Vascular endothelial growth factor-B-deficient mice show impaired development of hypoxic pulmonary hypertension

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Abstract

Objective: To test the hypothesis that Vegf-B contributes to the pulmonary vascular remodelling, and the associated pulmonary hypertension, induced by exposure of mice to chronic hypoxia. Methods: Right ventricular systolic pressure, the ratio of right ventricle / [left ventricle + septum] (RV/[LV+S]) and the thickness of the media (relative to vessel diameter) of intralobar pulmonary arteries (o.d. 50–150 and 151–420 μm) were determined in Vegfb knockout mice (Vegfb−/−; n=17) and corresponding wild-type mice (Vegfb+/+; n=17) exposed to chronic hypoxia (10% oxygen) or housed in room air (normoxia) for 4 weeks. Results: In Vegfb−/− mice hypoxia caused (i) pulmonary hypertension (a 70% increase in right ventricular systolic pressure compared with normoxic Vegfb−/− mice; P<0.001), (ii) right ventricular hypertrophy (a 66% increase in RV/[LV+S]; P<0.001) and (iii) pulmonary vascular remodelling (a 27–36% increase in pulmonary arterial medial thickness; P<0.05). In contrast, in Vegfb−/− mice hypoxia did not cause any increase in either right ventricular systolic pressure or pulmonary arterial medial thickness; also right ventricular hypertrophy (41% increase in RV/[LV+S]; P<0.001) was less pronounced (P<0.05) than in Vegfb−/− mice. Conclusion: Vegf-B may have a role in the development of chronic hypoxic pulmonary hypertension in mice by contributing to pulmonary vascular remodelling. If so, the effect of Vegf-B appears to be different from that of Vegf-A which is reported to protect against, rather than contribute to, hypoxia-induced pulmonary vascular remodelling.

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1. Introduction

Vascular endothelial growth factors (VEGF) are a family of closely related peptides, the mammalian members of which are VEGFs-A, -B, -C and -D and placental growth factor. VEGF-A (often referred to simply as VEGF) is the most extensively studied member of the family; it binds with high affinity to two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), and also to neuropilin-1 [1]. VEGF-A is an endothelial cell mitogen involved in the growth and proliferation of endothelial cells and in the maintenance of endothelial cell function [1]. It plays a critical role in the regulation of vasculogenesis and angiogenesis [2], and consequently VegfA
gene knock-out mice (Vegf$^{−/−}$) do not survive to term due to impairment of blood vessel development in the embryo [3,4]. VEGF-B has 45% sequence homology with VEGF-A and its tissue distribution overlaps with that of VEGF-A [5–7]. It binds to two of the same receptors as VEGF-A, namely VEGFR-1 and neuropilin-1, but not to VEGFR-2 [6,8,9]. VEGF-B, unlike VEGF-A, is not critical for vascular development [6,10] and Vegfb gene knock-out mice (Vegfb$^{−/−}$) survive to term and are outwardly healthy [11]. Vegfb$^{−/−}$ mice have a deficiency in the maintenance of coronary vascular function following an ischaemic insult [11] or a defect in atrial conduction characterised by a prolonged PQ interval [12] but, apart from these, the functions of this member of the VEGF family remain to be established [6].

VEGF-A is induced by hypoxia and, in rats, chronic hypoxia leads to increases in VEGF-A mRNA and protein [13]. Hypoxia also up-regulates VEGFR-1 and VEGFR-2 [13–16]. Consequently it has been proposed that VEGF-A may have a role during the development of chronic hypoxic pulmonary hypertension. Initially it was considered that VEGF-A might contribute to the pathophysiology of the disease [17]. However, more recent data have shown that a reportedly selective VEGFR-2 antagonist exacerbates the pulmonary vascular remodelling and pulmonary hypertension seen in hypoxic rats [18]. A similar trend was seen with a VEGF-A neutralising antibody [19,20]. Also, adenovirus-mediated over-expression of VEGF-A in rat lungs has been shown to attenuate hypoxia-induced pulmonary vascular remodelling and pulmonary hypertension [21]. Together these data raise the possibility that VEGF-A may have a protective role against some of the adverse effects of chronic hypoxia.

