The aortic ring model of angiogenesis: a quarter century of search and discovery

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Abstract

The aortic ring model has become one of the most widely used methods to study angiogenesis and its mechanisms. Many factors have contributed to its popularity including reproducibility, cost effectiveness, ease of use and good correlation with in vivo studies. In this system aortic rings embedded in biomatrix gels and cultured under chemically defined conditions generate arborizing vascular out-growths which can be stimulated or inhibited with angiogenic regulators. Originally based on the rat aorta, the aortic ring model was later adapted to the mouse for the evaluation of specific molecular alterations in genetically modified animals. Viral transduction of the aortic rings has enabled investigators to overexpress genes of interest in the aortic cultures. Experiments on angiogenic mechanisms have demonstrated that formation of neovessels in aortic cultures is regulated by macrophages, pericytes and fibroblasts through a complex molecular cascade involving growth factors, inflammatory cytokines, axonal guidance cues, extracellular matrix (ECM) molecules and matrix-degrading proteolytic enzymes. These studies have shown that endothelial sprouting can be effectively blocked by depleting the aortic explants of macrophages or by interfering with the angiogenic cascade at multiple levels including growth factor signalling, cell adhesion and proteolytic degradation of the ECM. In this paper, we review the literature in this field and retrace the journey from our first morphological descriptions of the aortic outgrowths to the latest breakthroughs in the cellular and molecular regulation of aortic vessel growth and regression.

Keywords:
neovascularization ● endothelial cells ● mural cells ● angiogenic factors ● integrins ● proteolytic enzymes ● fibronectin ● laminin ● collagen ● fibrin

Introduction

Angiogenesis, the growth of new blood vessels, plays a critical role in the progression of many diseases [32, 63]. Studies conducted during the past four decades have identified key mecha-
cancer and wet age related macular degeneration of the retina [61]. Instrumental in the attainment of this milestone have been the many experimental models developed to study the mechanisms of the angiogenic process and test the efficacy of anti-angiogenic drugs [14]. In vivo models such as the corneal micropocket and the chorioallantoic membrane of the chick embryo have provided invaluable information on the growth of blood vessels in the complex setting of the live animal [179, 194]. In vitro models with isolated endothelial cells have enabled investigators to further analyse mechanisms of angiogenesis in the simplified environment of the culture dish [77].

One of the most commonly used assays of angiogenesis is the aortic ring model. This model is based on the capacity of rat or mouse aortic explants to form new vessels in gels of collagen, fibrin or basement membrane [11]. The angiogenic outgrowth produced by the aortic rings consists of a mixed population of native cells that interact through paracrine mechanisms under chemically defined culture conditions. As such the aortic ring model bridges the gap between in vivo and in vitro models of angiogenesis, combining advantages of both systems. The purpose of this paper is to review the literature on this unique model and illustrate how the use of aortic cultures has contributed to our current understanding of the angiogenic process and the development of novel anti-angiogenic drugs.

**Early history of the aortic ring model**

The original observation that aortic rings have the capacity to produce microvessels in vitro dates back to the early 1980s when I was working in the research laboratory of Joseph Leighton at the Medical College of Pennsylvania in Philadelphia [170]. Leighton (Fig. 1), whose primary interest was cancer, was attempting to reproduce in vitro physiological gradients of oxygen and nutrient diffusion and had developed ingenious models for the three-dimensional growth of cancer cells. I was interested in the biology of blood vessels, a passion I had developed in medical school while working with an animal model of arterial injury and repair [213]. Intrigued by my fondness for endothelial cells, Leighton introduced me to the old tissue culture literature that described formation of capillaries in vitro. As I reviewed these pioneering papers, I learned that Warren Lewis in the 1930s had carefully documented formation of blood vessels in plasma clot cultures of chick embryo skin explants [133]. Leighton himself had observed capillary sprouting from different tissue explants but had never published these observations. He had long been interested in the mechanisms by which tumour aggregates replicate and spread, and suspected that the tumour stroma was involved in this process [127, 128]. My request to work in a vascular biology project rekindled Leighton’s interest in this area, and led to our first project in the field of angiogenesis.

Among the tissues potentially capable of an angiogenic response we chose the rat aorta which Leighton had already cultured in plasma clot and cultured in medium supplemented with foetal bovine serum generated a florid angiogenic response [170]. This model proved to be ideal to study the influence of the fibrovascular stroma on the growth and spread of cancer cells.

