}05$ was considered statistically significant.

Results

Haemangioma-derived endothelial cells respond abnormally to serum starvation

One of the most peculiar characteristics of ECs is that they undergo apoptosis in the absence of serum. Therefore, we established this system to efficiently and reproducibly induce apoptosis in HaemECs and HUVECs. Subconfluent cells were serum-starved for 12–48 h. To quantify apoptosis the number of TUNEL-positive cells was counted, and the annexin-V/PI apoptosis detection assay was performed (Fig. 1a,b). Serum-deprived HUVECs had a much higher prevalence of apoptosis and demonstrated typical apoptotic nuclei when cultured for 12 h in comparison with cells grown in 10% serum-containing medium. In contrast, HaemECs resisted apoptosis when cultured under serum-free conditions for 12 h. When HaemECs were cultured in serum-free medium for 24 and 48 h, an increasing fraction of the cells became apoptotic, although the apoptotic rate of the HaemECs remained significantly lower than that of the HUVECs (Fig. 1c). Therefore, these data indicate that a survival mechanism rescued the HaemECs from apoptosis under serum-free conditions.

Vascular endothelial cell growth factor receptor-2 activation promotes haemangioma-derived endothelial cell survival

A previous study reported that VEGF/VEGFR-2-dependent signal transduction is upregulated in proliferating HaemECs compared with normal ECs,[8] and similar results were obtained

