Angiogenesis (2019) 22:421–431
https://doi.org/10.1007/s10456-019-09667-z

ORIGINAL PAPER

The plaque-aortic ring assay: a new method to study human atherosclerosis-induced angiogenesis

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Received: 11 October 2018 / Accepted: 26 March 2019 / Published online: 9 April 2019

Abstract

Progression of atherosclerotic plaques into life-threatening lesions is associated with angiogenesis which contributes to intraplaque hemorrhages and plaque instability. The lack of adequate models for the study of human plaque-induced angiogenesis has limited progress in this field. We describe here a novel ex vivo model which fills this gap. Plaques obtained from 15 patients who underwent endarterectomy procedures were co-cultured in collagen gels with rat aorta rings which served as read-out of human plaque angiogenic activity. The majority of plaque fragments markedly stimulated angiogenic sprouting from the aortic rings while concurrently promoting the outgrowth of resident macrophages from the aortic adventitia. This stimulatory activity correlated with the presence of intraplaque macrophages. Proteomic analysis of plaque secretomes revealed heterogeneity of macrophage-stimulatory cytokine and angiogenic factor production by different plaques. VEGF was identified in some of the plaque secretomes. Antibody-mediated blockade of VEGF had significant but transient inhibitory effect on angiogenesis, which suggested redundancy of plaque-derived angiogenic stimuli. Pharmacologic ablation of adventitial macrophages permanently impaired the angiogenic response of aortic rings to plaque stimuli. Our results show that human plaque-induced angiogenesis can be reproduced *ex vivo* using rat aortic rings as read-out of plaque angiogenic activity. This model can be used to identify key cellular and molecular mechanisms responsible for the neovascularization of human plaques.

Keywords Atherosclerosis · Neovascularization · Endothelial cells · Assay · Collagen

Introduction

Atherosclerotic cardiovascular disease is a major cause of morbidity and mortality in the US [1]. Major disability or death is caused by progression of atherosclerotic plaques into unstable lesions. Plaque instability is associated with intraplaque bleeding [2] which is an important predictor of plaque vulnerability and mortality [3, 4]. Hemorrhages cause plaque disruption and oxidative injury, contribute to cholesterol accumulation, and deliver leukocytes which further damage the vessel wall through inflammatory mechanisms [5]. Ultimately, intraplaque hemorrhages lead to plaque rupture, thrombosis, and cessation of blood flow to vital organs [5]. Intraplaque hemorrhages are often caused by rupture of the fibrous cap overlying the lipid-rich and necrotic core of the plaques [6], but they may also be the result of bleeding from angiogenic neovessels that have penetrated into the plaque [7]. Hemorrhages from intraplaque angiogenesis have been attributed to the dysmorphic nature of intraplaque neovessels which lack adequate pericyte coverage and extracellular matrix (ECM) support [5, 8].

The neovasculature of atherosclerotic plaques originates primarily from adventitial vasa vasorum which proliferate and invade the intima through sites of media disruption [8, 9] in response to hypoxic and inflammatory stimuli originating from the plaque [10, 11]. Although angiogenesis is recognized as an important source of intraplaque hemorrhage and plaque vulnerability, the regulatory mechanisms of the angiogenic response induced by human atherosclerotic

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10456-019-09667-z) contains supplementary material, which is available to authorized users.

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plaques are poorly understood. Progress in this field has been hampered by the lack of suitable models for the study of human plaque-induced angiogenesis and its regulatory mechanisms.

To fill this gap, we have developed a new *ex vivo* model to study the angiogenic response of the aortic wall to atherosclerotic plaques. We have used this model to evaluate the angiogenic activity of plaque explants obtained from patients who underwent endarterectomy procedures at the VA Puget Sound Health Care System. Results of this study show that rat aortic ring cultures can be reproducibly used to assay human plaque angiogenic activity and investigate the cellular and molecular mechanisms of plaque-induced angiogenesis.

Materials and methods

Preparation of human atherosclerotic plaque explants for angiogenesis assays

Human plaques were obtained from patients undergoing carotid endarterectomy at VA Puget Sound Health Care System (VAPSHCS), Seattle, WA. All studies were performed with approval from the VAPSHCS Institutional Review Board. Excised plaques were placed in sterile serum-free Endothelial Basal Medium (EBM, Lonza) in the operating room, accessioned in the pathology laboratory, and examined under sterile conditions in our laboratory under a dissecting microscope. Distinct plaque domains (fibroatheroma, disrupted plaque, intimal thickening, thrombus) were identified, dissected into 1–2 mm^3 pieces, rinsed in EBM, and tested for angiogenic activity. Adjacent plaque regions were placed in buffered formalin and processed for histologic and immunohistochemistry studies. Plaque morphology was evaluated with hematoxylin and eosin and Masson trichrome stains [12]. A subset of plaque fragments was used to prepare plaque-conditioned medium (see below).

