Impact of Arachidonic Acid and the Leukotriene Signaling Pathway on Vasculogenesis of Mouse Embryonic Stem Cells

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Key Words

Arachidonic acid · Embryonic stem cells · Reactive oxygen species · Vasculogenesis

Abstract

Embryonic stem (ES) cells can differentiate into various kinds of cells, such as endothelial and hematopoietic cells. In addition, some evidence suggests that inflammatory mediators such as leukotrienes (LTs), which include the 5-lipoxygenase (LOX) family, can regulate endothelial cell differentiation. In the present study, the eicosanoid precursor arachidonic acid (AA) stimulated vasculogenesis of ES cells by increasing the number of fetal liver kinase-1+ vascular progenitor cells as well as vascular structures positive for platelet endothelial cell adhesion protein-1 and vascular endothelial cadherin. The stimulation of vasculogenesis and expression of the rate-limiting enzyme in the LT signaling pathway, 5-LOX-activating protein (FLAP), was blunted upon treatment with the FLAP inhibitors AM643 and REV5901. Vasculogenesis was significantly restored upon exogenous addition of LTs. Downstream of FLAP, the LTB₄ receptor (BLT1) blocker U75302, the BLT2 receptor blocker LY255283 as well as the cysteinyl LT blocker BAY-u9773 inhibited vasculogenesis of ES cells. AA treatment of differentiating ES cells increased

reactive oxygen species (ROS) generation, which was not affected upon either FLAP or cyclooxygenase-2 inhibition. Prevention of ROS generation by either the free radical scavengers vitamin E and N-(2-mercaptopropionyl)glycine or the NADPH oxidase inhibitor VAS2870 downregulated vasculogenesis of ES cells and blunted the provasculogenic effect of AA. In summary, our data demonstrate that proinflammatory AA stimulates vasculogenesis of ES cells via the LT pathway by mechanisms involving ROS generation.

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Introduction

Recent evidence suggests that in human pathologies, processes of inflammation and angiogenesis are interconnected. Furthermore, using an in vitro model of embryonic stem (ES) cells, we have recently demonstrated that vasculogenesis, i.e. the de novo formation of vascular structures during embryonic development, is closely connected to leukopoiesis [Hannig et al., 2010]. ES cells dif-

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Abbreviations used in this paper

AA	arachidonic acid
BLT	leukotriene B4 receptor
COX	cyclooxygenase
CysLT	cysteinyl leukotriene
DCF	2',7'-dichlorofluorescein
eGFP	enhanced green fluorescent protein
ES	embryonic stem
FLAP	5-lipoxygenase-activating protein
Flk-1	fetal liver kinase-1
H ₂ DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
HETE	hydroxyeicosatetraenoic acid
HpETE	hydroperoxyeicosatetraenoic acid
LT	leukotriene
LOX	lipoxygenase
NMPG	N-(2-mercaptopropionyl)glycine
PECAM	platelet endothelial cell adhesion protein
ROS	reactive oxygen species
VE	vascular endothelial
VEGF	vascular endothelial growth factor

ferentiate within the three-dimensional tissues of embryoid bodies. These can be considered as mini-organs in which various cell types of all three germ layers are present and interact via autocrine, juxtacrine, and paracrine signaling pathways, thereby closely mimicking the *in vivo* situation. Inflammation is mediated by a well-orchestrated interplay of proinflammatory cytokines which are interacting in different signaling pathways. One of the key players in the inflammation process is arachidonic acid (AA), which is a major constituent of the cell membrane, where it is released by the enzyme phospholipase A₂, which cleaves off the fatty acid moiety. Furthermore, it can also be generated from diacylglycerol by diacylglycerol lipase [Reisenberg et al., 2012]. Free AA in the cytosol can be enzymatically metabolized to eicosanoids through three major pathways: cytochrome P₄₅₀, cyclooxygenase (COX), and/or 5-lipoxygenase (LOX) pathways. In the cytochrome P₄₅₀ pathway, AA is metabolized to epoxyeicosatrienoic acids, hydroxyeicosatetraenoic acids (HETEs), and hydroperoxyeicosatetraenoic acids (HpETEs). In the COX pathway, AA is enzymatically converted to the intermediate prostaglandin H₂, which is then metabolized to prostanoids, including prostaglandins, and thromboxanes by specific prostaglandin and thromboxane synthases. In the LOX pathway (fig. 1), AA is metabolized by 12- and 15-LOX to 8-, 12-, and 15-HpETE, or by 5-LOX and 5-LOX-activating protein (FLAP) to intermediary 5-HpETE. 5-HpETE is

