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Uncovering a new role for peroxidase enzymes as drivers of angiogenesis

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ABSTRACT

Peroxidases are heme-containing enzymes released by activated immune cells at sites of inflammation. To-date their functional role in human health has mainly been limited to providing a mechanism for oxidative defence against invading bacteria and other pathogenic microorganisms. Our laboratory has recently identified a new functional role for peroxidase enzymes in stimulating fibroblast migration and collagen biosynthesis, offering a new insight into the causative association between inflammation and the pro-fibrogenic events that mediate tissue repair and regeneration. Peroxidases are found at elevated levels within and near blood vessels however, their direct involvement in angiogenesis has never been reported. Here we report for the first time that myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are readily internalised by human umbilical vein endothelial cells (HUVEC) where they promote cellular proliferation, migration, invasion, and stimulate angiogenesis both *in vitro* and *in vivo*. These pro-angiogenic effects were attenuated using the specific peroxidase inhibitor 4-ABAH, indicating the enzyme's catalytic activity is essential in mediating this response. Mechanistically, we provide evidence that MPO and EPO regulate endothelial FAK, Akt, p38 MAPK, ERK1/2 phosphorylation and stabilisation of HIF-2α, culminating in transcriptional regulation of key angiogenesis pathways.

These findings uncover for the first time an important and previously unsuspected role for peroxidases as drivers of angiogenesis, and suggest that peroxidase inhibitors may have therapeutic potential for the treatment of angiogenesis related diseases driven by inflammation.

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1. Introduction

Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are heme-containing enzymes, well-known for their pro-oxidative properties. To date their functional role in human health has mainly been limited to providing a mechanism for oxidative defence against invading bacteria and other pathogenic microorganisms (Klebanoff, 2005). Released by infiltrating neutrophils and eosinophils respectively, peroxidases are found in both physiological and pathological conditions (Davies et al., 2008). Infiltrating inflammatory cells are known to have a causative role in inflammation-driven angiogenesis (Kim et al., 2013). Angiogenesis is the process of new blood vessel formation from pre-existing vasculature. This leads to the generation of a new vascular network which is key to many physiological processes in organ development, wound healing and reproduction (Hoeben et al., 2004). This highly orchestrated process is driven by a balance of pro- and anti-angiogenic factors that control neovascularisation (Carmeliet, 2005). Disruption to this balance culminates in uncontrolled angiogenesis, a common feature seen in various pathological conditions including macular degeneration, rheumatoid arthritis, tumour growth, and metastasis (Felmeden et al., 2003; Lu et al., 2006).

Myeloperoxidase (MPO) represents 1–5% of total protein in human neutrophils (Bos et al., 1978; Klebanoff, 1999), while eosinophil peroxidase (EPO) is the most abundant protein and accounts for more than 40% of the eosinophil total mass (Abu-Ghazaleh et al., 1992). While MPO and EPO have been reported to be found at elevated levels within and near blood vessels in inflammatory conditions (Klinke et al., 2011; Blumenthal et al., 2000), their potential role in angiogenesis has never been contemplated and remains to be determined. Neutrophil derived MPO is considered to be a major determinant of inflammation and oxidative stress in coronary artery disease (Churg et al., 2012), vascular inflammation (Lau and Baldus, 2006) and cancer (Gross et al., 2009). Eosinophils

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are the principal effector cells of allergic inflammation, and contribute to airway remodelling in asthma (Flood-Page et al., 2003; Wenzel et al., 1999). Importantly, there is evidence for a role of eosinophils in the induction of angiogenesis (Puxeddu et al., 2005). While increased deposition of EPO from eosinophil degranulation has been reported within and near blood vessels in solid tumours (Samoszuk et al., 1996a), to-date, no mechanistic link in the induction of angiogenesis by EPO has been made. MPO and EPO share a 70% amino acid homology (Ten et al., 1989), are highly cationic and bind avidly to the anionic membranes of endothelial cells, where they are rapidly internalised (Zabucchi et al., 1989) and remain catalytically active generating intracellular reactive oxygen species (ROS) (Yang et al., 2001). This suggests that peroxidase enzymes and their highly reactive metabolites likely modulate cellular signalling pathways, consequently effecting endothelial function. Important in this context is our recently published data showing for the first time the ability of MPO and EPO to directly stimulate fibroblast migration/invasion and collagen biosynthesis both *in vitro* and *in vivo*, suggesting a causative role for peroxidases at sites of normal tissue repair and fibrosis (DeNichilo et al., 2015).

These studies and the increasing evidence that peroxidases may have a causative role on mesenchymal cellular function in tissue growth and repair, prompted us to investigate the possible contribution of peroxidases on angiogenesis. Here we report for the first time that peroxidase enzymes like MPO and EPO possess the capacity to promote vascularisation *in vitro* and *in vivo* and play an important role in the progression of angiogenesis related diseases driven by inflammation.