Aortic ring—atherosclerotic plaque angiogenesis assay

All animal procedures were performed with approval from the Veterans Administration Puget Sound Health Care System Institutional Animal Care and Use Committee and followed National Institutes of Health Guidelines. Aortic rings obtained from 1 to 2 month old Fischer 344 male rats were cultured, with or without human plaque explants, in collagen gels and serum-free EBM [13, 14]. Individual plaque explants were embedded in a 30 µl collagen drop in 4-well Nunc dishes, with or without an aortic ring which was placed at ~ 0.5–1 mm distance from each explant. The collagen drop was then spread with a pipette tip to form a thin wafer and allowed to gel at 37 °C in a humidified CO_2 incubator. Following collagen gelation, each culture was supplemented with 500 µl of serum-free EBM and returned to the incubator. At least three anatomically distinct regions were sampled from each plaque including the lipid-rich core of the plaque, calcified areas, and disrupted portions of the plaque, and each was tested in quadruplicate. Separate cultures prepared with plaque explants alone were incubated in serum-free EBM with or without 10 ng/ml vascular endothelial growth factor (VEGF) and 50 ng/ml basic fibroblast growth factor (bFGF) or 10% FBS. Collagen was prepared from rat tails as reported [15]. Angiogenesis was quantified by counting microvessels sprouted from vessel explants over time [15]. In selected experiments, aortic rings were depleted of adventitial macrophages by overnight incubation in a 1:10 dilution of liposomal clodronate (ClodronateLiposome.org) followed by washing in EBM [16]. PBS liposome-treated rings were used as controls. A separate set of aortic ring cultures was treated with plaque-conditioned EBM diluted 1:2 with fresh medium. For the preparation of conditioned medium, plaque fragments were rinsed up to 5 times in 5 ml of serum-free EBM and incubated in the same volume of medium for 6–8 days. Following centrifugation at 2000 rpm the supernatant was stored at − 80°C until use for aortic ring assay, immunoblot studies, and VEGF ELISA. The activity of plaque-derived VEGF in aortic ring-plaque co-cultures was blocked with anti-human VEGF (1 µg/ml) (R&D Systems). The efficacy of the anti-VEGF antibody was confirmed in aortic ring cultures treated with recombinant VEGF (R&D Systems) by neutralizing the activity of VEGF with the blocking antibody. Control cultures were treated with non-immune IgG of left untreated.

ELISA

Human VEGF in plaque-conditioned media was measured with an Invitrogen ELISA kit (ThermoFisher Scientific).

Immunocytochemistry

Aortic ring-derived macrophages in whole mount preparations of control and plaque-containing cultures were highlighted by immunoperoxidase with anti-rat CD68 monoclonal antibody, as reported [15, 17]. The cellular composition of human plaques including plaque fragments used in the aortic ring assay was characterized with The Ventana BenchMark immunohistochemistry detection system using anti-human specific antibodies against α-SMA, CD3 or CD163. Densitometric analysis of immunostained macrophages, T lymphocytes, and smooth muscle was performed using NIH Image J software [18].

Protein arrays

Serum-free medium conditioned for 7 days by human plaque explants obtained from 7 patients was analyzed by immunoblotting with a Human Cytokine XL Array (R&D Systems) targeting 102 growth factors/cytokines. Briefly, arrays were incubated with plaque-conditioned medium for 18 h at 4 °C, washed, exposed to chemiluminescent reagents (Pierce), and imaged with a Chemidoc XL chemiluminescent detection system (BioRad). Densitometric analysis of immunoblots was performed with NIH ImageJ software. Quantitative protein analysis in these blots was performed by normalizing the intensity of each analyte to internal reference controls.

Statistical analysis

Data from each experiment were analyzed using Prism software (GraphPad, Inc). Differences between experimental groups were evaluated by unpaired two-tailed t-tests. Statistical significance between experimental groups was set at $p < 0.05$. For the aortic ring co-culture assay, individual plaque explants were considered angiogenic if their stimulatory activity scored 2 standard deviations above the average of untreated controls from our archive of > 200 aortic ring cultures. Plaque domains scoring below this threshold were classified as non-angiogenic.

Results

Clinical data from enrolled patients

Plaques were obtained from 15 patients undergoing endarterectomy. One of these patients underwent both right and left carotid endarterectomy. A total of 16 plaques were therefore used for this study. Enrolled patients were all males, 55–74 years old. Ten patients presented with clinical manifestations of cerebral ischemia whereas the remaining five where asymptomatic. Risk factors included hyperlipidemia (100%, no data available for one patient), obesity (40%), hypertension (87%), diabetes (60%), smoking (53%), and sleep apnea (20%). Eleven patients had additional manifestations of cardiovascular disease including coronary artery disease, peripheral artery disease, aortic valve disease, and congestive heart failure. Thirteen patients were on statin therapy (Table 1).

Human plaque explants have limited capacity to produce angiogenic sprouts

Atherosclerotic plaque fragments cultured in collagen gels under serum-free conditions remained acellular or produced sparse outgrowths of mostly single cells. Isolated endothelial sprouts were observed in rare cultures (1–3 endothelial sprouts per plaque explant in 5% of cultures). Addition of serum or exogenous angiogenic factors (bFGF, VEGF, or both) failed to significantly increase the extent and frequency of angiogenic sprouting in these cultures. Histologic examination of samples obtained from regions of the plaque adjacent to the cultured plaque fragments showed fibrous cap disruption, fibrin deposition, calcifications, inflammation, and angiogenesis in 80–95% of tested plaques.

Human plaque explants potently stimulate angiogenesis from aortic explants

The inability of most of the plaque fragments to sprout was likely due the focal distribution of angiogenic endothelial cells and the dysfunctional nature of the plaque endothelium [5, 8]. We postulated that plaques, despite this intrinsic limitation, were still capable of stimulating angiogenesis from an external source of viable endothelial cells. We therefore tested the angiogenic properties of plaques in the rat aortic ring assay. In this assay, aortic rings cultured in collagen gel under serum-free conditions produce a self-limited angiogenic response characterized by sprouting of branching microvessels during the first week of culture. The angiogenic response is followed by vascular regression characterized by gradual reabsorption of the neovessels through proteolytic mechanisms [19, 20]. Evaluation in this assay of 190 plaque explants from 15 patients showed that plaques markedly stimulated angiogenic sprouting from the aortic explants. Plaque-induced neovessels were more numerous and longer than control and frequently polarized toward the plaque explants. A dense outgrowth of aorta-derived non-endothelial single cells was typically associated with the angiogenic neovessels. Potent stimulation of angiogenesis was also obtained using plaque-conditioned medium (Fig. 1). Thus, although human plaques were unable to produce an endogenous angiogenic response, they could potently stimulate angiogenesis from an intact arterial wall. All plaque domains exhibited angiogenic activity. However, fibroatheromas, disrupted plaque fragments, and thrombosed plaques were significantly more angiogenic than intimal thickenings.