further processed to form leukotrienes (LTs), the first of which is the unstable LTA₄. LTA₄ is subsequently converted to LTB₄ by LTA₄ hydrolase or together with glutathione to LTC₄ by LTC₄ synthase and glutathione-S-transferase. LTC₄ is converted by ubiquitous enzymes to form LTD₄ and LTE₄ [Savari et al., 2014]. Interestingly, states of inflammation are also involved in processes of early embryogenesis since embryo implantation is associated with an active T-helper type 1 inflammatory response while a T-helper type 2 humoral inflammation is required for pregnancy maintenance [Dekel et al., 2010; Granot et al., 2012]. These observations suggest that very early events in embryogenesis, e.g. the development of the cardiovascular system, require a proinflammatory state within the embryo. Furthermore, proinflammatory states, e.g. during wound healing, are associated with neoangiogenesis, which may besides sprouting of already existing blood vessels involve vasculogenesis from stem cells residing within the vessel wall or recruited from tissues or the bone marrow.

The aim of the present study was to investigate whether AA would stimulate vasculogenesis from ES cells and to identify reactive oxygen species (ROS) in the LT-mediated signaling pathway involved.

Materials and Methods

Materials

The FLAP inhibitor 3-{3-tert-butylsulfanyl-1-[4-(5-methoxy-pyrimidin-2-yl)-benzyl]-5-(5-methyl-pyridin-2-ylmethoxy)-1H-indol-2-yl}-2,2-dimethyl-propionic acid (AM643) was a generous gift from Panmira Pharmaceuticals (San Diego, Calif., USA). The FLAP inhibitor α -pentyl-3-(2-quinolinylmethoxy)benzenemethanol (REV5901) and LTD₄ were purchased from Biomol (Hamburg, Germany). The LTB₄ receptor (BLT1) inhibitor 6-[6-(3R-hydroxy-1E,5Z-undecadien-1-yl)-2-pyridinyl]-1,5S-hexanediol (U75302), the BLT2 inhibitor 1-[5-ethyl-2-hydroxy-4-[[6-methyl-6-(1H-tetrazol-5-yl)heptyl]oxy]phenyl]-ethanone (LY255283), the cysteinyl LT (CysLT) inhibitor 4-[[{(1R,2E,4E,6Z,9Z)-1-[(1S)-4-carboxy-1-hydroxybutyl]-2,4,6,9-pentadecatetraenyl]thio]-benzoic acid (BAY-u9773), LTB₄, and 12(S) HETE were obtained from Cayman Chemicals (Ann Arbor, Mich., USA). The free radical scavengers N-(2-mercaptopropionyl)glycine (NMPG) and 3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-carboxylic acid (trolox) were obtained from Sigma Aldrich (Deisenhofen, Germany). The NADPH oxidase inhibitor VAS2870 was a generous gift from Vasopharm (Würzburg, Germany).

Methods

Cell Culture of ES Cells and Embryoid Body Formation

Mouse ES cells (line CCE) were grown on mitotically inactivated feeder layers of primary murine embryonic fibroblasts in Iscove's medium (Gibco, Live Technologies, Helgerman Court,

Fig. 1. Pathway for the biosynthesis of LTs from AA. AA passes through the cell membrane by a 'flip-flop' mechanism and moves rapidly to the nuclear membrane. In parallel, AA activates NADPH oxidase (inhibited by VAS2870) in the cell membrane, thereby generating ROS (scavenged by trolox, NMPG), which may interfere with the LT signaling pathway by so far unknown mechanisms. 5-LOX in conjunction with its activating protein FLAP (inhibited by AM643 and REV5901) oxygenates AA to produce 5-HpETE. Thereafter, 5-LOX and FLAP catalyze the dehydration of 5-HpETE to the intermediate LTA₄ which is reacting either to LTB₄ by LTA₄ hydrolase or to LTC₄ by LTC₄ synthase. LTB₄ and LTC₄ can leave the cell and act in autocrine and paracrine manner on BLT1/BLT2 (inhibited by U75302 and LY255283) and – after conversion of LTC₄ to LTD₄ and LTE₄ – on CysLT receptor (inhibited by BAY-u9773), respectively.

Md., USA) supplemented with 15% heat-inactivated FCS (56°C, 30 min) (Sigma), 2 mM glutamine (PAA, Cölbe, Germany), 100 µM 2-mercaptoethanol (Sigma), 1% (v/v) non-essential amino acid stock solution (Biochrom, Berlin, Germany), 1% (v/v) MEM amino acids (Biochrom), 1 mM Na⁺-pyruvate (Biochrom), 0.4% penicillin/streptomycin (Biochrom), 2.5 µg/ml Plasmocin (InvivoGen, San Diego, Calif., USA) and 1,000 U/ml leukemia-inhibitory factor (Chemicon, Chandler's Ford, UK) in a humidified environment containing 5% CO₂ at 37°C and passaged every 2–3 days. Adherent cells (in the following defined as day 0) were enzymatically dissociated using 0.05% trypsin-EDTA in PBS (Gibco) and seeded at a density of 3 × 10⁶ cells/ml in 250 ml siliconized spinner flasks (CellSpin; Integra Biosciences, Fernwald, Germany) containing 125 ml Iscove's medium supplemented as described above but devoid of leukemia-inhibitory factor and Plasmocin. After 24 h, 125 ml of medium were added to give a final volume of 250 ml. The spinner flask medium was stirred at 20 rpm using a stirrer system (Integra Biosciences); 125 ml cell culture medium was exchanged every day.