However, the possibility that VEGF-B might have a role (contributory or protective) in hypoxic pulmonary hypertension has not previously been investigated. VEGF-B, unlike VEGF-A, is not induced by hypoxia, at least in in vitro studies [10,22], but it binds to VEGFR-1, one of the receptors upregulated by hypoxia, where it competes with VEGF-A for binding [6,9]. Moreover VEGF-B forms heterodimers with VEGF-A leading to the suggestion that VEGF-B may regulate the bioavailability of VEGF-A [5]. Therefore VEGF-B might conceivably influence the development of hypoxic pulmonary hypertension either directly, via a mechanism involving VEGFR-1, or indirectly, via an effect on VEGF-A availability or signalling. In the present study we have used Vegfb$^{−/−}$ mice and corresponding wild-type littermates (Vegfb$^{+/+}$) to test the hypothesis that Vegf-B contributes to the pulmonary vascular remodelling, and the related pulmonary hypertension, induced by exposure of mice to chronic hypoxia. A recent study has shown that both of the isoforms of Vegf-B are expressed in the lungs of adult mice, with Vegf-B$^{167}$ predominating [23].

2. Methods

2.1. Animals

The investigation conforms to the Code of Practice for Animal Experiments issued by the National Health and Medical Research Council of Australia.

Mice (strain, 129T2/SvEms) lacking the vascular endothelial growth factor gene (Vegfb$^{−/−}$ mice) were bred and genotyped as described in detail previously [11]. These mice were generated by standard gene targeting techniques using a targeting construct that replaced most of the coding sequence of the Vegfb gene with a β-geo selection cassette. To definitively show that the Vegfb gene was deleted and no Vegfb transcript was produced from the locus we analysed total RNA derived from both Vegfb$^{+/+}$ and Vegfb$^{−/−}$ animals by RT-PCR to detect both Hprt and Vegfb transcripts. The total RNA was isolated from heart tissue using Trizol reagent (Sigma) according to the manufacturer's instructions and the cDNA synthesised using Superscript II Reverse Transcriptase Kit (Gibco-BRL). The RT-PCR was performed on RNA extracts, under the following conditions: 1 μg RNA was incubated at 70°C for 10 min with 100 ng random hexamers in a total volume of 11 μl. The samples were placed on ice and a 9-μl reverse transcription mixture (0.5 mM dNTPs, 10 mM DTT, 200 units Superscript II, 40 units Rnasin and Superscript buffer) was added to the samples which were incubated at 45°C for 1 h, followed by 95°C for 5 min, then 30 μl of water was added to bring the final volume to 50 μl. An aliquot of the cDNA product (1 μl) was then PCR amplified in a 50 μl reaction mixture (2.5 units AmpliTaq Gold, 1.5 mM MgCl₂, 0.2 mM dNTPs. PCR buffer and 10 pmol of each of the two oligonucleotide primers necessary to amplify either Hprt or Vegfb. PCR was performed for 1 cycle of 12.5 min at 94°C, followed by 30 cycles of: 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min. The primers used were as follows: for Hprt, Hprtrnaf, 5′-gtc aaa caa cta gtg ccc ag-3′ and Hprtmar, 5′-gtc aag ggc ata tcc aac aac c-3′; for Vegfb, AM1, 5′-gtc aaa cca cta gta gcc ccc ag-3′ and AM2, 5′-tgt cgg tgt gta gct cta ag-3′. RT-PCR products were then run out on 2% agarose gels with ethidium bromide staining for visualisation.