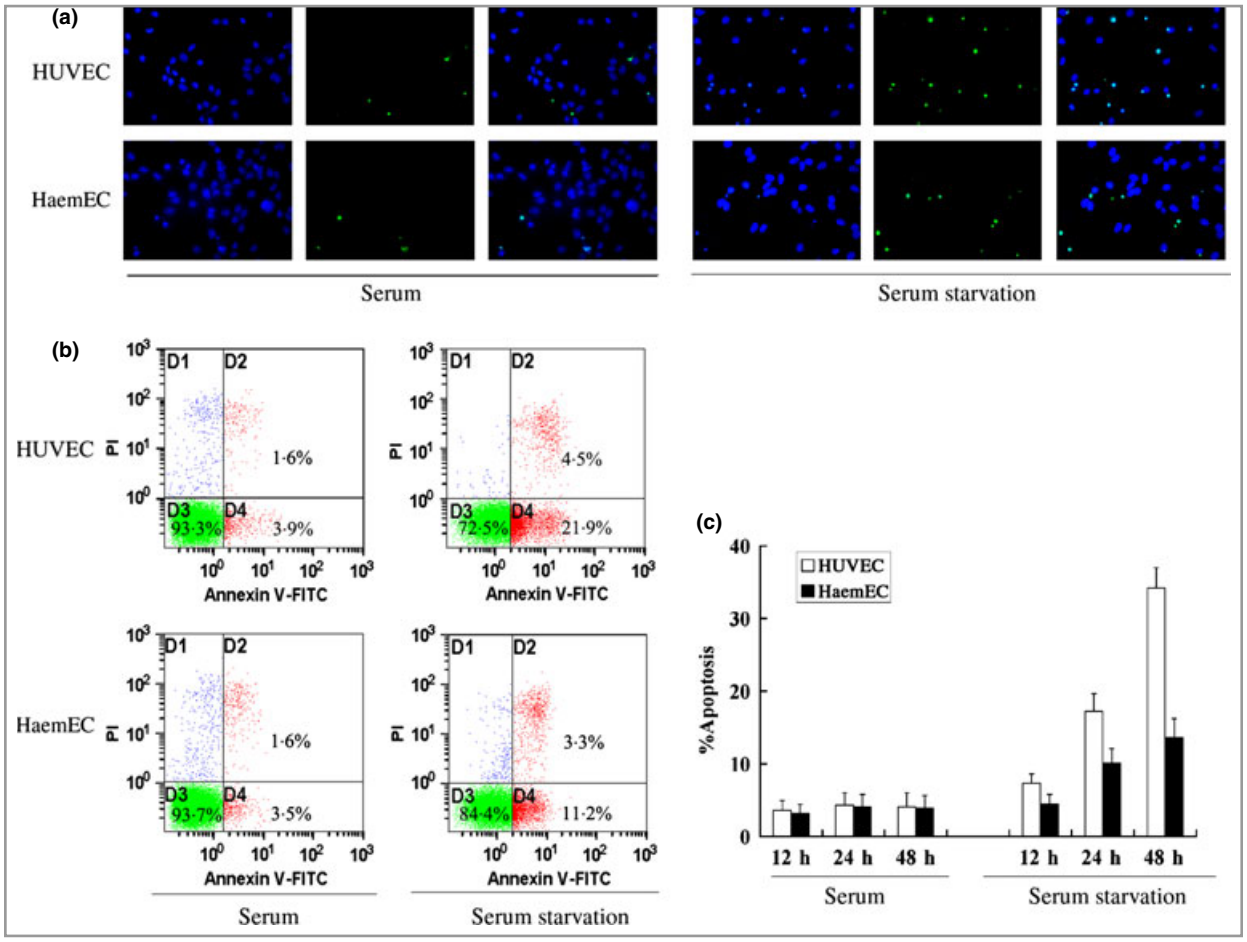

Fig 1. Resistance to apoptosis in HaemECs compared with HUVECs. (a) Representative fields of cultured ECs under serum or serum-free conditions for 24 h. Each culture was simultaneously stained for nuclear morphology (Hoechst 33343 and TUNEL). (b) Typical quadrant analysis of annexin-V/PI flow cytometry to evaluate EC apoptosis. ECs were cultured in the presence of 10% FBS or in serum-free conditions for 24 h. The data for $3{\cdot}0 \times 10^4$ cells were collected in the list mode. (c) Percentage of apoptotic (fragmented) nuclei in ECs. Apoptosis was evaluated using the TUNEL assay after 12–48 h of serum starvation. The results are shown as the mean ± SD of duplicated assays from one of three identical experiments. EC, endothelial cell; FBS, fetal bovine serum; HaemEC, haemangioma-derived endothelial cells; HUVEC, human umbilical vein endothelial cells; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling method.

by Western blot, as shown in Figure 2a. The activation of VEGFR-2 was markedly increased in HaemECs compared with HUVECs in serum-free conditions. To gain further insight into VEGFR-2 signalling in HaemECs, we assessed VEGF-A secretion by HaemECs and HUVECs. These cell subsets were placed in growth factor-free media for 24 h, and the VEGF-A levels of the resulting conditioned media were then analysed by ELISA. VEGF-A protein production was undetectable in conditioned media from HUVECs. However, elevated levels of VEGF were detected in the media from HaemECs (data not shown).

Constitutively active VEGFR-2 signalling enables the increased proliferation and altered migratory properties of HaemECs,[8] which suggests that the resistance to apoptosis in HaemECs may also be attributed to upregulated VEGFR-2 signalling. To test this hypothesis, we pretreated HaemECs with PTK787, a tyrosine kinase inhibitor that potently targets VEGFR-2. We also inhibited VEGF/VEGFR-2 signalling using the neutralizing VEGF Ab. As shown in Figure 2b, treatment of HaemECs with PTK787 or the VEGF Ab abolished the basal level of VEGFR-2 autophosphorylation and Akt activity. We further demonstrated that neither PTK787 nor the VEGF Ab had an effect on cell survival when HaemECs were cultured in 10% serum-containing medium (data not shown), whereas both agents increased cell sensitivity to serum starvation-induced apoptosis. In contrast to the inhibition of survival mediated by VEGF Ab treatment in HaemECs, this agent did not have a similar effect on control ECs (Fig. 2c). Together, these findings indicate that VEGF/VEGFR-2-mediated survival signals can override cell death signals in response to serum deprivation in HaemECs.

Haemangioma-derived endothelial cells maintain higher Bcl-2 levels after serum starvation

Given the observation that external VEGF promotes the survival of serum-starved ECs by increasing the level of