Immunohistochemistry

As primary antibodies, rat monoclonal anti-platelet endothelial cell adhesion protein (PECAM)-1 antibody (Merck-Millipore, Billerica, Mass., USA) (dilution 1:100) and rat monoclonal anti-vascular endothelial (VE)-cadherin antibody (BD Biosciences, Heidelberg, Germany) were used. Whole-mount embryoid bodies were fixed in ice-cold methanol for 20 min at –20°C and washed three times with PBST (PBS containing 0.01% Triton X-100) (Sig-

ma). Blocking against unspecific binding was performed for 60 min with 10% nonfat milk powder dissolved in 0.01% PBST. The cells were subsequently incubated for 120 min at room temperature with primary antibodies dissolved in PBS, supplemented with 10% milk in 0.01% PBST. Thereafter, the cells were washed three times with PBST and reincubated with a Cy5-conjugated goat anti-rat IgG (H+L; Dianova, Hamburg, Germany) at a concentration of 3.8 µg/ml in PBS containing 10% FCS in 0.01% PBST. After washing three times in PBST, cells were stored in PBS until assayed. Fluorescence recordings were performed by means of a confocal laser scanning setup (Leica TCS SP2; Leica, Bensheim, Germany). The confocal setup was equipped with a 5-mW helium/neon laser, single excitation 633 nm (excitation of Cy5). Emission was recorded at ≥665 nm.

Quantification of Vasculogenesis and Fetal Liver Kinase-1+ Endothelial Progenitor Cells in Embryoid Bodies

For the quantification of capillary areas within embryoid bodies, an optical sectioning routine based on confocal laser scanning microscopy was used. Images (512 × 512 pixels) were acquired from PECAM-1-stained embryoid bodies using the extended depth of focus algorithm of the confocal setup. In brief, 5 full frame images, separated by a distance of 20 µm in z-direction, were recorded, which included the information of the capillary area and spatial organization in a 100-µm-thick tissue slice. The acquired images were processed to generate a single in-focus image projection of the vascular structures in the scanned tissue slice. Using the image analysis facilities of the confocal setup, the branching points

of vascular structures within the three-dimensional projection were identified and counted in relation to the size of the respective embryoid body.

For visualization of fetal liver kinase (Flk)-1+ cells, a reporter ES cell line was used, which was transfected with a lentiviral vector based on the pGZ-CMV vector (System Biosciences, Palo Alto, Calif., USA) containing enhanced green fluorescent protein (eGFP) under the control of the Flk-1 promoter. The Flk-1-eGFP ES cell line displays a bright green fluorescence in Flk-1+ cells when excited by the 488-nm laser line of the confocal microscope (Em 515–530 nm). As a counter stain (red), the cytopainter deep red fluorescence dye (Ex/Em = 630/650 nm) (Abcam, Cambridge, UK) was used. Flk-1+ cells were counted in a 100- μ m-thick optical tissue slice in either untreated 4-day-old embryoid bodies or embryoid bodies treated for 24 h with 100 μ M AA using the Meta-morph image analysis software (Visitron Systems, Puchheim, Germany).

Western Blot Analysis

Western blot assays were carried out after washing the embryoid bodies in PBS and lysing in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Nonidet P-40], supplemented with protease inhibitor cocktail (Biovision, Hannover, Germany) for 20 min on ice. Samples were centrifuged at 13,000 g for 10 min to pellet the debris. After determination of the protein concentration using a Lowry protein assay, 30 μ g of protein samples were boiled, separated in 10% SDS polyacrylamide gels and transferred to PVDF membranes at 20 V for 7 min. Membranes were blocked with 5% (wt/vol) dry fat-free milk powder in TBST (Tris-buffered saline with 0.1% Tween) for 60 min at room temperature. Incubation with primary antibody was performed at 4°C overnight. The primary antibodies used were a monoclonal rat anti-mouse PECAM-1 (Merck-Millipore) antibody, a monoclonal rat anti-mouse VE-cadherin (BD Biosciences), a monoclonal rat anti-mouse Flk-1 antibody (New England Biolabs, Frankfurt, Germany), and a polyclonal goat anti-mouse FLAP (Novus Biologicals, Cambridge, UK). After washing with 0.1% TBST, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 60 min at room temperature. The blot was developed using an enhanced chemiluminescence detection kit (Perkin Elmer, Freiburg, Germany) to produce a chemiluminescence signal. ImageJ software [Schindelin et al., 2015] was used to quantify the protein bands from Western blot images. For quantification, the density of protein bands on the Western blot image, which was acquired using the PEQLAB gel documentation system (VWR, Erlangen, Germany), was assessed by ImageJ. The final quantification reflects the relative amounts of protein as a ratio of each protein band to the respective housekeeping protein.

