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Suppression of angiogenic response in local vein wall is associated with reduced thrombus resolution

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ABSTRACT

Introduction: The formation of new vascular channels within and around venous thrombus contributes to its resolution. Neovascularisation arising from the surrounding vein may facilitate this process. Treatment of cancer patients with anti-angiogenic agents can lead to increased incidence of venous thromboembolic events, but the effect of these agents on the processes that govern thrombus resolution are unclear. The aim of this study was to determine the effect of anti-angiogenic treatment with 2-methoxyestradiol (2ME) on (i) angiogenic response in the thrombosed vein and (ii) venous thrombus resolution.

Materials and methods: Venous thrombus was induced in the inferior vena cava (IVC) of 36 adult male BALB/C mice. Thrombosed mice received either the anti-angiogenic agent, 2ME (150 mg/kg/day, i/p), or vehicle control ($n = 18$/group). In the thrombosed IVC of both groups: hypoxia-inducible factor (HIF) 1α, and its angiogenic targets, vascular endothelial growth factor (VEGF) and placental growth factor (PLGF), were quantified using enzyme-linked immunosorbent assays at days 1 and 10 post-thrombus induction ($n = 6$/group); and inflammatory cell content, cell proliferation, and vein recanalisation were quantified using immunostaining and image analysis at day 10 ($n = 6$/group).

Results: In the IVC of mice treated with 2ME compared with control: HIF1α ($P < 0.005$ and $P < 0.02$), VEGF ($P < 0.005$ and $P < 0.02$), and PLGF levels ($P < 0.01$ and $P < 0.001$) were reduced at days 1 and 10 post-thrombus induction respectively, and macrophage content ($P < 0.005$), neutrophil content ($P < 0.01$), vein recanalistion ($P < 0.05$), and thrombus resolution ($P < 0.001$) were also reduced at day 10.

Conclusions: Anti-angiogenic treatment with 2ME supressed the HIF1-mediated angiogenic drive in local vein wall and attenuated venous thrombus resolution. The potential pro-thrombotic effect of anti-angiogenic agents should be carefully considered when managing venous thromboembolic events in cancer patients.

Introduction

Venous thromboembolism resulting from deep vein thrombosis is a common complication in cancer patients [1,2]. Management is normally by anticoagulation, which prevents thrombus propagation but does not accelerate thrombus resolution. Natural resolution occurs by a slow process of organisation that involves retraction of the thrombus away from the surrounding vein, and neovascularisation within and around the thrombus, including the formation of new channels that arise from the local vein wall [3].

Intra-thrombus neovascularisation and subsequent vein recanalisation may be regulated by an angiogenic response arising from the local vein, e.g. via production of angiogenic factors and infiltration of angiogenic inflammatory cells [3,4]. Given that poor thrombus resolution is associated with the development of debilitating post-thrombotic syndrome (which is characterised by chronic leg pain and swelling) [5,6], it is undesirable to restrict this angiogenic response. Conversely, increasing local angiogenic drive accelerates thrombus resolution [7–9], but nevertheless, anti-angiogenic agents are being investigated for the prevention of tumour growth [10], and these agents may increase the incidence of post-thrombotic complications in cancer patients [11]. For example, 2-methoxyestradiol (2ME) prevents nuclear accumulation and activation of the transcription factor that stimulates the angiogenic remodelling response to hypoxia (i.e. hypoxia-inducible factor 1, HIF1) [12]. Given that (i) angiogenic (including HIF1-mediated) activity in the local vein increases thrombus resolution [4], and (ii) the mechanisms by which anti-angiogenic agents increase

Abbreviations: 2ME, 2-methoxyestradiol; HIF, hypoxia-inducible factor; IVC, inferior vena cava; PLGF, placental growth factor; VEGF, vascular endothelial growth factor.

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thrombosis are unknown, the primary aim of this study was to determine whether anti-angiogenic treatment with 2ME affects angiogenic drive in the local vein wall and venous thrombus resolution.

Methods and Materials

Animal experiments were performed under the Animals (Scientific Procedures) Act with approval from the institutional ethics committee. Thrombus was induced in the inferior vena cava (IVC) of 36 male BALB/C mice using an established flow and endothelial disturbance model of venous thrombosis as previously described [7,13]. Thrombosed mice received 2ME or vehicle control (n = 18/group): 2ME (Enzo Life Sciences, UK) was administered by intraperitoneal injection at a dose of 150 mg/kg prepared in 1% DMSO, 5 hours after induction and once daily thereafter as previously described [14]. HIF1α (the oxygen-regulated and active subunit of HIF1), vascular endothelial growth factor (VEGF), and placental growth factor (PLGF) expression in the thrombosed IVC were quantified at days 1 and 10 after thrombogenesis by enzyme-linked immunosorbent assay as described previously (n = 6/group) [4]. At day 10 after thrombus formation: cell proliferation [15] and neutrophil and macrophage content [7] in the IVC, and IVC recanalisation were quantified by immunostaining and image analysis as previously described (n = 6/group) [9,16]. Differences between groups were compared using unpaired t-tests; $P < 0.05$ was considered significant. Data are means ± standard error.

