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Letter to the Editors-in-Chief

Local accumulation of hypoxia-inducible factor 2 alpha during venous thrombus resolution

Dear Editors,

Venous thrombus resolution occurs by a process of organisation, which includes the infiltration of neutrophils and macrophages, and the formation of new vascular channels within and around the thrombus [1]. Rapid thrombus resolution is associated with reductions in the incidence of post-thrombotic syndrome [2,3]. Characterisation of the cellular and molecular mechanisms that control venous thrombus resolution could therefore lead to the development of novel therapies for patients with deep vein thrombosis.

The tissue remodelling response to hypoxia is controlled primarily by activation of hypoxia-inducible factors (HIFs) 1 and 2 [4]. Accumulation of HIF1α and 2α (the hypoxia-dependent subunits of HIF1 and HIF2 respectively) leads to HIF activation, and subsequent upregulation of a variety of factors that mediate vascular remodelling [4]. We previously showed that: (i) naturally resolving venous thrombus is hypoxic compared with venous blood; (ii) HIF1α is expressed in distinct spatial and temporal patterns throughout resolution; and (iii) this process is accelerated when HIF1α levels are enhanced in the thrombus and surrounding vein [5–7]. Although it is known that the 2 HIFα isoforms regulate an over-lapping but distinct catalogue of target genes, and that these isoforms can act in co-operation or opposition (depending on the cell type, tissue type, and condition studied) [8], the role of HIF2α in venous thrombus resolution is unknown. Our primary aim was to determine whether HIF2α is expressed in the newly formed and naturally resolving thrombus and surrounding vein. Given that thrombus resolution is increased by treatment with a HIF agonist, L-mimosine [6,7], we also wished to determine whether these increases could be partly mediated by HIF2.

Methods

Studies were performed under the Animals (Scientific Procedures) Act, 1986. Venous thrombus was induced in the inferior vena cava (IVC) of 8-10week old male BALB/C mice using an established model of blood flow reduction and endothelial disturbance [9]. Thrombus and surrounding vein were excised, sectioned, and immunostained for HIF2α at days 1, 7, 10, and 14 after thrombus induction (n = 3/group) as described [6] using an anti-HIF2α primary antibody (Novus, UK). Positive staining was quantified in the thrombus and surrounding vein by image analysis as described [6] and expressed as % area of nucleated cells. Contiguous tissue sections were also stained for the macrophage marker, Mac2, as described [6] at day 10 post-thrombus induction.

An additional sub-group of thrombosed mice received the HIF agonist, L-mimosine, or vehicle control, as described (n = 6/group) [6,7]. At day 10 after thrombus induction, thrombus and surrounding vein were excised and weighed then HIF2α was detected by immunostaining as described above and positive staining quantified as described previously [6].

One-way analysis of variance with Bonferroni post hoc was used to test whether there was a relationship between HIF2α in the thrombus or IVC and time after thrombus induction. Unpaired t-tests were used to identify statistically significant ($P < 0.05$) differences between L-mimosine- and vehicle-treated mice. Data are expressed as mean +/− standard error.

Results

We observed that HIF2α is discretely expressed in nucleated cell-dense regions of vein wall surrounding newly formed thrombus at day 1 post-induction, while staining for HIF2α in nucleated cells within the thrombus at this time (largely neutrophils) was weak or absent (Fig. 1A). At day 7, when thrombus resolution is underway in this model, nucleated cells within the thrombus and surrounding vein also stained positively for HIF2α, and by day 10, staining was strongest in nucleated cell-dense regions at the periphery of the thrombus, while positive staining in the IVC appeared weak or absent (Fig. 1B). Similarly at day 14, when thrombus resolution is well advanced in this model, positive staining appeared in nucleated cell-dense regions of the thrombus, and in association with areas of neovascularisation within the thrombus, but staining appeared weak or absent in the surrounding vein wall (Fig. 1C). While HIF2α levels were greater in the 10-day-old compared with the 1-day-old thrombus (2.2 +/− 0.5 versus 0.5 +/− 0.2% respectively, $P < 0.05$, Fig. 1D), levels of HIF2α in the surrounding vein did not change significantly with respect to time ($P > 0.05$, Fig. 1E). When HIF2α levels peaked in the thrombus at day 10, comparable Mac2-positive staining was observed on contiguous tissue sections (Fig. 2). HIF2α in the thrombus or surrounding IVC did not change significantly when values were compared throughout the length of the tissue at different longitudinal levels.

Although L-mimosine treatment reduced thrombus weight at day 10 (9.1 +/− 1.2 versus 13.5 +/− 0.9 mg in controls, $P < 0.05$), there were no significant increases between HIF2α levels in the thrombus ($P > 0.05$) or surrounding vein ($P > 0.05$) of mice treated with L-mimosine compared with vehicle.