Measurement of ROS Generation

Intracellular ROS levels were measured using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA; Life Technologies, Darmstadt, Germany), which is a nonpolar compound that is converted into a nonfluorescent polar derivative (H₂DCF) by cellular esterases after incorporation into cells. H₂DCF is membrane impermeable and is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of intracellular ROS [Sharifpanah et al., 2008]. For the experiments, embryoid bodies were incubated in serum-free medium, and 20

μ M H₂DCF-DA dissolved in DMSO was added. After 30 min, intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 3,600- μ m² regions of interest using an overlay mask unless otherwise indicated. For fluorescence excitation, the 488-nm argon ion laser band of the confocal laser scanning microscope was used. Emission was recorded at an emission band of 515–550 nm.

Statistical Analysis

Data are given as means $\pm$ SEM, with *n* denoting the number of experiments unless otherwise indicated. In immunohistochemical analyses of vascular structures, at least 30 embryoid bodies were analyzed in each experiment. One-way ANOVA for unpaired data and Student's *t* test were applied as appropriate. A value of *p* $\leq$ 0.05 was considered significant.

Results

Stimulation of ES Cell-Derived Vasculogenesis and FLAP Expression by AA

To investigate whether activation of the LT signaling pathway would stimulate vasculogenesis, ES cells were treated with different concentrations (50, 100, and 150 μ M) of AA from days 3 to 10 of differentiation, and protein expression of PECAM-1 (fig. 2a) and VE-cadherin (fig. 2c) as well as the differentiation of PECAM-1-positive vascular structures (fig. 2b) were assessed. It was apparent that AA stimulated PECAM-1 expression, with maximum effects achieved at concentrations of 50–100 μ M and a decrease at 150 μ M (fig. 2a). Likewise, AA at a concentration of 100 μ M stimulated protein expression of VE-cadherin (fig. 2c). Consequently, the number of PECAM-1-positive branching points in embryoid bodies was increased upon treatment with 50 and 100 μ M AA (fig. 2b). Since FLAP is a key enzyme in the metabolism of AA towards LTs, FLAP expression was monitored under conditions of AA treatment. As expected, this treatment resulted in upregulation of FLAP protein expression (fig. 2d).

Vasculogenesis is the de novo blood vessel formation from progenitor cells. To assess whether AA treatment increased the number of Flk-1+ vascular progenitor cells, a reporter gene transgenic Flk-1+ eGFP ES cell line, where eGFP is under control of the Flk-1 promoter, was used (fig. 3). It was shown that 24-hour treatment of differentiating embryoid bodies on day 3 with 100 μ M AA resulted in augmented numbers of Flk-1+ vascular progenitor cells on day 4 of cell culture (fig. 3a, b) and increased vascular areas on day 9 (fig. 3c), suggesting that indeed AA stimulated vasculogenesis of ES cells.

Fig. 2. Stimulation of vasculogenesis and FLAP expression of ES cells by AA. **a** Western blot analysis of PECAM-1 expression upon treatment of embryoid bodies from days 3 to 10 of cell culture with different concentrations of AA. The bar chart shows the means $\pm$ SEM of 5 independent experiments. **b** Stimulation of vascular branch differentiation upon treatment with different concentrations of AA (50–150 μ M) as evaluated by determination of branch-

ing points in embryoid bodies immunolabeled with an antibody directed against PECAM-1. The images show representative embryoid bodies. The bar represents 300 μ m. The bar chart shows the means $\pm$ SEM of 4 independent experiments. Protein expression of VE-cadherin (**c**) and FLAP (**d**) upon stimulation with AA. β -Actin and vinculin were used as loading controls for VE-cadherin and FLAP, respectively. * $p < 0.05$ vs. the untreated control.

Inhibition of AA-Mediated Stimulation of Vasculogenesis by FLAP Inhibitors

FLAP is the rate-limiting enzyme in AA metabolism. We therefore treated embryoid bodies from days 3 to 10 with the FLAP inhibitors AM643 (5 μ M) and REV5901 (2 μ M) either in the absence or presence of AA (100 μ M) and assessed vascular differentiation by immunohistochemistry of PECAM-1-positive vascular structures (fig. 4a). Moreover, we determined protein expression of PECAM-1 and VE-cadherin in Western blot assays under the same experimental conditions (fig. 4b). Our data demonstrated that FLAP inhibitors significantly downregulated the number of branching points as well as protein expression of PECAM-1 and VE-cadherin,

indicating inhibition of AA-stimulated vasculogenesis of ES cells upon pharmacological inactivation of FLAP. Notably, functional inhibition of FLAP by AM643 and REV5901 downregulated FLAP protein expression (fig. 4c).