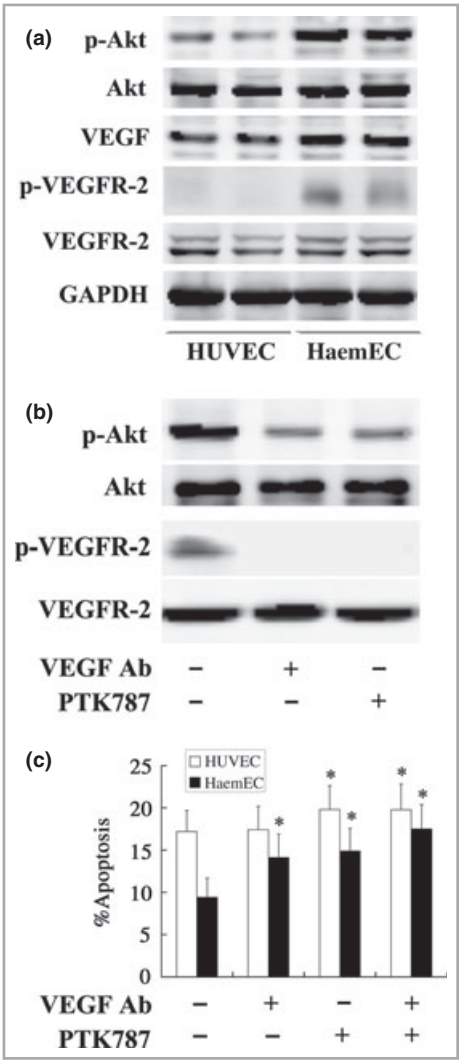

Fig 2. VEGFR-2 activation protects HaemECs from serum deprivation-induced apoptosis. (a) Western blot analysis of VEGF, VEGFR-2, phospho-VEGFR-2 (Tyr1175), Akt and phospho-Akt (Ser473) levels in HaemECs and HUVECs after 24 h in serum-free conditions. The blot was stripped and reprobed with an antibody that recognizes GAPDH to confirm equal loading. Upregulation of VEGFR-2 activation was accompanied by upregulation of the active form of Akt in HaemECs. (b) VEGFR-2 activity was undetectable in HaemECs when treated with either the neutralizing VEGF Ab or PTK787. The Akt activity was also significantly inhibited following treatment with these agents. Serum-deprived HaemECs were treated with the VEGF Ab (2 μg mL^{-1}) for 24 h or with PTK787 (10 nmol L^{-1}) for 2 h, and VEGFR-2 activity was analysed by Western blot. (c) ECs were grown for 24 h under serum-free conditions, in the absence or presence of the VEGF Ab, or were treated with PKT787 for 2 h or the combination of VEGF Ab and PTK787. The percentage of TUNEL-positive nuclei for each treatment was calculated as described in 'Materials and methods'. The results are shown as the mean ± SD and were statistically analysed by ANOVA. *$P < 0.05$ when compared with the control group. Ab, antibody; EC, endothelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HaemEC, haemangioma-derived endothelial cells; HUVEC, human umbilical vein endothelial cells; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling method; VEGF, vascular endothelial cell growth factor; VEGFR, VEGF receptor.

the anti-apoptotic protein Bcl-2,[22] we investigated whether the autocrine VEGF loop exerts a similar function on the expression of Bcl-2. To do so, we examined the levels of Bcl-2 in ECs. As shown in Figure 3a, a dramatic decrease in Bcl-2 level was consistently observed in HUVECs compared with HaemECs under serum-free conditions. Next, the neutralizing VEGF Ab and PTK787 were used to examine the involvement of VEGF/VEGFR-2 on Bcl-2 expression. As shown in Figure 3a,b, the protein levels of Bcl-2 were decreased when HaemECs were treated with either the VEGF Ab or PTK787.

Akt activation is necessary for VEGF/VEGFR-2-mediated resistance to apoptosis in haemangioma-derived endothelial cells

As shown in Figure 2, Akt is activated in HaemECs as a result of constitutive VEGFR-2 signalling in serum-free conditions. This finding suggests that VEGF/VEGFR-2-mediated cell survival may occur through the activation of general survival pathways, such as the PI3K/Akt pathway. To test this possibility, a specific inhibitor of PI3K was used, and the results indicated that LY294002 potently restored the sensitivity of HaemECs to serum deprivation-induced apoptosis as measured by TUNEL staining (Fig. 4a). In addition, treatment of cells with LY294002 also reversed the inhibitory effects of recombinant VEGF on the frequency of TUNEL-positive cells, and the inhibition of PI3K/Akt signalling in HaemECs was accompanied by a reduction in Bcl-2 expression and increased caspase-3 and caspase-9 activation (Fig. 4b,c). These findings highlight the involvement of the PI3K/Akt pathway in the survival of HaemECs.