Human plaque explants release a heterogeneous mixture of angiogenic factors, cytokines, and chemokines

To characterize the plaque angiogenic secretome, medium conditioned for 7 days by plaque explants obtained from 7 patients was analyzed by immunoblotting. This study showed that human plaques secrete a variety of pro-angiogenic and macrophage-stimulatory growth factors, cytokines, and chemokines. It also demonstrated that

Table 1 Clinical data of enrolled patients

Plaque #	Age	Sex	Clinical data								
			Symptoms	Hyperlipidemia	Obesity (BMI)	Hypertension	Diabetes	Smoking	Other cardiovascular problems	Statins	Sleep apnea
1	64	M	Staggering, feeling unsteady, right eye blurriness	Yes	Yes (34.2)	Yes	Yes	Yes	Peripheral vascular disease	Yes	No
2	74	M	Stroke/acute infarct, left pons/RLE weakness	Yes	Yes (32)	Yes	No	Yes	Chronic brain ischemia, aortic valve insufficiency	Yes	No
3	68	M	Syncope, aphasia, right sided weakness	Yes	No (27.7)	No	No	Yes	Deep vein thrombosis	Yes	No
4	68	M	Asymptomatic	Yes	Yes (33.5)	Yes	No	No	Coronary artery disease, congestive heart failure, cerebrovascular accident	Yes	No
5	67	M	Asymptomatic	Yes	Yes (34.8)	Yes	Yes	No	Stenosis of left internal carotid artery	Yes	Yes
6[a]	59	M	Asymptomatic	Yes	No (18.9)	Yes	No	Yes	No	Yes	No
7	68	M	Infarct in right parietal lobe and caudate nucleus	Yes	Yes (30.2)	Yes	Yes	Yes	Coronary artery disease, peripheral vascular disease	Yes	No
8	68	M	Stroke: left sided hand and leg weakness	Yes	No (21.2)	Yes	No	Yes	Peripheral vascular disease	Yes	No
9	70	M	Stroke: left arm and leg weakness	Yes	Yes (37.4)	Yes	yes	No	Coronary artery disease	Yes	Yes
10	55	M	Right side numbness	No data available	No (29.1)	No	Yes	No	No	Yes	No
11	68	M	Asymptomatic	Yes	Yes (31)	Yes	Yes	No	No	No	No
12	63	M	No reported symptoms	Yes	No (26.7)	Yes	Yes	No	Coronary artery disease, peripheral vascular disease	No	No
13	64	M	Left arm weakness, left face numbness	Yes	Yes (30.31)	Yes	Yes	Yes	No	Yes	No
14	73	M	Tunnel vision/dizziness	Yes	Yes (48.6)	Yes	No	No	No	Yes	No
15	69	M	Asymptomatic	Yes	Yes (34.8)	Yes	Yes	No	Stenosis of right carotid artery	Yes	Yes
16[a]	63	M	Right arm numbness and weakness	Yes	No (23.6)	Yes	Yes	Yes	Peripheral vascular disease	Yes	No

[a]Plaque 6 and 16 were obtained from right and left carotid arteries of the same patient

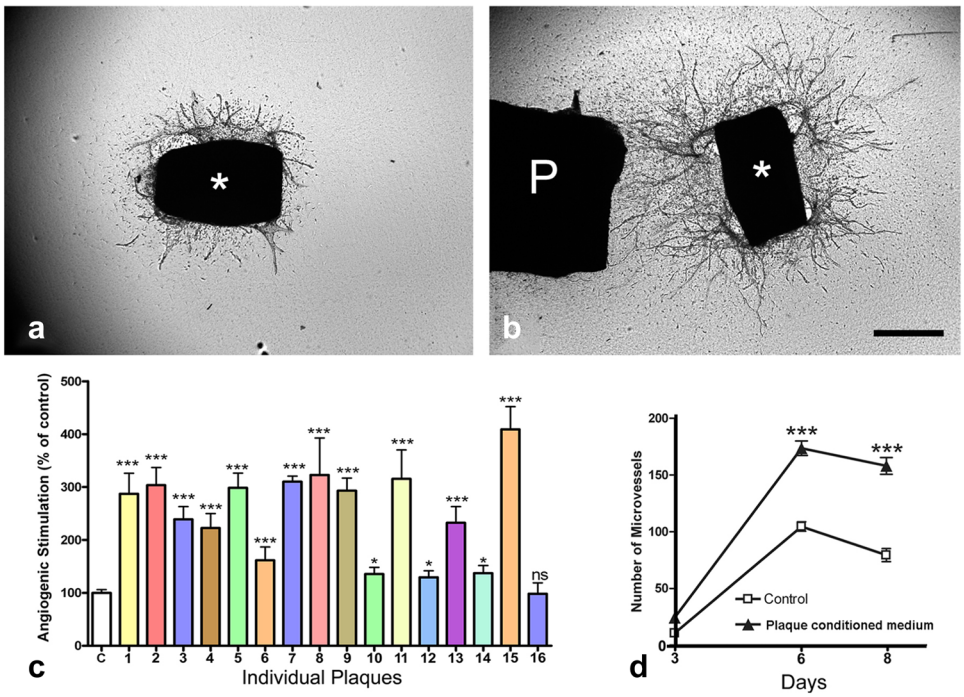

Fig. 1 Human plaques potently stimulate angiogenesis from rat aortic rings in collagen gel culture. Photographs show rings of rat aorta (asterisks) cultured without (**a**) or with (**b**) human plaque (P, scale bar = 1 mm). Bar graph (**c**) shows angiogenic response of aortic rings to 16 plaques (labeled 1–16) obtained from 15 patients ($N = 4$–20 plaque fragments/experiment; one patient underwent bilateral endarterectomy); control (C) in this graph represents the average angiogenic score of all control aortic ring cultures ($N = 68$). Line graph (**d**) shows the angiogenic response over time of aortic ring cultures treated with plaque-conditioned medium (obtained from plaque 7) or left untreated ($N = 4$). $^{*}p < 0.05$, $^{***}p < 0.001$