In the meantime Judah Folkman’s hypothesis that tumour growth is angiogenesis dependent, originally formulated in 1971 [62], was beginning to take hold and an increasing number of investigators were joining this field. Folkman’s group successfully isolated microvascular endothelial cells from bovine adrenal glands and demonstrated that these cells were capable of forming capillary tubes in vitro [64]. Shortly thereafter, Shing, Folkman and Klagsbrun reported the isolation and purification of the first tumour angiogenic factor from a chondrosarcoma [209]. This molecule turned out to be basic fibroblast growth factor (bFGF) which Denis Gospodarowicz had purified earlier from the bovine pituitary gland [78]. Thus, the birth of the aortic ring model occurred at a very propitious time when the angiogenesis field was entering the age of molecular biology and new discoveries were creating a need for assays that could characterize the activity, potency and mechanism of action of angiogenic regulators.

**Morphology of the angiogenic response**

We originally cultured aortic rings in plasma clots with serum-supplemented growth medium [170]. Clots were obtained by mixing the supernatant of homogenized chick embryos with a chick plasma solution. In later studies we grew aortic rings under serum-free conditions in gels of purified bovine or human fibrin or rat interstitial collagen [34, 70, 168, 174]. The elimination of serum reduced the number of non-endothelial mesenchymal cells, greatly facilitating the observation and measurement of the angiogenic outgrowths [168]. The development of a whole mount staining method further simplified the morphological analysis of the

![Fig. 1 Joseph Leighton, physician and cancer researcher (1921–1999).](image_url)
cultures, obviating the need for the labour-intensive techniques initially used to prepare histological sections [245]. The following description reflects our current understanding of how vessels develop and are remodelled in aortic cultures. This knowledge is based on studies conducted over time in our laboratory and by others with constantly improving methodologies.

Aortic cultures are composed of a mixed population of endothelial cells, pericytes, fibroblasts, macrophages and dendritic cells (Fig. 2). The first cells to migrate out of the aortic rings after 1–2 days are fibroblasts and macrophages. Fibroblasts appear as spindly or triposar cells with finely tapered cytoplasmic processes. Macrophages are characteristically rounded and have a granular and vacuolated cytoplasm with short pseudopods. Both macrophages and fibroblasts originate from the adventitia. Macrophages accumulate at the edges of resection where the aortic wall has been transected for the preparation of the rings ([70] and unpublished observations).

Endothelial sprouts first appear at the severed edges of the explants after 2–3 days of culture. They can be distinguished from fibroblasts by their thicker appearance and cohesive pattern of growth [168]. The neovessel tips are made of highly migratory cells which probe the surrounding matrix with filopodia-like processes. Stains for cell proliferation markers such as Ki-67 or phospho-histone demonstrate that endothelial tip cells migrate without dividing whereas trailing endothelial cells actively proliferate [73]. Endothelial cell proliferation is critical for optimal angiogenic responses but not necessary for minimal sprouting which can occur exclusively through endothelial migration when cultures are treated with mitomycin C, an inhibitor of cell mitosis [162, 170]. As the outgrowth expands and matures, vessels develop a visible lumen [168, 170]. Over time neovessels become surrounded by pericytes, which migrate and proliferate along the endothelium. Pericytes are readily recognized by their dendritic morphology and close association with the endothelium [174] (Fig. 2).

Neovessels express the endothelial markers von Willebrand factor (vWF), CD31 and Tie2 [169, 245], bind the Griffonia simplicifolia isoelectin-B4 [245], and take up Dil-acetylated LDL [208]. Pericytes are highly immunostained by immunostaining the cultures for α-smooth muscle actin (α-SMA), desmin, calponin or the proteoglycan NG2 [98, 168, 245]. Fibroblasts stain for vimentin, which is also expressed in pericytes and endothelial cells [34]. Some fibroblasts also express α-SMA (myofibroblasts). Macrophages react for CD45, CD68 and CD163 [12, 70]. Dendritic cells stain for CD45 and CD54 (ICAM) (Fig. 2).

Electron microscopy shows that immature sprouts are composed of solid endothelial cords. Primitive lumens formed through an intercellular mechanism appear as slit-like spaces sealed by junctional complexes. Lumens also develop by coalescence of cytoplasmic vacuoles in adjacent endothelial cells [168, 170]. Endothelial cells rest on a thin basement membrane which is best visible in serum-supplemented cultures [170]. Endothelial organelles include mitochondria, free ribosomes, rough endoplasmic reticulum, Golgi complex, pinocytic vesicles and occasional lysosomes. Weibel–Palade bodies are rarely observed. Pericytes have an electron dense cytoplasm and abundant rough endoplasmic reticulum. Pericyte cell processes are focally connected with the endothelium through cell junctions [168, 170].