Discussion

Nucleated cells in the thrombus at day 1 did not stain strongly for HIF2α, unsurprising given that the predominant nucleated cell type in the thrombus at this time point (i.e. neutrophils) [1] already express high levels of HIF1α [6], and that the expression patterns of the 2 major HIFα isoforms are often temporally distinct [8]. It indeed appears that the temporal expression patterns of these 2 isoforms are distinct in the naturally resolving thrombus: HIF1α levels

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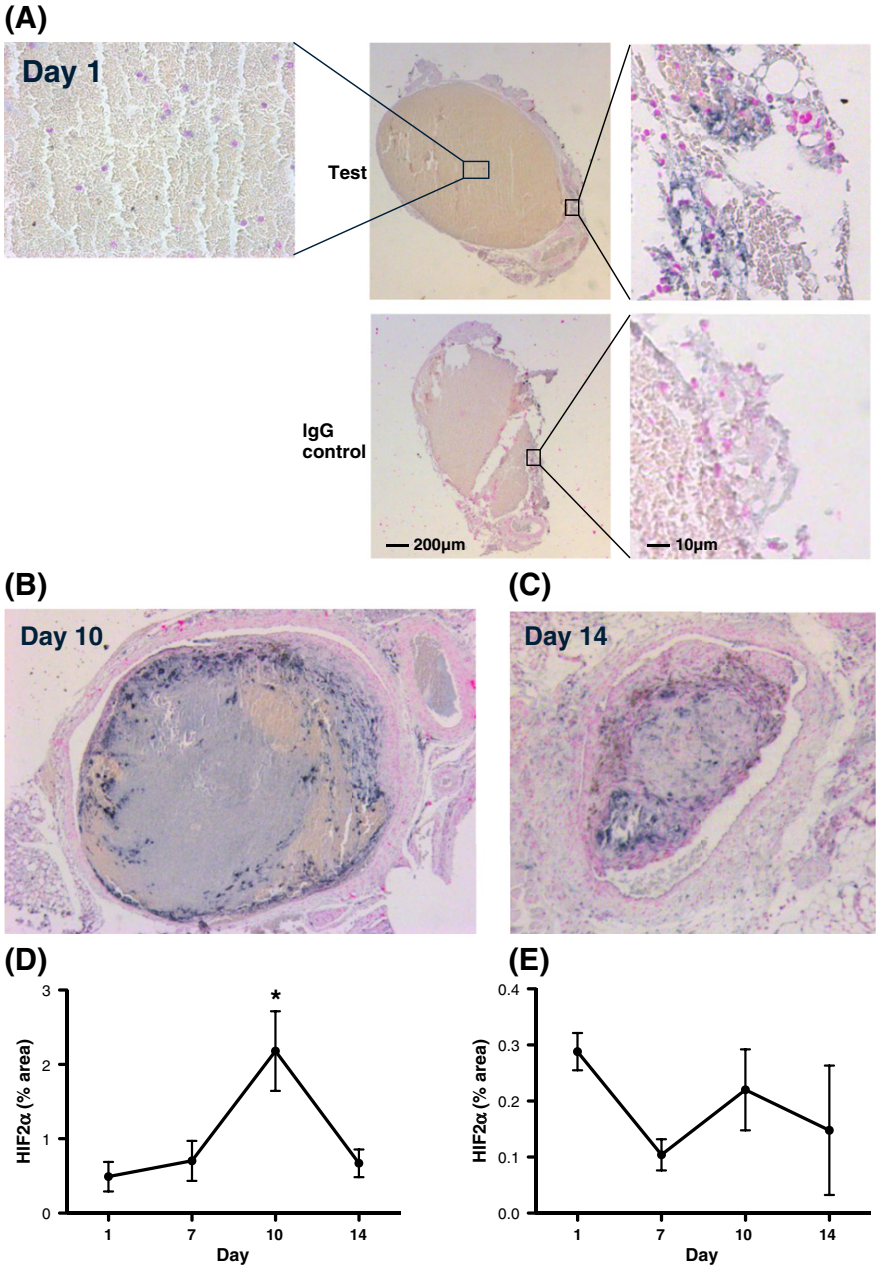

Fig. 1. Expression of HIF2α during venous thrombus resolution. Thrombus and surrounding vein wall were stained for HIF2α (black) at days 1 (A), 7, 10 (B), and 14 (C) after thrombus induction. Contiguous tissue sections exposed to isotype-matched IgG acted as negative controls. Images are representative (n = 3/group). HIF2α levels in the thrombus (D) were greater at day 10 compared with day 1, while HIF2α levels in the surrounding vein (E) did not alter significantly with time ($P > 0.05$). *$P < 0.05$ versus day 1.

decreased with time in a previous study of resolution using this model [6], while HIF2α levels increased in the current study (from day 1 until day 10 post-induction).

Vein wall surrounding new (day 1) and resolving thrombus (day 7) stained weakly for HIF2α in distinct, localised, and nucleated cell-dense regions, suggesting that the temporal expression patterns of HIF-mediated angiogenic factors found in the vein wall surrounding naturally resolving thrombus in a previous study [7] could be controlled by HIF2. HIF2α levels in the IVC did not, however, change significantly with time throughout resolution, and similarly, HIF1α expression in the IVC did not change significantly with time in a previous study [7], supporting the possibility that cells within blood vessel walls are chronically hypoxic [10].