plaque secretomes are heterogeneous and variable in different plaque samples. Angiogenic molecules released by plaques included angiogenin [21], hepatocyte growth factor (HGF) [22], and VEGF [23], as well as pro-angiogenic cytokines and chemokines including growth differentiation factor 15 (GDF15) [24], interleukin-6 (IL-6) [25], IL-8 [26] monocyte chemotactic protein 1 (MCP1) [27], macrophage migration inhibitory factor (MIF) [28], YKL-40 [26], and stromal cell-derived factor-1 (SDF1-alpha/CXCL12) [29]. Some of these factors such as angiogenin, MCP1, and GDF-15 were consistently found in the plaque secretome from different patients whereas others such as VEGF were detectable in some of the secretomes (Fig. 2, Supplement 1). To confirm that production of VEGF varied in different plaques, we measured VEGF levels by ELISA in conditioned media obtained from all tested plaques ($N = 16$). VEGF concentration ranged from 0 to 702 pg/ml. Measurable levels of VEGF were identified in 75% of plaque secretomes.

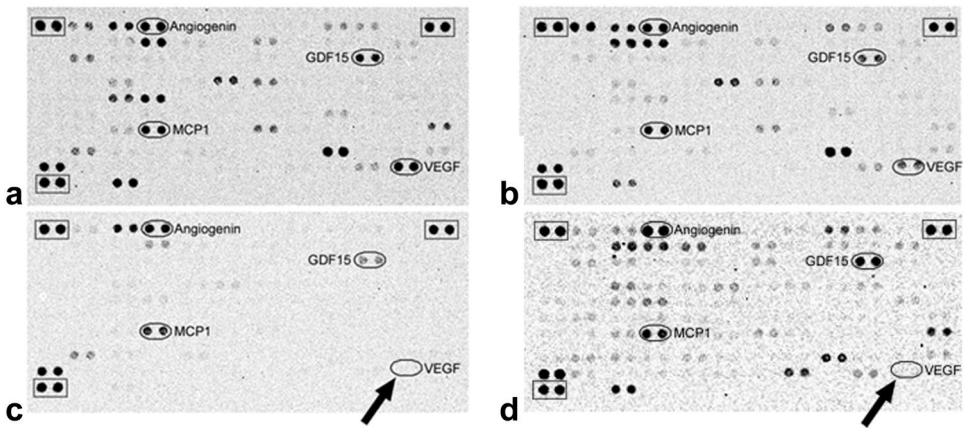

Fig. 2 Atherosclerotic plaques produce a heterogeneous mixture of pro-angiogenic growth factors, cytokines and chemokines. Representative dot blots hybridized to plaque-conditioned medium (CM) from four different patients (**a**–**d**). On these arrays, each factor is represented by duplicate spots. Selected growth factors/cytokines are shown by ovals. Rectangles indicate control spots. Note that VEGF is present in the CM from two plaques (**a**, **b**), but is absent in the others (**c**, **d** arrow) whereas angiogenin, MCP1 and GDF-15 are present in all CMs. Cross-reference with bar graph of Fig. 1c: **a** CM from plaque 7; **b** CM from plaque 6; **c** CM from plaque 4; **d** CM from plaque 5

Although VEGF was detected in the conditioned medium of most angiogenic plaques, no good correlation was observed between VEGF levels and number of microvessels. For example, whereas VEGF was detected by both immunoblotting and ELISA (428 pg/ml) in the conditioned medium of plaque 7, no detectable VEGF was identified by either assay in the conditioned medium of plaque 8 which was equally angiogenic (Fig. 1, bar graph). Angiogenic factors other than VEGF may have contributed to the angiogenic response in these cultures. In fact, several of the angiogenic factors/cytokines identified in the plaque secretomes have been previously shown to stimulate angiogenesis in the aortic ring assay [13, 30–34].

Angiogenic activity of plaque explants correlates with intraplaque macrophages

Stimulation of angiogenesis was observed in the vast majority of plaque-aortic ring co-cultures. However, using very stringent criteria of angiogenic stimulation (> 2 SD over unstimulated aortic ring control cultures) we identified a subset of plaque fragments with low or absent stimulatory activity. To define whether differences in plaque angiogenic activity were due to specific cell types, plaque fragments tested in the aortic ring assay were excised from the collagen gels, processed for paraffin embedding, and further analyzed by immunohistochemistry for the presence of macrophages, T lymphocytes and smooth muscle cells/myofibroblasts. This additional study showed that angiogenic plaque fragments contained significantly higher number of CD163 positive macrophages compared to non-angiogenic or minimally angiogenic plaque fragments. CD3+ lymphocytes were also increased in the angiogenic fragments, but without definite statistical significance. There was no noticeable difference in α-SMA+ cells between angiogenic and non-angiogenic plaque fragments (Fig. 3). Thus, stimulation of aortic angiogenesis by human plaques correlated with the presence of immune cells and, specifically, plaque-associated macrophages.