Female Vegfb$^{+/+}$ and Vegfb$^{−/−}$ mice were housed either in room air (control, normoxic mice) or in a hypoxic chamber containing 10–11% oxygen (hypoxic mice) for 4 weeks, as previously described for hypoxic rats [24]. The weights of the mice at the completion of the period of exposure to normoxia (n=8) or hypoxia (n=9) were (g): Vegfb$^{+/+}$ normoxic 20±0.5, hypoxic 18±0.4; Vegfb$^{−/−}$ normoxic 19±0.8, hypoxic 18±0.8. During the 4-week exposure to normoxia, mice from both groups had gained weight (Vegfb$^{+/+}$ 15±1.7%; Vegfb$^{−/−}$ 10±1.0%). In contrast, during the 4-week exposure to hypoxia mice had
lost weight (Vegf \(b^{+/+}\) \(-6\pm1.4\%; \) Vegf \(b^{-/-}\) \(-6\pm1.3\%\)). Mice were removed from the hypoxic chambers before anaesthetising them for measurement of right ventricular pressure, as described below. In preliminary experiments it was shown that a shorter period of hypoxic exposure (2 weeks), which is sufficient to induce pulmonary hypertension in rats [25], did not cause pulmonary hypertension in Vegf \(b^{+/+}\) mice, inasmuch as there was no elevation in right ventricular systolic pressure (RVSP). Elevations in RVSP parallel elevations in pulmonary artery pressure [26] (Wanstall, unpublished data).

2.2. Assessment of hypoxia-induced pulmonary hypertension

Normoxic and hypoxic Vegf \(b^{+/+}\) and Vegf \(b^{-/-}\) mice were anaesthetised with pentobarbitone (60 mg/kg i.p.; Rhone Merieux, Brisbane, Australia). The mice were artificially ventilated with room air (60 strokes/min) with a Ugo Basile Rodent Ventilator, via a tracheal cannula, modified to deliver a stroke volume of 0.5 ml. The thorax was opened, a heparin-filled hypodermic needle (23 G) was inserted into the right ventricle and right ventricular pressure was rapidly (in less than 30 s after opening the thorax) recorded on a Ugo Basile Gemini recorder. Six Mean values, calculated from data obtained from aconsecutive values ... was inserted into the right ventricle and right ventricular
diameter was defined as the distance between two diametrically opposed external elastic laminae; two measurements were made, (i) along the line corresponding to the longest distance and (ii) along the line perpendicular to it, and these two values were averaged to give ‘mean arterial diameter’. Medial wall thickness was defined and measured as the distance between internal and external elastic laminae; four measurements were obtained, one in each of the four quadrants defined by the perpendicular lines used to measure mean arterial diameter (see above), and averaged to give ‘mean medial thickness’. Medial wall thickness was then expressed as a percentage of mean arterial diameter according to the formula: ‘% medial wall thickness’=(2×mean medial wall thickness)×100÷mean vessel diameter. Vessels were selected on the basis that (i) they had both internal and external elastic laminae, (ii) were cut transversely (i.e., not obliquely) and (iii) had outer diameter (o.d.) \(\geq 50\) m. The data were divided into two groups, comprising arteries 50–150 and 151–520 \(\mu\)m o.d., respectively.

2.4. Statistical analyses

Mean values, calculated from data obtained from a number of different animals, are quoted together with their S.E. mean. The data were analysed by analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison post test using the statistics program, Graphpad Instat.

3. Results

3.1. Confirmation that the knockout mice do not produce Vegf \(b\) gene product

RT-PCR analysis (Fig. 1) for Vegf \(b\) gene product in total heart RNA derived from Vegf \(b^{+/+}\) mice showed the
expected products at 446 bp for Vegf-B\textsuperscript{186} and 345 bp for Vegf-B\textsuperscript{167}, the two alternative splice forms of Vegf\textsubscript{b} transcript (Fig. 1, lane 5). No Vegf\textsubscript{b} gene products were amplified in the RNA derived from Vegf\textsubscript{b}\textsuperscript{+/+} mice (Fig. 1, lane 6) confirming that the knockout had indeed generated a null allele of Vegf\textsubscript{b}.