After sprouting, branching and forming networks for approximately 1 week, vessels stop growing and begin to regress. This phenomenon is particularly pronounced in serum-free collagen gel cultures. As neovessels are reabsorbed, the gel inside the aortic lumen and around the explants lyases, causing formation of a periaortic halo. Matrix degradation also occurs at a much finer level around each microvessel, causing endothelial cells to detach from the underlying substrate and one another. The angiogenic outgrowth becomes fragmented and is gradually reabsorbed. The neovessel roots retract and are pulled back into the stalks of the thicker vessels. The combination of collagen lysis, vessel fragmentation and vessel retraction ultimately leads to complete vascular regression [241].

**Source of microvessels in aortic cultures**

During the early 1980s the capacity to form vascular tubes was thought to be a specific feature of microvascular endothelial cells and not large vessel endothelium. Our observation with the aortic ring model suggested that large vessel endothelial cells were capable of sprouting as well [168]. Working with an *in vitro* colla- gen invasion assay, Montesano and Orci observed formation of microvessels in cultures of human umbilical vein and calf pulmonary artery endothelial cells [150] as previously reported by the same group for microvascular endothelial cells [149]. Meanwhile Diglio et al. reported that selective killing of the adventitia with ethanol abrogated angiogenesis in aortic cultures, but noticed that some ethanol treated rings retained the capacity to sprout [47]. The angiogenic response of these rings was completely eliminated only by killing the intimal endothelium. These authors concluded that both aortic adventitia and intima were needed for the angiogenic response.

To evaluate the capacity of the aortic endothelium to form neovessels we studied the angiogenic behaviour of aortic tubes that had been turned inside out prior to collagen embedding. This method directly exposed intimal cells to collagen while blocking the outgrowth of adventitial cells. The intimal endothelial cells that survived the eversion procedure reorganized into microvessels and sprouted into the collagen gel [164]. Using a similar approach Mori et al. noticed that aortic endothelial sprouting occurred at sites of intimal injury [152]. We later observed that isolated rat aortic endothelial cells formed capillary networks when cultured between two layers of collagen, as reported for microvascular endothelial cells [151], and sprouted following stimulation with angiogenic factors [175]. Moreover rat carotid artery explants failed to generate an angiogenic response when completely de-endothelialized with a balloon catheter whereas control carotid arteries with an intact intimal endothelium produced microvessels from their ends of resection (unpublished observations in collaboration with Michael Reidy, University of Washington).

Taken together these studies indicated that the intimal endothelium of the aorta and its collaterals had full angio...
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forming spheroids with smooth outlines and necrotic centres.
Cancer cells cultured in plasma clot grew slowly by expansion
with the rat bladder carcinoma cell line NBT-II-81. Aggregates of
cancer cells. For these studies we co-cultured rat aortic rings
Our initial work focused on the interactions between microvessels
and cancer cells. For these studies we co-cultured rat aortic rings
with the rat bladder carcinoma cell line NBT-II-81. Aggregates of
cancer cells cultured in plasma clot grew slowly by expansion
forming spheroids with smooth outlines and necrotic centres. Aortic rings placed at 300–500 μm from the aggregates gave rise
to branching vessels. When the vessel outgrowth contacted the
tumour aggregates, cancer cells invaded the perivascular spaces,
disrupted the endothelium and used the denuded vascular tunnels
to spread into the plasma clot [171]. Normal bladder epithelium
instead formed cystic structures and did not invade after being contacted by the angiogenic outgrowths [129]. Studies with iso-
lated endothelial cells showed that cancer cells preferentially adhered to the subendothelial matrix compared to plasma clot or
gelatin. This suggested that favourable adhesive gradients gener-
ated by sprouting microvessels were promoting the spread of can-
cer in our model [172].