Targeting of VEGF has transient inhibitory effect on plaque-induced angiogenesis

To evaluate the role of VEGF in plaque-induced angiogenic activity, aortic ring-plaque co-cultures prepared with VEGF-producing plaque fragments were treated with anti-human

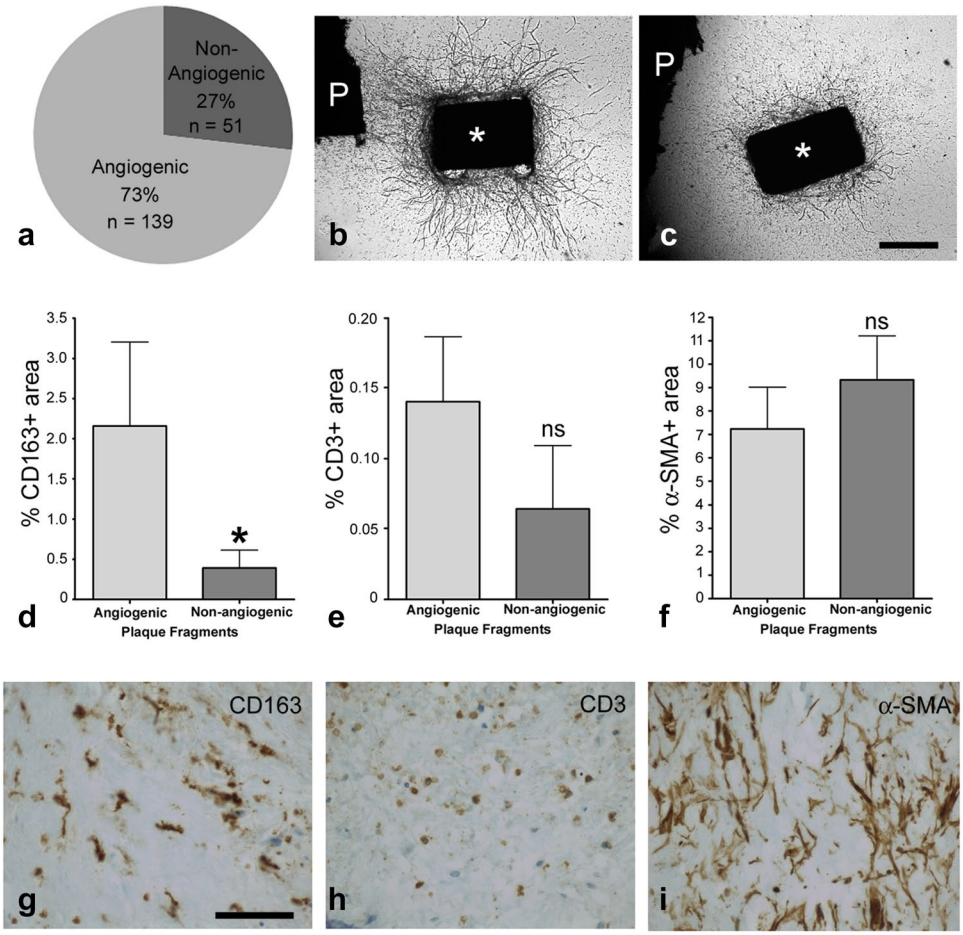

Fig. 3 Plaque angiogenic activity correlates with the presence of CD163+ macrophages within plaques. Pie chart showing percentage of plaque fragments with angiogenic activity from 190 aortic ring-plaque co-culture assays (**a**). Photographs show examples of sprouting response of aortic rings (asterisk) to angiogenic (**b**) and non-angiogenic (**c**) plaque samples (P, scale bar = 1 mm). Immunostaining of cultured plaque fragments harvested at day 7 following completion of the angiogenesis assay for specific cell markers (**d**, **e**, **f**) demonstrates significantly higher number of CD163+ macrophages in angiogenic plaques (**d**); CD3+ lymphocytes also appear to be increased in angiogenic plaques, but this finding is not statistically significant (**e**). No difference is observed in α-SMA+ cells between angiogenic and non-angiogenic plaques (**f**). *$p < 0.05$, $N = 5–10$. Representative images of angiogenic plaque samples immunostained for CD163 (**g**), CD3 (**h**), and α-SMA (**i**), scale bar = 50 μM

VEGF blocking antibody or non-immune IgG. The anti-VEGF antibody significantly suppressed the plaque-induced angiogenic response of the aortic rings during the first 5 days of culture. However, this inhibitory effect gradually waned and became statistically insignificant over time (Fig. 4). This finding suggests that plaque-induced angiogenesis is mediated by a redundant system of angiogenic stimuli, and that pharmacologic blockade of individual growth factors/cytokines is insufficient to completely and permanently suppress the angiogenic response to human plaques.

The ex vivo angiogenic response of the aortic wall to plaque stimuli is associated with polarized outgrowth of adventitial macrophages toward the plaque

Angiogenic outgrowths in aortic-plaque co-cultures contained numerous rounded cells with granular and vacuolated cytoplasm. Daily monitoring of the cultures demonstrated that these cells originated from the aortic rings and were chemotactically attracted toward the plaque fragments, while no significant outgrowth was observed from the plaques themselves. Over time, some of the rounded cells acquired translucent cytoplasmic droplets consistent with lipid material, as seen in foam cells. These cells showed features of macrophages which are a component of the aortic outgrowths. Macrophages in the aortic ring assay originate from the aortic adventitia, are closely associated with angiogenic neovessels, and can be highlighted by immunohistochemistry using antibodies against CD68 or CD163 [16, 17, 35, 36]. Immunostaining of aortic ring-plaque co-cultures for CD68 confirmed the macrophage nature of the rounded cells (Fig. 5).