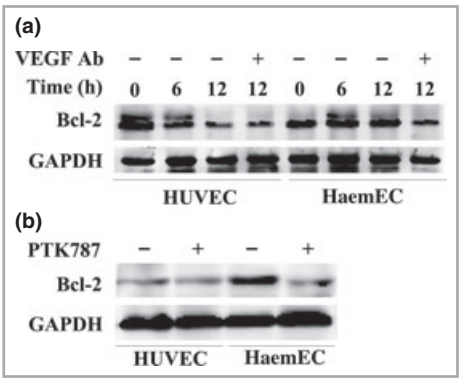

Fig 3. Autocrine VEGF/VEGFR-2 loop maintains Bcl-2 expression in HaemECs. (a) ECs were cultured in serum-free conditions in the presence or absence of 2 μg mL^{-1} of VEGF Ab. The expression of Bcl-2 protein was then detected. GAPDH was used as an internal control. (b) PTK787 treatment decreased the Bcl-2 expression in HaemECs. Immediately after exposing HaemECs to serum starvation conditions, PTK787 (10 nmol L^{-1}) was added to the cells. The cells were harvested after 24 h, and Bcl-2 expression was analysed by Western blot. Data are representative of three independent experiments. Ab, antibody; EC, endothelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HaemEC, haemangioma-derived endothelial cells; VEGF, vascular endothelial cell growth factor; VEGFR, VEGF receptor.

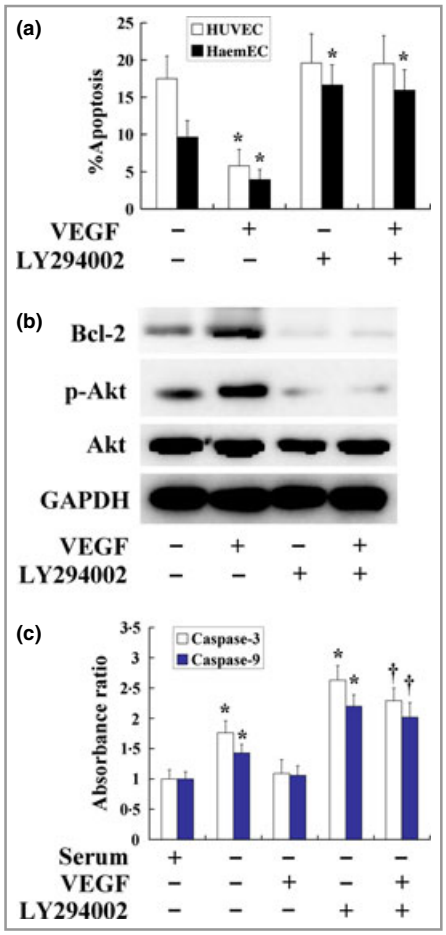

Fig 4. The survival activity of the autocrine VEGF/VEGFR-2 loop is inhibited by the PI3K inhibitor LY294002. (a) ECs were cultured in the absence of serum with recombinant VEGF (100 ng mL^{-1}) or LY294002 (100 nmol L^{-1}), or the combination of VEGF and LY294002, for 24 h. Apoptotic cell death was determined by TUNEL staining. *$P < 0{\cdot}05$ when compared with the control group. (b) Western blot of HaemEC extracts cultured in serum-free conditions, with or without VEGF and/or LY294002. LY294002 significantly inhibited Bcl-2 expression in HaemECs. (c) Caspase-3 and caspase-9 activity was increased in HaemECs treated with LY294002. ECs were cultured in serum-free conditions and were treated with 100 nmol L^{-1} of LY294002. After 24 h, the cleavage of caspase-3 and caspase-9 was assayed using a caspase colorimetric assay. Cells cultured in 10% FBS medium were used as a normal control. The results are shown as the mean ± SD of duplicated assays from one of three identical experiments. *$P < 0{\cdot}05$ when compared with the serum-treated group; †$P < 0{\cdot}05$ when compared with the serum starvation-treated control group. EC, endothelial cells; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HaemEC, haemangioma-derived endothelial cells; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling method; VEGF, vascular endothelial cell growth factor; VEGFR, VEGF receptor.