In the early 1960s Pietro Gullino had observed that when nor-
mal renal tissue was replaced by cancer in an experimental animal
model, the blood flow fell to very low levels even though the
tumour was growing and becoming richly vascularized. He con-
cluded that there was no direct correlation between the tumour
promoting effect of angiogenesis and blood flow [80, 81]. Our
observations with the aortic ring model, which lacks blood flow,
corroborated Gullino’s observations indicating that angiogenesis
could stimulate tumour spread not only by providing oxygen and
nutrients but also by secreting molecules that facilitated the
growth and invasive behaviour of cancer cells. Studies by other
investigators later confirmed the hypothesis that cancer and
endothelial cells can reciprocally influence each other’s behaviour
in a paracrine or juxtacrine manner [188].

Regulation of angiogenesis by the extracellular
matrix

Our observation that the microvascular extracellular matrix (ECM)
was involved in the spread of cancer cells was made at a time
when ECM molecules were found to be key regulators of endothel-
ial cell behaviour and capillary morphogenesis [66]. Intrigued by
the adhesive properties of the endothelial ECM [172], we set out
to study its composition in the aortic ring model. Immunohistochemical analysis of plasma clot cultures showed that immature endothelial sprouts were enveloped by fibronectin and type V collagen fibrils, and by patchy deposits of laminin and type IV collagen. As microvessels matured they became sur-
rounded by abundant laminin and type IV collagen, interstitial col-
lagens types I and III, and type V collagen [166]. Treatment with
ascorbic acid enhanced the synthesis and perivascular accumu-
lation of collagen. Microvessels formed in the absence of ascorbic
acid became markedly dilated, whereas microvessels treated with
ascorbic acid remained small throughout the angiogenic process
[160]. Inhibition of perivascular collagen accumulation with cis-
hydroxyproline, a proline analogue that interfered with collagen
secretion, blocked the angiogenic response. This effect was
reversed by supplementing the cultures with L-proline, which
restored endogenous collagen synthesis and secretion [160].
To investigate the role of the ECM in angiogenesis, we cultured aortic rings in basement membrane-like gels or collagen gels supplemented with purified basement membrane molecules. Hynda Kleinman and coworkers had developed a method to obtain a basement membrane-like gel composed of laminin, type IV collagen, heparan sulphate and entactin from a mouse sarcoma cell line [117]. Using this matrix, which was later termed Matrigel, they were able to induce rapid reorganization of endothelial cells into networks of ‘tube-like’ structures [123]. We found that aortic rings cultured in Matrigel produced polygonal networks of highly branched endothelial cords. Luminal spaces and DNA synthesis were however significantly reduced in Matrigel compared to plasma clot, fibrin or collagen [169]. These observations suggested that premature exposure of aortic outgrowths to high concentrations of basement membrane components interfered with vessel growth and maturation which instead occurred unimpeded in interstitial collagen and fibrin gels.

To define the effect of individual basement membrane molecules in angiogenesis we cultured aortic rings in gels of interstitial collagen supplemented with fibronectin, laminin, entactin or type IV collagen. Each of these molecules caused a dose-dependent elongation of microvessels [26, 162, 163]. Fibronectin-treated microvessels grew longer through an increased migratory recruitment of endothelial cells. This effect did not require cell proliferation because it occurred also in cultures treated with mitomycin C, an inhibitor of cell mitosis [162]. Our results were consistent with earlier studies indicating that fibronectin promoted endothelial cell migration [7, 144] and that endothelial cell proliferation was not required for in vivo angiogenic sprouting [212].

In addition to promoting vascular elongation, type IV collagen and the laminin–entactin complex had a bimodal effect on vascular proliferation. Intermediate concentrations of these molecules stimulated angiogenesis whereas low concentrations were ineffective and high concentrations were anti-angiogenic [26, 163]. In keeping with these observations Kleinman's group later identified 13 synthetic laminin peptides with pro-angiogenic effects in the aortic ring model [140]. Others demonstrated that oncothain, a peptide from the NC1 domain of type IV collagen inhibited aortic ring model [140]. Using this matrix, which was later termed Matrigel, Kleinman and coworkers had developed a method to obtain a basement membrane-like gel composed of laminin, type IV collagen, heparan sulphate-collagen hybrid molecule whose proteolytic degradation generates endostatin, an inhibitor of angiogenesis [148, 180]. Li and Olsen found that aortic rings from collagen XVIII deficient mice generated twice as many microvessels as control explants [134], unless the cultures were treated with recombinant endostatin which reduced angiogenesis to control levels. Collagen XVIII probably interfered with cell adhesion by competing with fibronectin for heparan sulphate groups on the cell surface. The increased adhesiveness of collagen XVIII-deficient endothelial cells to endogenous fibronectin seemingly promoted angiogenesis through enhanced vessel stabilization and reduced vascular regression [134]. The anti-angiogenic effect of endostatin was confirmed in a separate study in which mouse aortic rings were transduced with an adenoviral vector carrying the endostatin gene [85]. Inhibition of angiogenesis in aortic cultures was also obtained by overexpressing angiostatin, an anti-angiogenic molecule derived from proteolytic degradation of plasminogen [181].