3.2. Right ventricular systolic pressure (RVSP), right ventricular hypertrophy and haematocrit

Hypoxic Vegf\textsubscript{b}\textsuperscript{+/+} mice had elevated RVSP, when compared with the value in corresponding normoxic Vegf\textsubscript{b}\textsuperscript{+/+} mice (Figs. 2a and 3a). They also had right ventricular hypertrophy as evidenced by significant increases in the ratios RV/(LV+S) and RV/body wt (Fig. 3b,c). In contrast hypoxic Vegf\textsubscript{b}\textsuperscript{+/−} mice did not have elevated RVSP (Figs. 2b and 3a) and, although there was significant right ventricular hypertrophy, the values for RV/(LV+S) and RV/body wt in hypoxic Vegf\textsubscript{b}\textsuperscript{+/−} mice were significantly less than the corresponding value in hypoxic Vegf\textsubscript{b}\textsuperscript{+/+} mice (Fig. 3b,c).

Haematocrit was elevated in hypoxic Vegf\textsubscript{b}\textsuperscript{+/+} mice (normoxic, 46±1.1, n=8; hypoxic, 53±0.8, n=9; \( P \leq 0.001 \)) and hypoxic Vegf\textsubscript{b}\textsuperscript{+/−} mice (normoxic, 48±1.3, n=8; hypoxic, 55±1.4, n=9; \( P < 0.01 \)), but in both normoxic and hypoxic mouse there was no difference between the Vegf\textsubscript{b}\textsuperscript{+/+} and Vegf\textsubscript{b}\textsuperscript{+/−} groups (\( P > 0.05 \)).

3.3. Medial thickness of intralobar pulmonary arteries

In Vegf\textsubscript{b}\textsuperscript{+/+} mice, chronic hypoxia caused significant increases in medial wall thickness of intralobar pulmonary arteries 50–150 and 151–420 \( \mu \text{m} \) o.d. (Fig. 4). This was not seen in hypoxic Vegf\textsubscript{b}\textsuperscript{+/−} mice and, consequently,
Fig. 3. (a) Right ventricular systolic pressure (RVSP), (b) RV/(LV+S) and (c) RV/body wt in normoxic (open bars) and hypoxic (filled bars) Vegfb+/+ and Vegfb−/− mice (n=8–9 mice in each group). *Value significantly greater than corresponding value in normoxic mice (**P<0.01, ***P<0.001). #Value significantly less than corresponding value in Vegfb+/+ mice (#P<0.05, ##P<0.01).

Fig. 4. Medial wall thickness (expressed as a percentage of vessel diameter) in intralobar pulmonary arteries, (a) o.d. 151–420 and (b) 50–150 μm, from normoxic (open bars) and hypoxic (filled bars) Vegfb+/+ and Vegfb−/− mice (n=15–35 vessels, seven to nine mice in each group). *Value significantly greater than corresponding value in normoxic mice (*P<0.05, ***P<0.001). #Value significantly less than corresponding value in Vegfb+/+ mice (#P<0.05, ##P<0.01).

values for medial thickness in hypoxic Vegfb−/− mice were significantly smaller than the corresponding values in hypoxic Vegfb+/+ mice (Fig. 4).

4. Discussion

The results of this study have shown that mice lacking the Vegfb gene, unlike their wild-type littermates, did not develop sustained pulmonary hypertension in response to chronic hypoxia. For the purposes of this study an eleva-
tion in RVSP was taken as indicative of pulmonary hypertensive correlations since RVSP closely parallels pulmonary artery pressure [26] (Wanstall, unpublished). In Vegfb+/- mice, the 70% increase in RVSP caused by 4 weeks of hypoxia was within the range of increases reported in other studies in mice after 3–5 weeks of hypoxia, viz. 42–100% increase [26–30]. The different values for RVSP in hypoxic Vegfb-/- and hypoxic Vegfb+/+ mice could not be attributed to differences in blood viscosity since both groups of mice showed the same degree of polycythemia.