Effects of BLT1, BLT2, and CysLT Receptor Inhibition on Vasculogenesis of ES Cells

LTs act on target cells via specific receptors. CysLTs (e.g. L T E $_4$) are acting via CysLT receptors, which can be inhibited by BAY-u9773. The L T A $_4$ metabolite L T B $_4$ is acting on cells via the receptors BLT1 and BLT2, which can be antagonized by the specific BLT1 inhibitor U75302 and the BLT2 inhibitor LY255283. To evaluate the spe-

Fig. 3. Stimulation of the differentiation of Flk-1+ vascular progenitor cells by AA. Three-day-old embryoid bodies remained either untreated, or were treated either for 24 h or 6 days with AA (100 μ M). On day 4, the number of Flk-1+ green GFP cells was counted in a 100- μ m thick optical slice either in untreated (**a**) or AA-treated (**a'**) embryoid bodies. **b** Bar chart of Flk-1 cell numbers representing the means $\pm$ SEM of 3 independent experiments. * $p < 0.05$ vs. the untreated control. **c, c'** Vascular areas of Flk-1+ cells in plated outgrown embryoid bodies after 9 days of cell culture either in the absence (**c**) or presence of AA (**c'**). The embryoid bodies were counterstained with cell tracker deep red. **a** Bar = 100 μ m. **c** Bar = 300 μ m.

cific participation of LT receptors on vasculogenesis of ES cells, differentiating embryoid bodies were treated either with BAY-u9773 (1 μ M), U75302 (1 μ M), or Ly255283 (1 μ M) from days 3 to 10 of differentiation, and branching points of vessel-like structures (fig. 5a) as well as protein expression of PECAM-1 and Flk-1, which are early markers of cardiovascular progenitor cells (fig. 5b, c), were assessed by Western blot technique. It was apparent that all inhibitors significantly inhibited the formation of vascular structures in embryoid bodies (fig. 5a) and downregulated PECAM-1 as well as Flk-1 expression (fig. 5b, c),

thus indicating that the CysLT receptor as well as BLT1 and BLT2 are involved in the regulation of vasculogenesis of ES cells.

Effects of Exogenous Addition of LTs and 12(S)HETE on Vasculogenesis of ES Cells

The data of the present study suggest that AA-mediated vasculogenesis of ES cells could be inhibited upon pharmacological inactivation of FLAP, indicating the involvement of LTs in vasculogenesis of ES cells. To corroborate these data, we incubated embryoid bodies from

Fig. 4. Inhibition of vasculogenesis of ES cells by FLAP antagonists. Embryoid bodies were incubated from days 3 to 10 with AA (100 μ M) either in the absence or presence of the FLAP inhibitors AM643 (5 μ M) or REV5901 (2 μ M). Subsequently, branching points of vascular structures were determined. * $p < 0.05$ vs. the untreated control. **a** Representative embryoid bodies stained against PECAM-1. Bar = 300 μ m. The bar chart shows the means $\pm$ SEM of 3 independent experiments. # $p < 0.05$ vs. the AA-treated sample. **b** Treatment of differentiating embryoid bodies from days 3 to 10 of cell culture with either AM643 or REV5901 significantly inhibited protein expression of PECAM-1 and VE-cadherin. The upper panels show representative Western blots. The lower panel shows a bar chart of the means $\pm$ SEM of 3 experiments for AM643/PECAM-1 and REV5901/VE-cadherin, and 4 experiments for REV5901/PECAM-1 as well as AM643/VE-cadherin, respectively. **c** Downregulation of FLAP expression upon treatment with either AM643 or REV5901. The upper panel shows representative Western blots. The lower panel shows a bar chart of the means $\pm$ SEM of 3 experiments.

Fig. 5. Effects of BLT1, BLT2, and CysLT receptor inhibition on vasculogenesis of ES cells. Embryoid bodies were treated from days 3 to 10 of differentiation with either the BLT1 receptor inhibitor U75302 (1 μ M), the BLT2 receptor inhibitor LY255283 (1 μ M), or the CysLT receptor inhibitor BAY-u9773 (1 μ M) and PECAM-1 positive vascular structure formation was assessed (**a**). Shown are representative embryoid bodies stained against PECAM-1. The bar chart shows the means $\pm$ SEM of 4 experiments for LY255283, 5 experiments for U75301 and 3 experiments for BAY-u9773. Bar = 300 μ m. **b** Protein expression of PECAM-1 and Flk-1 upon treatment with either U75302 or LY255283. Shown are representative Western blots. The bar charts show means $\pm$ SEM of 4 experiments for PECAM-1 and 3 experiments for Flk-1. **c** Protein expression of PECAM-1 and Flk-1 upon treatment with BAY-u9773. Shown are representative Western blots. The bar charts show means $\pm$ SEM of 4 experiments for PECAM-1 and 3 experiments for Flk-1. * $p < 0.05$ vs. untreated control.