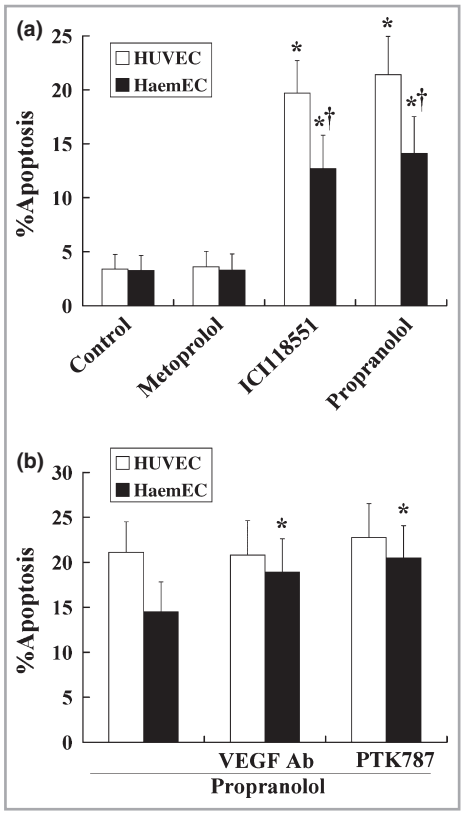

Fig 5. Blockade of the autocrine VEGF/VEGFR-2 loop promotes β-blocker-induced HaemEC apoptosis. (a) ECs were left untreated (in the presence of 10% FBS) or treated with 100 μmol L^{-1} different β-blockers, and were then stained with Hoechst 33343 and TUNEL. The results show the percentage of viable cells in the different conditions. *$P < 0{\cdot}05$ when compared with the control group; †$P < 0{\cdot}05$ when compared with the HUVEC group. (b) Treatment of HaemECs with the VEGF Ab or PTK787 clearly promoted propranolol-induced apoptotic cell death. HaemECs were exposed to 100 μmol L^{-1} propranolol for 48 h. The results are shown as the mean ± SD of duplicated assays from one of three identical experiments. *$P < 0{\cdot}05$ when compared with the control group. Ab, antibody; EC, endothelial cells; HaemEC, haemangioma-derived endothelial cells; HUVEC, human umbilical vein endothelial cells; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling method; VEGF, vascular endothelial cell growth factor; VEGFR, VEGF receptor.

VEGF/VEGFR-2 inhibition sensitizes haemangioma-derived endothelial cells to β-blocker-induced apoptosis

Propranolol, which inhibits EC proliferation, angiogenesis and tumour growth, concomitantly stimulates EC death.[23–25] To test whether VEGF/VEGFR-2 signalling can lead to the general suppression of apoptosis in HaemECs, besides preventing serum deprivation-induced apoptosis, ECs were treated with 100 μmol L^{-1} of different β-blockers in the presence of 10% FBS for 48 h. The use of 100 μmol L^{-1} concentration of β-blockers is based on previous studies.[23–25] The culture medium was replaced daily with fresh medium plus the indicated drugs. The cells were then analysed for apoptosis using TUNEL staining. The results indicated that propranolol (nonselective β-blocker) and ICI118551 (selective β_2-blocker) induced similar changes in HaemECs even in the presence of 10% FBS (Fig. 5a). Surprisingly, however, the pro-apoptotic effects of the β-blockers were EC type-independent. We found that

propranolol and ICI118551 more significantly affected the survival rate of HUVECs than HaemECs, although metoprolol (selective β_1-blocker) had no effect on the survival of either EC type. In addition, we inhibited VEGF/VEGFR-2 signalling by pretreatment with the VEGF Ab or PTK787, and as expected, these agents induced a significant increase in propranolol-induced apoptotic HaemEC death (Fig. 5b). Taken together, these findings suggest that autocrine VEGF/VEGFR-2 signalling in HaemECs can induce general resistance to apoptotic stimuli.

Discussion

The present study found that proliferating HaemECs possess similar basic characteristics but enhanced survival in comparison with normal ECs. The finding that HaemECs responded abnormally to apoptotic stimuli (that otherwise elicit extensive apoptosis in normal ECs) suggests that HaemECs may progressively acquire altered phenotypes as a result of an abnormal microenvironment. In IHs, a multistep process involving mutations to both the interstitial and mesenchymal compartment has been reported, suggesting a possible reciprocal interaction between HaemECs and surrounding cells.[26] Alternatively, it could be speculated that HaemECs originate from progenitor-like cells, which are known to display robust proliferative and clonogenic capabilities.[27]

The stimulation of EC apoptosis may inhibit the tumour blood supply and result in tumour regression. Furthermore, blockade of VEGF in tumours was shown to lead to EC detachment and apoptosis, followed by vascular collapse, haemorrhage and tumour necrosis.[11,13] Induction of cancer-associated EC apoptosis can be achieved by inhibitors directed against the VEGFR-2 by either conditional switching or administration of inhibitors against VEGFR-2.[15,28] In the present study, a small-molecule inhibitor of VEGFR-2 dramatically reduced HaemEC survival, and blockade of VEGF with an anti-VEGF antibody induced similar effects. These findings support the involvement of a VEGF/VEGFR-2-dependent autocrine signalling loop in HaemEC survival. Our findings suggest that, similar to malignant cells and cancer-derived ECs, HaemECs may be able to adapt to the abnormal physical environment of the tumour (e.g. stress) by undergoing a form of reprogramming that involves an increase in apoptosis resistance and upregulating a VEGF autocrine survival feedback loop to sustain these effects and stabilize the aberrant phenotype.[29–31]