The absence of pulmonary hypertension in the Vegfb-/- knockout mice did, however, reflect the absence of pulmonary vascular remodelling, as assessed by increases in pulmonary arterial medial thickness. An increase in pulmonary arterial medial thickness is a characteristic feature of hypoxic pulmonary hypertension, reflecting both hypertrophy of the smooth muscle and an increase in extracellular connective tissue [31,32]. Thickening of the media was seen in vessels from hypoxic Vegfb+/+ mice but not hypoxic Vegfb-/- mice. The data suggest that Vegf-B may be one of the factors responsible, directly or indirectly, for the alterations in pulmonary vascular structure caused by chronic hypoxia in vessels of the size range examined. Alternatively, the absence of Vegf-B may promote various compensatory changes, one or more of which might hinder hypoxic vascular remodelling.

Despite the absence of any elevation in RVSP, the hypoxic Vegfb-/- mice did exhibit some right ventricular hypertrophy, albeit less pronounced than in the pulmonary hypertensive wild-type mice. In pulmonary hypertensive animals, right ventricular hypertrophy is considered to reflect an increase in afterload and there is generally a good correlation between right ventricular hypertrophy and elevations in pulmonary artery pressure or RVSP [33,34]. However, there are occasions when these two changes do not occur together. For example, rats exposed to intermittent hypoxia (4 h/day, for a total of 24 exposures) had right ventricular hypertrophy with no increase in RVSP [35]. There are various possible explanations for the presence of right ventricular hypertrophy in the hypoxic Vegfb-/- mice, in the absence of a sustained elevation in RVSP. For example, there may have been a temporary elevation in pulmonary artery pressure, occurring only while the mice were in the hypoxic chamber and due to acute hypoxic pulmonary vasoconstriction rather than vascular remodelling. The resulting temporary increase in afterload could lead to right ventricular hypertrophy [35], even though there was no sustained increase in pressure once the mice were removed from the chamber and ventilated with room air. Alternatively right ventricular hypertrophy may reflect, in part, a direct effect of hypoxia on the right ventricle, i.e., part of the hypertrophy may be unrelated to an increase in pressure. Finally we cannot conclude the possibility that the RVSP values may have been under-estimated in the hypoxic Vegfb-/- mice. This would theoretically be possible if there was a greater hypotensive effect of the anaesthetic in the knockout mice, or even if there was more pronounced blood loss (although in all mice blood loss was negligible). However we think this third explanation is unlikely in view of the fact that there was not only no increase in RVSP in the hypoxic Vegfb-/- mice but also no pulmonary vascular remodelling. It should be emphasised that the right ventricular hypertrophy in these mice was significantly less than seen in hypoxic wild-type mice. The possibility that this reflects a specific role for VEGF-B in right ventricular hypertrophy cannot be excluded.

Our data suggest that VEGF-B contributes to the pathology of hypoxic pulmonary hypertension. If this conclusion is correct, then it appears that VEGF-B has a very different role from that of VEGF-A in the response to chronic hypoxia. Recent studies in rats have indicated that VEGF-A may be protective in hypoxic pulmonary hypertension. Partovian et al. showed that over-expression of VEGF-A in lungs (achieved with adenovirus-mediated gene transfer) attenuated the rise in pulmonary artery pressure and the pulmonary vascular remodelling induced by chronic hypoxia, and suggested that this beneficial effect was due in part to protection of endothelial cell function [21]. Taraseviciene-Stewart et al. showed that in hypoxic rats treated with SU5416, a reportedly selective antagonist at VEGFR-2 receptors (which bind VEGF-A but not VEGF-B), elevations in pulmonary artery pressure and thickening of the medial layer of pulmonary arteries were exaggerated when compared with data in untreated hypoxic rats [18]. Also, clusters of proliferating endothelial cells obstructing the lumen were observed in the SU5416-treated rats [18]. Pulmonary hypertension was also enhanced in hypoxic rats treated with a monoclonal antibody to VEGF-A [19,20]. Furthermore SU5416 caused the development of pulmonary hypertension even in normoxic rats [18]. Treatment with a broad-spectrum caspase inhibitor (at a concentration previously shown to inhibit caspase-3) prevented the exacerbation of hypoxic pulmonary hypertension induced by this VEGFR-2 antagonist [18]. The authors hypothesised that in the absence of VEGF-A signalling via VEGFR-2 and the presence of chronic hypoxia, pulmonary endothelial cell dysfunction and cell death occurs, leading to the emergence of an apoptosis-resistant, proliferating endothelial cell phenotype that causes obstruction of the vascular lumen. They also suggested a link between the perturbation of endothelial cell function and an increase in vascular smooth muscle in the media [36]. Collectively, the findings from these studies imply that VEGF-A, via an action on VEGFR-2, may have a protective effect against pulmonary vascular remodelling induced by hypoxia.