Results and Discussion

In the thrombosed IVC of mice treated with 2ME compared with control: HIF1α expression was 2-fold lower at day 1 ($P < 0.005$) and also reduced at day 10 ($P < 0.02$, Table 1); VEGF expression was 3-fold lower at day 1 ($P < 0.005$) and 2-fold lower at day 10 ($P < 0.02$, Table 1); and PLGF expression was 2-fold lower at days 1 ($P < 0.01$) and 10 ($P < 0.001$, Table 1). Cells that stained positively for the macrophage marker Mac-2 were present in the thrombosed IVC of 2ME-treated mice and vehicle controls at day 10; macrophage content was decreased by approximately 2-fold in mice treated with 2ME versus vehicle control (6.3 ± 0.8 versus 11.9 ± 0.6% in controls, $P < 0.005$, Fig. 1A). Cells that stained positively for the neutrophil marker NIMP R14 were also present in the thrombosed IVC of 2ME- and vehicle-treated mice at day 10; neutrophil content was similarly decreased by 2-fold in mice treated with 2ME compared with vehicle (2.9 ± 0.4 versus 5.6 ± 0.6% in controls, $P < 0.01$, Fig. 1B). Cells in the thrombosed IVC also stained positively for PCNA in 2ME- and vehicle-treated mice at day 10, but the decrease in cell proliferation in 2ME-treated mice versus controls did not reach significance (1.4 ± 0.7 versus 2.5 ± 1.2% in controls, $P = 0.4$, Fig. 1C). There was also no significant difference in thrombus weight at 1 day after formation in 2ME-treated mice (16.7 ± 1.3 mg) compared with controls (17.9 ± 1.8 mg, $P > 0.05$). At day 10 after thrombus induction, however, 2ME treatment resulted in increased thrombus weight (10.9 ± 0.6 versus 6.0 ± 0.8 mg in controls, $P < 0.001$, Fig. 2A) and a 2-fold decrease in vein recanalisation (0.1 ± 0.01 versus 0.2 ± 0.03 mm^2 in controls, $P < 0.005$, Fig. 2B).

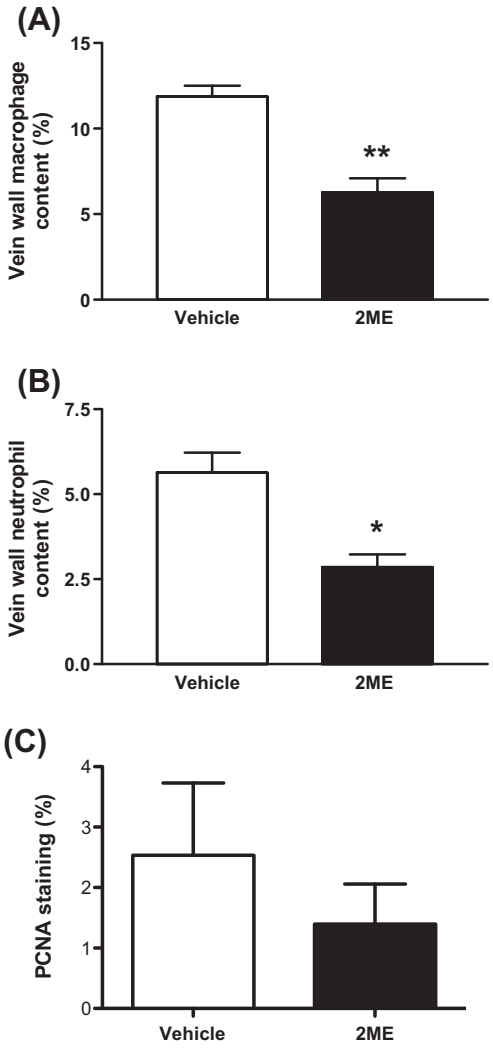

Fig. 1. Macrophage and neutrophil content and cell proliferation in the thrombosed vein of mice treated with 2-methoxyestradiol. At day 10 post-thrombus induction, (A) macrophage and (B) neutrophil content but not (C) cell proliferation were significantly reduced in the thrombosed vein (IVC) of mice treated with 2-methoxyestradiol (2ME) compared with control. Macrophage content, neutrophil content, and cell proliferation is expressed as a percentage of IVC stained positively for Mac-2, NIMP R14, and PCNA respectively. *P < 0.05 vs. control. **P < 0.005 vs. control.

Table 1
HIF1α and angiogenic HIF1 targets in the thrombosed vein of mice treated with 2-methoxyestradiol
Values are pg/mg.

		Vehicle control	2ME	Significance
Day 1	**HIF1α**	157.6 ± 23.9	72.3 ± 7.3	P < 0.005
	VEGF	116.7 ± 20.9	37.6 ± 8.6	P < 0.005
	PLGF	107.7 ± 16	53.6 ± 5.8	P < 0.01
Day 10	**HIF1α**	113 ± 17.4	65.6 ± 9.6	P < 0.02
	VEGF	26.3 ± 3.3	12.3 ± 2.3	P < 0.02
	PLGF	100.6 ± 5.7	51.3 ± 6.7	P < 0.001

Venous thrombus resolution involves an angiogenic response in the surrounding vein [3,4]. Angiogenic factors are expressed in distinct temporal patterns in the resolving thrombus and surrounding vein, and enhancing the levels of these factors accelerates resolution [4,8,9,17]. We previously showed that enhancing the HIF1-mediated angiogenic response in thrombus and surrounding vein stimulates its resolution [4,7,18]. In the current study, administration of the anti-angiogenic agent, 2ME, to thrombosed mice decreased the levels of angiogenic growth factors and inflammatory cells in the thrombosed IVC, and attenuated thrombus resolution and vein recanalisation. We have recently shown that anti-angiogenic agents can inhibit venous thrombus resolution [14], and our current findings suggest that at least part of this effect occurs via the thrombosed vein, e.g. by suppression of angiogenic targets including VEGF.

Treatment with 2ME led to reductions in the expression of angiogenic factors VEGF and PLGF in the thrombosed IVC. These factors are