Fig. 6. Reversal of FLAP inhibitor effects on vasculogenesis of ES cells by exogenous LTs. Embryoid bodies were treated from days 3 to 10 of differentiation with FLAP inhibitors in the absence or presence of exogenous LTB_4 (50 nM), LTD_4 (50 nM), or 12(S)HETE (50 nM). Subsequently, protein expression of vascular markers was assessed by Western blot, and differentiation of vascular structures was analyzed by the determination of PECAM-1-positive branching points. **a–c** Reversal of REV5901 (2 μM) effects on PECAM-1 expression upon treatment with LTB_4 (n = 4) (**a**), LTD_4 (n = 3) (**b**), and 12(S)HETE (n = 4) (**c**). Shown are representative Western blots as well as the means $\pm$ SEM of individual experiments. * $p < 0.05$ vs. untreated control.

days 3 to 10 of cell culture with either the FLAP inhibitor REV5901 (2 μM) or AM643 (5 μM) either in the presence or absence of LTB_4 (50 nM) (fig. 6a, d, e), LTD_4 (50 nM) (fig. 6b, d, e) or 12(S)HETE (50 nM) (fig. 6c–e). Subsequently, protein expression of PECAM-1 as well as PECAM-1-positive vascular structures were assessed. As expected, REV5901 downregulated PECAM-1 expression,

which was partially restored upon exogenous addition of LTs (fig. 6a, b). Moreover, reversal of PECAM-1 expression was achieved upon exogenous addition of 12(S)HETE (fig. 6c). Comparable results were achieved with AM643 (data not shown). Notably, REV5901 and AM643 impaired the formation of vascular structures, which was reversed upon exogenous addition of either LTB_4 , LTD_4 ,

Fig. 6. Reversal of FLAP inhibitor effects on vasculogenesis of ES cells by exogenous LTs. Embryoid bodies were treated from days 3 to 10 of differentiation with FLAP inhibitors in the absence or presence of exogenous LTB₄ (50 nM), LTD₄ (50 nM), or 12(S)HETE (50 nM). Subsequently, protein expression of vascular markers was assessed by Western blot, and differentiation of vascular structures was analyzed by the determination of PECAM-1-positive branching points. **d** Representative embryoid bodies stained against PECAM-1 are shown. Bar = 300 μ m. **e** The bar chart shows the means $\pm$ SEM of 3, 7, and 8 independent experiments for LTB₄, LTD₄, and 12(S)HETE, respectively. * $p < 0.05$ (as indicated).

or 12(S)HETE (fig. 6d, e). In conclusion, these data support the assumption that LTs as well as 12(S)HETE are involved in vasculogenesis of ES cells.

ROS Generation in Embryoid Bodies upon AA Treatment

Previous studies have indicated that AA induces ROS generation in cells [Chien et al., 2013] and cell-free systems [Souabni et al., 2012] via interaction of the p67(phox)-Rac complex with the phagocyte oxidase Nox2 [Matono et al., 2014]. Moreover, our studies have demonstrated that ROS are critically involved in vasculogenesis of ES cells [Sauer et al., 2005; Bekhite et al., 2010]. To investigate whether AA exerted its provasculogenic effects through ROS generation in differentiating ES cells, embryoid bodies (4 days old) were treated with AA (100 μ M), and ROS generation was evaluated using the ROS-sensitive fluorescence indicator H₂DCF-DA (fig. 7a). AA treatment for 30 min increased ROS

generation and reached maximum levels after 6 h of treatment. Even 20 h after AA treatment, an elevated plateau of ROS generation was observed in ES cells. Application of AA in the presence of either the FLAP inhibitors REV5901 or AM643 or in the presence of nimesulide (100 μ M), which inhibits COX-2 downstream of AA, failed to inhibit ROS generation, suggesting that ROS are neither generated downstream of FLAP nor downstream of COX-2 (fig. 7b). To link ROS generation by AA to the stimulation of vasculogenesis by this compound, embryoid bodies were treated from days 3 to 10 of differentiation with AA (100 μ M) either in the presence or absence of the ROS scavengers trolox (100 μ M) and NMPG (100 μ M) or the NADPH oxidase inhibitor VAS2870 (50 μ M) (fig. 7c, d). This treatment totally abolished the stimulation of vasculogenesis by AA as assessed by PECAM-1 immunohistochemistry, and indicated that ROS play a determining role in the vasculogenic stimulation of ES cells.