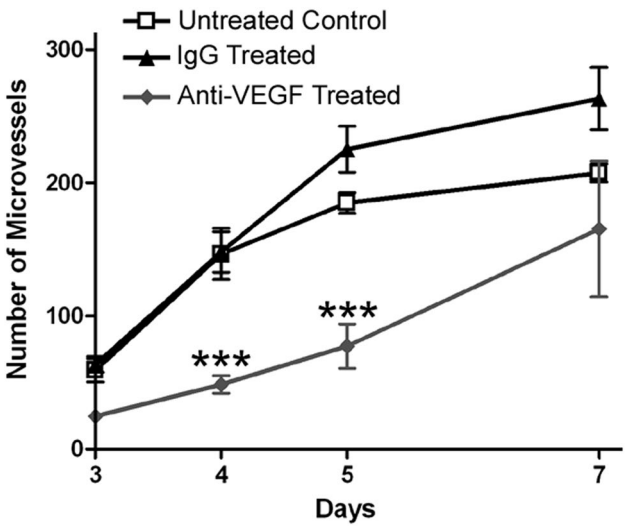

Fig. 4 Effect of VEGF inhibition on angiogenesis in aortic ring-human plaque co-culture. Line graph demonstrating that treatment of aortic ring-plaque co-cultures with anti-human VEGF antibody has significant but transient inhibitory effect on the angiogenic response. Controls include untreated cultures and cultures treated with non-immune IgG (***$p < 0.001$, $N = 4$). Cultures were prepared with VEGF-producing plaque fragments (plaque #7 in bar graph of Fig. 1, and blot "**a**" in Fig. 2; VEGF concentration: 428 pg/ml by ELISA)

Ablation of adventitial macrophages severely hampers the angiogenic response of the aortic wall to plaque stimuli

To evaluate the role of adventitial macrophages in the plaque-induced angiogenic response, aortic rings were pre-incubated overnight with liposomal clodronate, rinsed in serum-free medium, embedded in collagen and treated with plaque-conditioned media. Exposure to liposomal clodronate ablated the vast majority of macrophages from the aortic adventitia [16]. Macrophage depletion caused marked reduction in the angiogenic response of aortic rings to plaque stimuli (Fig. 6). Conversely pre-incubation with control PBS liposomes, which left adventitial macrophages intact, had no effect. The extent of angiogenesis inhibition following macrophage depletion in plaque-conditioned media-stimulated cultures was comparable to that of the untreated control. These findings indicate that adventitial macrophages are critically important not only for the physiologic angiogenic response of the aortic rings to injury, as reported [16, 17], but also for the stimulation of angiogenesis by atherosclerotic plaques.

Discussion

The mechanisms that regulate angiogenesis in human atherosclerosis are complex and poorly understood. Hypoxia, inflammation, and hemodynamic factors have been implicated in the generation of angiogenic stimuli from the lipid-rich core of the plaque [37]. In response to these stimuli, adventitial vasa vasorum produce neovessels which vascularize discrete regions of the plaque through a pathologic angiogenic response [38]. The angiogenic neovessels that vascularize the plaque are dysmorphic and prone to bleeding and have been implicated in the formation of hemorrhages which disrupt the plaque and contribute to the complications of atherosclerosis [39]. Thus, understanding the regulatory mechanisms of plaque-induced angiogenesis may provide new clues to inhibit the pathologic neovascularization of the arterial intima and stabilize the plaque neovasculature toward the goal of reducing the vulnerability of atherosclerotic plaques.

Herein we describe a new method to evaluate the angiogenic activity of human plaques and mechanisms of human plaque-induced angiogenesis. This method takes advantage of the capacity of rat aortic rings to produce a neovascular