At days 7, 10, and 14 post-thrombus induction, discrete staining for HIF2α was observed in association with nucleated cells particularly at the periphery of the thrombus (i.e. in regions where vein recanalisation including neovascularisation commonly occurs in this model) [1, 11–13]. Target genes of HIF2α include the angiogenic factor, vascular endothelial growth factor, and upregulation of HIF2α in the naturally resolving thrombus could facilitate the formation of new vascular channels via transcriptional upregulation of multiple angiogenic HIF2 targets. Macrophage content in the thrombus and surrounding vein also increases as natural resolution progresses, while neutrophil content decreases [14,15]. We showed that regions of the resolving thrombus that stained positively for HIF2α (when its level peaked at day 10) also stained positively for a macrophage-specific marker, Mac2. These

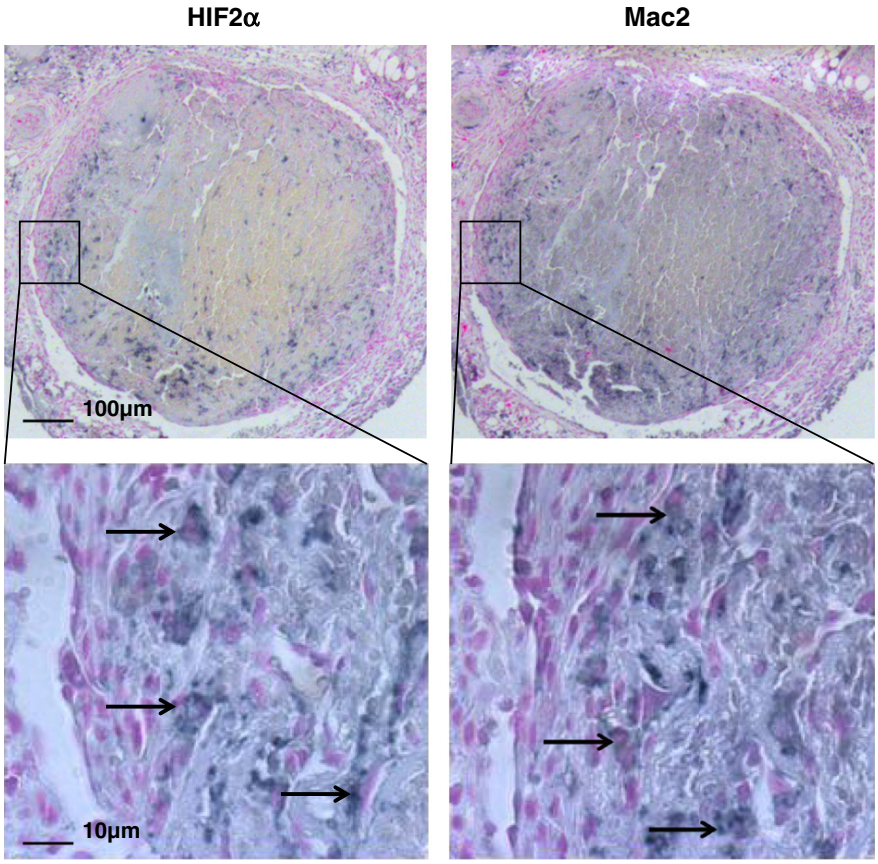

Fig. 2. Expression of HIF2α and Mac2 in resolving venous thrombus. Contiguous tissue sections of 10-day old thrombus and surrounding vein wall were stained for HIF2α (black, left column) and the macrophage marker Mac2 (black, right column) with nuclear fast red counterstain. Images are representative (n = 3).

observations are comparable with a previous study demonstrating that macrophages localise at the thrombus periphery at day 10 post-induction [11]. Macrophages are therefore likely to be the predominant cell type that expresses HIF2α in the resolving thrombus (e.g. at day 10), although the expression of HIF2α by other cell types cannot be discounted and could be investigated by co-localisation studies with other cell-specific markers (e.g. endothelial cell-specific CD31).

We previously showed that L-mimosine treatment induces HIF1α expression in the thrombus and surrounding vein and that these increases are associated with enhanced thrombus resolution [6,7]. Although L-mimosine treatment versus vehicle did not lead to significant increases in HIF2α levels in the thrombus or surrounding vein wall in this study, data presented do not preclude the possibility that L-mimosine-induced increases in resolution could be partly mediated by HIF2. The role of HIF2α in venous thrombus resolution could be thoroughly investigated in studies of larger mouse cohorts, e.g. by comparing resolution in conditional HIF2α knockout mice with wildtype controls [16].

In summary, we have shown that HIF2α is expressed in a distinct spatial and temporal pattern throughout venous thrombus resolution. The effect of targeting the HIF2α signalling pathway on venous thrombus resolution should be investigated in future studies.

Conflict of Interest Statement

The authors declare no conflict of interest.

Acknowledgements

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