2. Materials and methods:

2.1. Reagents

Native human eosinophil peroxidase (EPO) was obtained from Cell Sciences (Canton, USA), and Genway Biotech Inc. (San Diego, USA). Recombinant human myeloperoxidase (MPO), recombinant human vascular endothelial growth factor (VEGF) were purchased from R&D Systems (Minneapolis, USA). Growth Factor-Reduced Matrigel was obtained from BD Biosciences (San Jose, USA). Antibodies against HIF-1α, HIF-2α, phospho-Akt (Ser437), Akt, phospho-FAK (Tyr576/577), FAK, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), p44/42 MAPK (ERK1/2), were purchased from Cell Signalling Technology (Danvers, USA). The irreversible peroxidase inhibitor 4-aminobenzoic acid hydrazide (4-ABAH) was purchased from Sigma–Aldrich (St Louis, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) from adult donors were provided by Prof Andrew Zannettino (SAHMRI, The University of Adelaide) and Dr Claudine Bonder (Centre for Cancer Biology, University of South Australia). HUVEC were cultivated in Medium 200 supplemented with 20% heat inactivated-FCS and endothelial growth supplement, LSGS (Invitrogen). The cells were used between passages 2 and 4. For studies involving hypoxic conditions, cells were grown to confluence in T25 flasks and were treated with 1 μg/ml of EPO or MPO and cultured in a hypoxic incubator with 1% O_2/5% CO_2 at 37 °C for 24 h. Additionally these treatments were run in parallel under normoxic conditions (20% O_2/5% CO_2 at 37 °C for 24 h). Flasks were then promptly placed on ice to prevent degradation of HIF and cell lysates were prepared for Western analysis. For determination of cell signalling events, confluent HUVEC in T25 flasks were stimulated with EPO and MPO at 1 μg/ml over 5, 10,and 20 min. The peroxidase inhibitor 4-ABAH was prepared as per manufacturer's instructions and used at 50 μM and pre-incubated for 30 min prior to peroxidase treatment. Flasks were washed twice with phosphate buffered saline (PBS), and cell lysates were harvested.

2.3. Endothelial cell proliferation assay

Proliferation of sub-confluent HUVEC cultures was assessed by alamarBlue™ (Invitrogen), as previously described (Sivamurthy et al., 2001; Xin et al., 1999). Briefly, HUVEC (3×10^3 cells) were cultured in 96-well plates pre-coated with collagen (4 μg) in 50 μl of 10% heat inactivated-FCS Medium 200. Cells were treated with 50 μl of increasing concentrations of MPO (0.1–2 μg/ml) and EPO (0.05–0.1 μg/ml) at the time of cell plating and cultured for 48 h. The fluorescence was measured and quantified at wavelengths of 530 nm excitation and 595 nm emission using FLUOstar Optima plate reader (BMG Labtech).

2.4. Endothelial cell migration assay

Endothelial cell migration was determined using the 24 well transwell plate (BD Falcon FluoroBlok™) system with 8.0 μm pore PET membranes as previously described (Parri et al., 2014). Briefly, HUVEC were starved in 2% heat inactivated-FCS Medium 200, (endothelial base media; EBM), for 18 h. HUVEC (1×10^5) were then seeded in 100 μl of EBM into the upper wells and incubated at 37 °C in 5% CO_2 for 30 min to allow cell adhesion. Lower chambers were then filled with 700 μl of either EBM as the negative control, LSGS containing 10 ng/ml hEGF and 3 ng/ml bFGF serving as the positive control, or with the various peroxidase proteins for 18 h. The peroxidase inhibitor 4-ABAH was prepared as per manufacturer's instructions and used at 10 μM. Migrated cells were fixed in 6:1 ethanol/acetic acid for 10 min, stained with DAPI (1 μg/ml), and then photographed and quantified on a fluorescent inverted microscope (Observer Z1; Zeiss).

2.5. Tube formation assay for in vitro angiogenesis on Matrigel

The BD BioCoat™ 96 well Angiogenesis System (BD Biosciences) was used to assess the formation of tube-like structures according to the manufacturer's instructions. Briefly, HUVEC (2×10^4) in 100 μl of EBM were seeded into the wells of the Matrigel pre-coated assay plate followed by 100 μl of increasing concentrations of MPO and EPO (0.2, 2, 10 μg/ml), endothelial growth supplement LSGS serving as the positive control, or EBM as the vehicle control. The peroxidase inhibitor 4-ABAH was prepared as per manufacturer's instructions and used at 10 μM and pre-incubated for 30 min prior to peroxidase treatment of 2 μg/ml with VEGF (10 ng/ml) serving as the positive control. The cells were then incubated for 6 h at 37 °C in 5% CO_2 humidified atmosphere. Images of tube formation were captured on a light microscope (Eclipse 90i; Nikon). Triplicate wells were photographed for each treatment.

2.6. Tube formation assay for in vitro angiogenesis in the absence of Matrigel

HUVEC cells were seeded onto 15 mm diameter plastic coverslips placed in a 12 well plate and grown to 70% confluence in culture media. The cells were starved in EBM for 6 h at 37 °C in 5% CO_2 humidified atmosphere. Cells were then treated with 1 ml of MPO and EPO (10 μg/ml), VEGF (10 ng/ml) serving as the positive control, or EBM as the vehicle control and cultured for 5 days at 37 °C in a humidified incubator. The coverslips were fixed in 4% phosphate buffered formaldehyde containing 5% sucrose for 10 min. Non-specific binding sites were blocked by using 3% BSA in PBS containing 0.1% glycine and a 1:10 dilution of non-immune

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