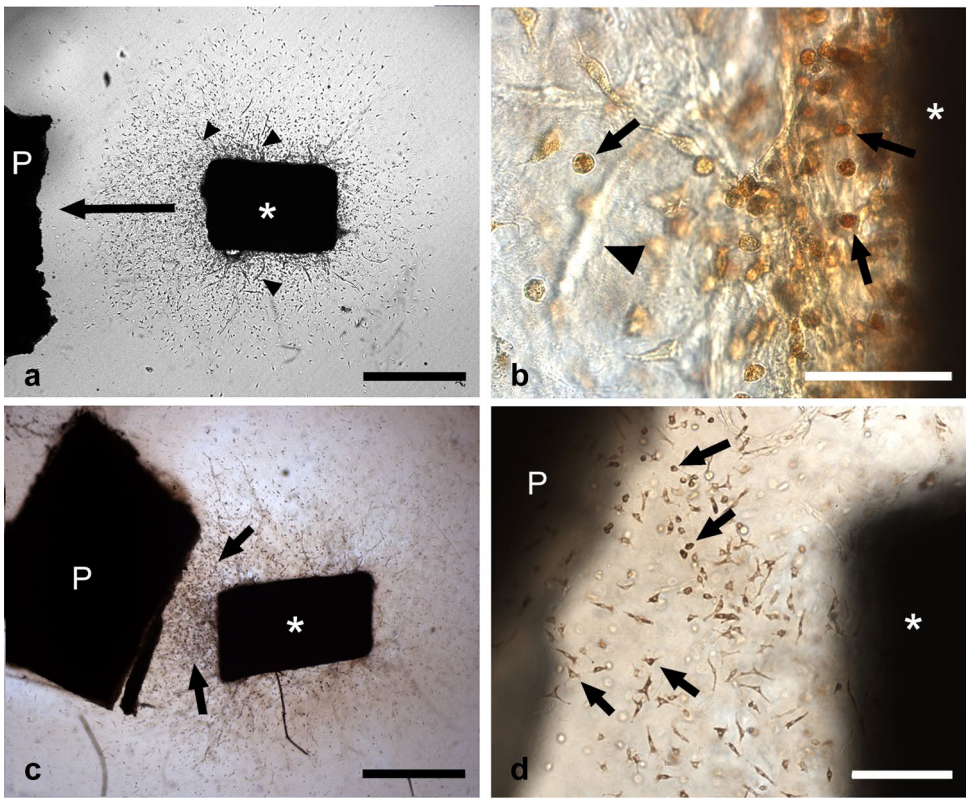

Fig. 5 Polarized growth of aortic macrophages toward atherosclerotic plaque in aortic ring-plaque co-cultures. A 4-day-old aortic ring-plaque co-culture shows a cellular outgrowth originating from the aortic explant (asterisk) and no outgrowth from the plaque fragment (P). The aortic origin of the outgrowth was established by daily monitoring of the co-culture: note that the outgrowth is denser in the gel facing the plaque. The arrow represents the vector of cell migration from the aorta toward the plaque; arrowheads highlight early endothelial sprouts (**a**). Immunohistochemical stain for CD68 highlights macrophages (arrows) migrating from the aortic ring (asterisk); a negatively stained endothelial sprout (arrowhead) is observed in a different focal plane (**b**). CD68 immunostain of a separate 5-day-old culture demonstrates a dense outgrowth of macrophages (arrows) between aortic ring (asterisk) and plaque fragment (P), photographed at low (**c**) and high (**d**) magnification. Again, note that the macrophage outgrowth is denser in the region between the aortic ring and the plaque. Scale bars: **a**, **c** = 1 mm; **b**, **d** = 200 μM

outgrowth in response to a variety of angiogenic stimuli including human angiogenic factors [13]. Human plaques obtained from endarterectomy procedures were co-cultured in collagen gels with rat aortic rings under serum-free conditions. Using this assay, we found that plaque explants stimulated angiogenic sprouting through paracrine mechanisms. Angiogenesis was stimulated in both aortic ring-plaque co-cultures and in cultures of isolated rings treated with plaque-conditioned medium. Proteomic analysis of the plaque secretome showed heterogeneity of pro-angiogenic growth factor and inflammatory cytokine production from different plaque explants. Thus, whereas MCP-1, and GDF-15 were identified in most tested plaques, VEGF was not always detected by immunoblot and/or ELISA.

To evaluate the role of VEGF in the angiogenic response of the aortic rings to plaque stimuli, the angiogenic activity of VEGF-producing plaque explants was tested in our assay in the presence or absence of a human-specific anti-VEGF blocking antibody. Inhibition of VEGF significantly suppressed the angiogenic response during the first 5 days of culture, but this inhibitory effect was transient and waned over time. These findings are consistent with previous studies showing that VEGF is an important mediator of plaque-induced angiogenesis [40, 41]. They also suggest that pharmacologic blockade of a single plaque-derived growth factor, even one as potent as VEGF, may be insufficient to completely suppress the angiogenic response to human plaques. This angiogenic resistance to VEGF inhibition is likely due to the presence in the plaque microenvironment of multiple pro-angiogenic molecules which limit the angiostatic effect of inhibitors directed against individual growth factors/cytokines. This hypothesis is supported by our finding that the plaque secretome contains a heterogeneous and variable mixture of angiogenic growth factors and cytokines including not only VEGF but also angiogenin [21], HGF [22], GDF15 [24], IL-6 [25], IL-8 [26], MCP1 [27], YKL-40 [26], MIF [28], and SDF1-alpha [29]. The angiogenic activity of most of these molecules in the aortic ring assay

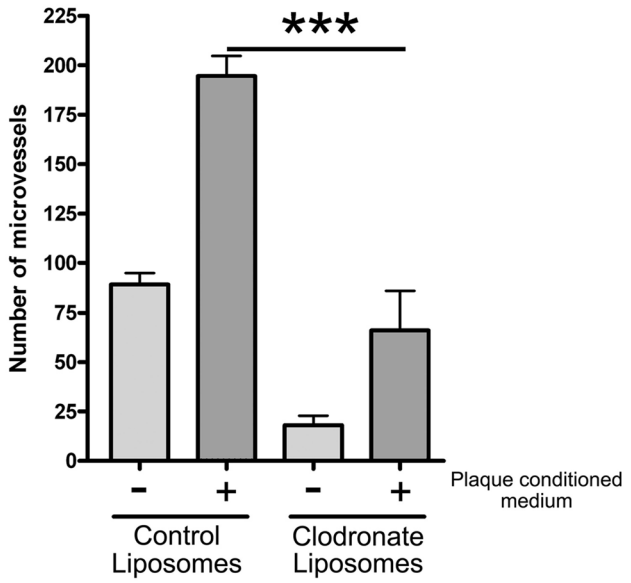

Fig. 6 The angiogenic effect of plaque-conditioned medium (CM) is markedly reduced following ablation of aortic macrophages. Plaque CM significantly stimulates angiogenesis from aortic rings pretreated with control PBS liposomes. Depletion of adventitial macrophages by pretreatment of aortic rings with clodronate-containing liposomes markedly reduces the angiogenic effect of plaque CM. ***$p < 0.001$, $N = 4$. No significant difference is observed in the percentage decrease in angiogenesis following macrophages ablation between control (80% decrease) and plaque CM-treated cultures (66% decrease, $p = 0.23$), indicating that adventitial macrophages are critically important for both physiologic angiogenesis and the angiogenic response of the aortic wall to atherosclerotic plaque stimuli

has been confirmed by our group and others in previous studies [13, 30–34]. In addition, angiogenic factors released by the aortic wall in response to plaque stimuli may have contributed to the stimulation of angiogenesis in this system.