Fig. 7. Generation of ROS upon treatment of embryoid bodies with AA, effects of FLAP and COX-2 inhibition as well as inhibition of AA-mediated vasculogenesis by free radical scavengers and the NADPH oxidase inhibitor VAS2870. **a** Four-day-old embryoid bodies were incubated with the fluorescent ROS indicator H₂DCF-DA, and the increase in DCF fluorescence following treatment with AA (100 μM) was analyzed. * *p* < 0.05 vs. 0 h. **b** AA-mediated ROS generation in the absence and presence of either the FLAP inhibitors REV5901, AM643, or the COX-2 inhibitor nimesulide. * *p* < 0.05 vs. w/o AA. **c, d** Analysis of branching points in PECAM-1-positive vascular structures upon incubation with AA either in the absence or the presence of the NADPH oxidase inhibitor VAS2870 (50 μM), or the ROS scavengers NMPG (100 μM) and trolox (100 μM). **c** Representative embryoid bodies are shown. Bar = 300 μm. **d** The bar chart shows the means ± SEM of 3 individual experiments. # *p* < 0.05, * *p* < 0.05 (as indicated).

Discussion

LTs are decisive players in inflammation and wound healing, which is generally associated with recruitment of blood vessels to the inflamed tissue and may include vascular differentiation of stem cells. Moreover, the early postimplantation embryo is subjected to non-life-threatening inflammatory episodes [Straub et al., 2011], which may be necessary for the induction of differentiation pathways in stem cells of the inner cell mass. In the present study, it is demonstrated that AA stimulated vasculogenesis of ES cells by increasing the number of vascular branches, protein expression of the endothelial markers VE-cadherin and PECAM-1, as well as by increasing the numbers of Flk-1+ endothelial progenitor cells. Notably, AA significantly increased expression of FLAP, which is an accessory protein for 5-LOX to oxidize AA in two steps to LTA₄. In human osteosarcoma cells lacking FLAP [Dixon et al., 1990], or where FLAP is pharmacologically inhibited, transformation of endogenous AA by 5-LOX is efficiently blocked [Peters-Golden and Brock, 2003].

Since we assumed that the effect of AA on vasculogenesis of ES cells involved LTs, we coadministered the known FLAP inhibitor REV5901 as well as the newly developed compound AM643 [Stock et al., 2010], which block the formation of both LTB₄ and the CysLTs (i.e. LTC₄, LTD₄, and LTE₄). FLAP inhibitors act as broad-spectrum LT-modifier drugs and may have a wide range of therapeutic applications in the treatment of asthma, chronic obstructive pulmonary disease, arthritis, cardiovascular disease, and cancer [Sampson, 2009]. Both FLAP inhibitors downregulated endothelial marker expression and abolished the effects of AA on vasculogenesis of ES cells. Notably, FLAP inhibitor treatment resulted in downregulation of FLAP expression which is – to the best of our knowledge – a novel feature of these compounds, and may contribute to the observed inhibition of vasculogenesis. Downregulation of FLAP presumably results in a decrease in provasculogenic LT synthesis with the consequent loss of vasculogenic capacity. Since inhibition of vasculogenesis by FLAP inhibitors strongly suggests the involvement of LTs in signal transduction, we supplemented the cell culture medium with exogenous LTB₄, LTD₄ as well as 12(S)HETE in the absence and presence of FLAP inhibitors. As assumed, treatment with LTB₄ and LTD₄ restored vasculogenesis towards the control level, despite the presence of FLAP inhibitors. The reversing effect of 12(S)HETE on vasculogenesis was somehow unexpected, since this compound is synthesized from AA via the 12-LOX pathway. However, previous studies have

shown that the highly selective FLAP inhibitor 655,238 inhibited 12(S)HETE production in human platelets, but had no effect on 12-LOX activity [Ozeki et al., 1999], which points towards a potential interaction between the 5-LOX and the 12-LOX pathway.