VEGF-B does not bind to VEGFR-2 but it does bind to VEGFR-1, and these receptors are up-regulated by hypoxia in various tissues including lungs [13,16]. The functions associated with activation of VEGFR-1 are still unclear, but a recent report suggested that VEGFR-1 might nega-
tively regulate the endothelial mitogenic effect mediated via VEGFR-2 [37]. In light of this, activation of VEGFR-1 by VEGF-B might conceivably have the same net effect as blockade of VEGFR-2 with SU5416 (see above). In other words, VEGF-B might contribute to hypoxic pulmonary vascular remodelling indirectly, by inhibiting the beneficial (protective) effect of VEGF-A.

An alternative (or additional) possibility is that activation of VEGFR-1 by VEGF-B might contribute to pulmonary vascular remodelling independently of the effects of VEGF-A. One possible mechanism could be via matrix metalloproteinase production, which has been linked with activation of VEGFR-1 in vascular smooth muscle cells [38]. Wang and Keiser showed that VEGF-A increased matrix metalloproteinase mRNA and protein in vascular smooth muscle cells [38]. This evidently occurred via activation of VEGFR-1, because (i) there were VEGFR-1 but no VEGFR-2 receptors present in the smooth muscle cells and (ii) a VEGFR-1-selective ligand mimicked the effect of VEGF-A [38]. Extracellular matrix-degrading enzymes facilitate the growth and migration of smooth muscle cells [38,39] and permit the release of growth factors bound to extracellular matrix proteins [40,41]. Therefore one pathway whereby VEGF-B, through its ability to bind to VEGFR-1, might conceivably contribute to pulmonary vascular remodelling is through matrix metalloproteinase production. Note that neither of the speculative explanations described above implies that VEGF-B, itself, necessarily has any direct mitogenic activity.

Placental growth factor is another member of the VEGF family that binds to VEGFR-1, and it has recently been stated (though the data were not shown) that placental growth factor-deficient mice, like the Vegf−/− mice examined in this study, had impaired hypoxic pulmonary vascular remodelling [42]. Also, data very similar to those obtained in the present study were obtained in a study in mice partially deficient in hypoxia-inducible factor 1α [27], which regulates, among other genes, the gene encoding VEGFR-1 [43].

The possibility that the differential response to chronic hypoxia in the Vegf−/− and Vegf+/+ mice may be a consequence of some compensatory mechanism(s) existing in the knockout mice, rather than a direct consequence of the absence of Vegf-B, cannot be ruled out; this is a limitation of any study in knockout animals. Nevertheless, the simplest conclusion from the data obtained in this study is that, in some way, Vegf-B contributes to pulmonary vascular remodelling during the development of hypoxic pulmonary hypertension in mice. Other growth factors that have been implicated in pulmonary vascular remodelling include platelet-derived growth factor, basic fibroblast growth factor and insulin-like growth factor-1, as well as various vasoactive factors that also have mitogenic properties, viz. angiotensin II, endothelin-1 and 5-hydroxytryptamine [44]. The present data suggest that VEGF-B, too, may have a role in this disease.

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