Angiogenesis in atherosclerotic plaques is characteristically focal and often localized to the shoulder regions of the plaque [42]. This may be due to the anatomy of the plaque and the presence within the plaque of inhospitable regions for vessel growth such as the necrotic and severely hypoxic core of the plaque [36]. Using aortic rings as read-out of plaque angiogenic activity, we found that although most plaque fragments were potently angiogenic, approximately one fourth of these lacked significant angiogenic activity. Plaque angiogenic activity correlated with the presence, within tested plaque fragments, of endogenous (human-derived) CD163+ macrophages. These findings are consistent with observations by others that CD163+ plaque macrophages promote angiogenesis in atherosclerotic plaques [41]. Pro-angiogenic plaque fragments also contained more CD3+ T lymphocytes compared to non-angiogenic fragments, though quantitative analysis of this finding did not reach statistical significance. Overall, our studies with the aortic ring-plaque co-culture model indicate that the angiogenic activity of atherosclerotic plaques correlates with the presence of immune cells, particularly CD163+ macrophages.

The response of the aortic ring to angiogenic plaques was associated with a polarized outgrowth of aortic macrophages toward the plaque. These cells originate from a resident population of CD68+ and CD163+ macrophages present in the aortic adventitia [16, 43]. Adventitial macrophages have the capacity to proliferate and play a critical role in the production of VEGF by the injured vessel wall [17, 43]. We evaluated the role of aorta-derived macrophages in plaque-induced angiogenesis, by co-culturing plaque explants with aortic rings previously depleted of macrophages by treatment with the macrophage-targeting toxin liposomal clodronate. Adventitial macrophage ablation markedly and permanently impaired angiogenic sprouting from the aortic wall toward the plaque. Our analysis of the plaque secretome demonstrates that human plaques have the capacity to produce a variety of macrophage-stimulatory cytokines including MCP1, GDF-15, MIF, YKL-40, and SDF1-alpha (CXCL12) which are also angiogenic. Based on results obtained with our model, we postulate that, resident macrophages migrate from the adventitia into the plaque in response to these chemotactic stimuli. As resident macrophages leave their adventitial niche, they are likely to amplify angiogenic signals originated from the plaque microenvironment by producing VEGF [17] and other angiogenic molecules, further potentiating the angiogenic response to plaque stimuli [43, 44]. By promoting plaque-induced angiogenesis, adventitial macrophages may contribute to the progression of atherosclerosis. However, *in vivo* studies are needed to further investigate this hypothesis as CD163+ macrophages have been shown to promote plaque angiogenesis [41] but they have also been attributed a protective role in atherosclerosis [45, 46]. Since macrophages polarization plays a critical role in macrophage function, it will be particularly important to define how environmental cues including different chemokines and cytokines will influence the ability of CD163+ macrophages to promote angiogenesis and vascularize the plaque.

The plaque-aortic ring assay offers several advantages over existing models. Previous in vivo and in vitro studies have tested the angiogenic activity of human plaques in the chorioallantoic membrane of the chick embryo [47] or the rabbit cornea assay [48] which do not reproduce the arterial vascular microenvironment in which plaque-induced angiogenesis takes place. In our model, human plaque angiogenic activity is assayed using aortic rings which contain all cellular components involved in the arterial angiogenic response to plaque stimuli, including vasa vasorum endothelial cells, intimal endothelial cells, pericytes, smooth muscle cells, and adventitial macrophages. Since angiogenesis takes place under chemically defined

culture conditions, molecules released by plaque fragments and aortic rings can be identified without the confounding effect of serum or exogenous growth factors. The role of plaque-derived angiogenic factors/cytokines in the angiogenic response can be tested in multiple assays (at least 20 cultures per animal) with blocking antibodies specifically directed against the human molecules. The role of specific cell types such as the adventitial macrophages can be evaluated by pharmacologically ablating these cells from the aortic wall prior to the co-culture assay.

Like all bioassays, our model has also limitations. Although previous studies from our laboratory and by others have shown that rat aortic rings respond effectively to human angiogenic factors, we cannot exclude that species differences may influence results obtained with this assay. Aortic rings routinely used in this model are from young animals whereas atherosclerosis becomes symptomatic in older individuals. It is possible that aging may affect the angiogenic response of the arterial wall to plaque stimuli; this aspect of plaque-induced angiogenesis has not been studied in animal models and requires further investigation. Angiogenesis in the aortic ring assay is directed outward whereas plaques become vascularized by penetrating vessels that sprout inward into the inner intima through the media. Finally, the dissection procedure used to obtain plaque fragments may have activated angiogenic stimuli which were otherwise silent in the original plaque. However, the observation that approximately one fourth of plaque fragments were not angiogenic supports the interpretation that the assay specifically identifies plaque domains that have the capacity to stimulate angiogenesis.

In summary our study demonstrates that the angiogenic activity of human plaques can be reproduced *ex vivo* using rat aortic rings as read-out of plaque angiogenic activity. This model can be used to investigate cellular and molecular mechanisms involved in the regulation of human plaque-induced angiogenesis. This approach may prove particularly valuable in elucidating the role of growth factors and inflammatory cytokines in plaque-induced angiogenesis and may help elucidate how plaque and adventitial macrophages cooperate to regulate the sprouting of angiogenic neovessels into the plaque. Gaining insight into these mechanisms may lead to new therapies for the prevention of the complications caused by pathologic neovascularization of plaques in human atherosclerosis.

Acknowledgements This work was supported in part by a Grant-in-aid (17GRNT33410141) from the American Heart Association and by the VA Puget Sound Health Care System. The contents of this paper do not represent the views of the U.S. Department of Veteran Affairs or the United States Government. We gratefully acknowledge the support of the Vascular Surgery Team of VA Puget Sound Health Care System for this project.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were performed with approval from the Veterans Administration Puget Sound Health Care System Institutional Review Board and comply with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Research involving animal rights All animal procedures were performed with approval from the Veterans Administration Puget Sound Health Care System Institutional Animal Care and Use Committee and followed National Institutes of Health Guidelines.

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