LTs act on target cells via G-protein-coupled LT receptors. In differentiating ES cells, the expression of BLT1, BLT2 as well as CysLT1 receptor and CysLT2 receptor has been shown previously [Finkensieper et al., 2010]. To investigate the involvement of specific receptors on vasculogenesis of ES cells, we used the specific BLT1 antagonist U75302, the specific BLT2 inhibitor LY255283 as well as the CysLT receptor inhibitor BAY-u9773. Each of these inhibitors partially downregulated the expression of vascular markers, indicating that not only one single LT receptor subtype was responsible for AA-mediated vasculogenesis, but presumably all receptor subtypes were involved. Previously, it has been evidenced that the CysLT1 receptor blocker montelukast, but not the CysLT2 receptor blocker BAYcyslt2, inhibited microvessel outgrowth from rat thoracic aortic rings [Xu et al., 2010]. The same group reported that LTD4 stimulated migration but not proliferation of human endothelial cells upon CysLT1 receptor activation [Yuan et al., 2009]. Moreover, BLT2 has been shown to be involved in proangiogenic processes since BLT2 inhibition by LY255283 almost completely blocked VE growth factor (VEGF)-induced vessel formation from human endothelial cells in Matrigel plug assays, and VEGF significantly upregulated BLT2 expression in human umbilical vein endothelial cells [Kim et al., 2009]. BLT2 as well as BLT1 are expressed on human endothelial cells [Kim et al., 2009; Johansson et al., 2010]. In human immature endothelial progenitor cells isolated from the bone marrow, LTB₄ stimulated cell migration, presumably via direct interaction with BLT1 receptors, which are prominently expressed on this cell type [Walenta et al., 2011]. VEGF and VEGF receptors are induced by hypoxia in tumor tissue. Moreover, an altered metabolism of AA by COX and LOX is a common feature of several epithelial-derived malignancies and has been shown to have crucial roles in cancer progression [Wang and DuBois, 2010]. This suggests a close interaction between AA- and VEGF-mediated signaling pathways.

Tissue inflammation is generally associated with the generation of ROS, which do not only play deleterious effects in cells by exerting oxidative stress, but also regulate the expression of a plethora of signaling pathways, regulating the expression of pro- and anti-inflammatory cytokines and growth factors like VEGF, fibroblast growth factor-2 and platelet-derived growth factor-BB [Sauer et

al., 2001], which are involved in processes of neoangiogenesis, e.g. during wound healing. Especially VEGF is well known to regulate vascular growth via generating ROS within the signaling cascade [Kim and Byzova, 2014]. In human eosinophils, LTB₄ induced intracellular ROS production and surface upregulation of the exocytosis marker protein CD63 via BLT1-mediated signaling [Min et al., 2014]. Furthermore, AA has been reported to increase ROS production, e.g. in small porcine coronary arteries [Oltman et al., 2003] and in human leukemia cells [Chien et al., 2013]. Oxidative stress has been shown to increase the synthesis of inflammatory LTs and prostaglandins, thereby perpetuating a vicious cycle of excessive and uncontrolled inflammation. Oxidative stress and peroxidation of membrane phospholipids enhance phospholipase A₂ [Balboa and Balsinde, 2006] and increase the release of AA from murine cell membranes [Han et al., 2003]. Moreover, expression and function of 5-LOX, the key enzyme in LT synthesis, is apparently redox regulated: catalysis by 5-LOX requires oxidation of Fe²⁺ to the active Fe³⁺ state by lipid hydroperoxides [Radmark et al., 2015], suggesting that the intracellular redox state is an important parameter of cellular 5-LOX activity. The mechanism by which AA increases ROS generation may involve different sources, which include LOX and COX as well as NADPH oxidases [Cho et al., 2011; Speed and Blair, 2011]. Recently, it has been demonstrated that AA induces superoxide generation in human HeLa cells via direct interaction of the p67(phox)-Rac complex with the phagocyte oxidase Nox2 [Matono et al., 2014]. Moreover, stimulation of ROS production downstream of BLT1 and BLT2 signaling has been reported in different leukocyte cell types and cancer cells, and was mainly attributed to the activation of the NOX1 isoform of NADPH oxidase [Cho et al., 2011]. In mice, selective inhibition of the

BLT2 receptor reduced the release of vascular ROS and improved endothelial function [Hoyer et al., 2012].

The data of the present study suggest the involvement of NADPH oxidase in ROS formation upon treatment of differentiating ES cells with AA, since the NADPH oxidase inhibitor VAS2870 as well as antioxidants decreased vascular structure formation achieved with AA. Notably, neither inhibition of FLAP nor inhibition of prostaglandin synthesis by the COX-2 antagonist nimesulide blunted AA-induced ROS generation. This suggests that ROS may arise from the direct stimulation of NADPH oxidase by AA, as has been previously demonstrated for NOX1 in human colon adenocarcinoma cells [Sadok et al., 2008], NOX2 in HeLa cells [Matono et al., 2014], and NOX4 in human fibroblasts [Rossary et al., 2007]. Our data confirm the results of our previous studies showing that ROS, namely from NOX1 and NOX4, are critically involved in the regulation of vascular differentiation of murine ES cells [Sauer et al., 2005; Schmelter et al., 2006; Bekhite et al., 2010]. Since accumulating evidence suggests that different diseases with excessive and uncontrolled vascular growth, e.g. psoriasis, arthritis, and cancer, are associated with excessive inflammation and overstimulated LT signaling, the knowledge of the participation of stem cells in pathological vascular growth will aid to develop novel therapeutic approaches to treat these severe and life-threatening diseases.

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