It should also be stressed that our findings do not exclude autocrine VEGF signalling in normal EC survival functions.[32] Instead, we found that the autocrine VEGF loop decreased cell death in cultured HUVECs under serum-free conditions. The experimental evidence for this conclusion was that blockade with the VEGFR-2-specific inhibitor also significantly promoted HUVEC apoptotic death. However, the anti-apoptotic effect of autocrine VEGF signalling in HUVECs was considerably lower than that observed in HaemECs. Our findings indicate that survival signalling in an autocrine mode alone may be insufficient for normal ECs under stress situations (e.g. serum starvation).

Mechanistically, VEGF activates the survival-promoting PI3K/Akt pathway. Activation of Akt in turn stimulates the expression of anti-apoptotic proteins, such as Bcl-2, and thereby inhibits the execution of apoptosis.[33] Moreover, Akt activates the endothelial NO synthase, leading to an enhanced synthesis of NO, which promotes EC survival by inhibiting the cysteine protease activity of caspase-3 via S-nitrosylation of the reactive cysteine residue.[34] In addition, VEGF, either endogenous or exogenous, can directly induce expression of Bcl-2 in primary vascular ECs.[14,22,32] Many reports have also demonstrated altered Bcl-2 levels in ECs engaged in physiological as well as pathological angiogenesis,[35,36] and an increase in Bcl-2 expression within the proliferating IH lesions has been observed. In contrast, Bcl-2 expression decreases in ECs with increasing age of the lesions, suggesting that Bcl-2 may be important in the pathogenesis of IHs.[5] In the present study, we found that maintaining Bcl-2 expression via VEGF/VEGFR-2 signalling in primary HaemECs blocked the cells from apoptotic death in the absence of external VEGF. In contrast, the inactivation of PI3k/Akt suppressed the VEGF/VEGFR-2-mediated anti-apoptotic effect and unleashed the inhibitory effect of VEGF/VEGFR-2 signalling over the reduction of Bcl-2 expression, thereby amplifying the activation of the caspase cascade. Taken together, the data presented here highlight a mechanism whereby the upregulated autocrine VEGF loop, via regulation of the PI3K/Akt/Bcl-2 pathway, promotes HaemEC survival.

The autocrine VEGF loop may also protect cells against apoptosis in response to other stimuli, as exemplified by the protective effects of the autocrine VEGF loop on ECs or embryonic stem cells against hypoxia-induced apoptosis or the inhibition of ionizing radiation- or chemotherapy-induced apoptosis of malignant cells.[16–18,32,36,37] In addition, the autocrine VEGF loop has been shown to protect epithelial ovarian carcinoma cells from anoikis.[38] Currently, propranolol is an extremely effective first-line treatment for proliferating IHs when administered early and continued throughout the proliferative phase.[39,40] It has been suggested that the benefits of propranolol treatment may primarily be due to the promotion of EC apoptosis.[41,42] This hypothesis was recently confirmed by several studies, which reported that the antihaemangioma activity of propranolol appears to be mediated via the induction of HaemEC apoptosis.[19,23,24] In the present study, however, we found that HaemECs exhibited increased resistance to apoptosis induced by propranolol in comparison to HUVECs. In addition, our study revealed the novel finding that propranolol-induced apoptosis could be partially overridden by an upregulation in VEGF, specifically an autocrine action involving VEGFR-2, in HaemECs. These data suggest that the reported propranolol-induced HaemEC apoptosis may not be EC type-specific but rather a more common phenomenon. Nonetheless, our findings do not rule out the possibility that propranolol can induce HaemEC apoptosis in the context of a tumour. Thus, further *in vivo* experiments are needed to extend these findings.

In conclusion, the present study demonstrated that the upregulated VEGF/VEGFR-2 signalling in HaemECs is maintained

by an autocrine loop, which allows for the persistent activation of downstream intracellular pro-survival pathways and thereby promotes HaemEC survival.

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