DeanneLee and coworkers recently demonstrated that microvessels in aortic cultures express syndecan-1, a cell membrane protein that binds to ECM molecules via heparan sulphate proteoglycan side chains. Synstatin, a synthetic peptide that interferes with syndecan-1 function, inhibited bFGF- and VEGF-induced angiogenesis [18].

### Role of integrin receptors in aortic angiogenesis

In 1987, Ruoslahti and Piershbacher identified the arginine–glycine–aspartic acid (RGD) amino acid sequence as the cell attachment domain of fibronectin [199]. The RGD motif was also found to mediate cell adhesion to vitronectin, fibrinogen and vWF [45]. RGD peptides interfered with cell adhesion by competing with natural RGD-containing molecules for cell receptor binding, causing detachment of endothelial cells [36]. Because sprouting endothelial cells produced RGD containing molecules [162], we hypothesized that angiogenesis could be an RGD-dependent process. Treatment of collagen gel cultures with a GRGDS peptide potently inhibited angiogenesis, whereas no effect was obtained with a GRGES control peptide. The effect of GRGDS was nontoxic and reversible because the angiogenic response of the aortic rings was restored following withdrawal of the drug [161]. The GRGDS peptide also induced vascular regression when added to the cultures after the angiogenic growth phase [162]. These observations implicated for the first time RGD sensitive integrin receptors as regulators of angiogenesis. These integrins probably mediated the attachment of endothelial cells not only to native fibronectin but also to degraded type I collagen and laminin whose cryptic RGD sequences could be unmasked by proteolysis [15, 39, 42].

Putative candidates of the RGD sensitive subfamily of integrin receptors included \( \alpha_5 \beta_1 \), \( \alpha_8 \beta_1 \), \( \alpha_9 \beta_1 \), \( \alpha_5 \beta_3 \), \( \alpha_5 \beta_5 \), \( \alpha_5 \beta_6 \) and \( \alpha_5 \beta_8 \) [102, 246]. Although progress was initially delayed by the limited availability of neutralizing reagents, knowledge of how integrins regulate angiogenesis in the aortic ring model has advanced...
significantly in recent years. Using double immunofluorescence microscopy to localize integrin receptors in specific cell types, we found that endothelial cells and pericytes expressed both β1 and β3 integrins [34]. Angiogenesis in collagen was blocked with an anti-β1 antibody but not with an anti-β3 antibody. Likewise, vascular regression was accelerated by blocking β1 but not β3 integrins. In keeping with these observations, aortic rings from β3 deficient mice cultured in collagen or Matrigel produced the same angiogenic response as normal rings [39, 139, 193]. β3 integrins, however, were found to participate in angiogenesis under selected culture conditions. We observed that antibody blockade of both β1 and β3 integrins was needed to effectively inhibit aortic angiogenesis and promote vascular regression when aortic rings were embedded in fibrin gels [34]. Using a >600 fold higher concentration of anti-β3 integrin antibody (20 mg/ml) than the one tested in our study (30 μg/ml), Hayashi et al. were able to reduce by ~50% angiogenesis in fibrin gel cultures [90]. Interestingly, aortic rings from mice expressing a mutated β3 integrin failed to form capillaries in response to VEGF, a key regulator of angiogenesis (see below), or serum in Matrigel culture. The difference in angiogenic behaviour between β3 deficient aortic rings (normal angiogenesis, see above) and aortic rings with mutated β3 integrin (reduced angiogenesis), was attributed to the overexpression of VEGFR2 that occurred following β3 integrin ablation [192, 193]. Stimulation of VEGFR2 expression and angiogenesis was recently obtained also by treating VEGF-stimulated aortic cultures with low (nanomolar) concentrations of synthetic αvβ3/αvβ5 inhibitors [191].

A number of studies have attempted to identify the α partners of the β integrin receptors involved in angiogenic signaling. Suda et al. observed that ‘migrating cells’ at the periphery of mouse foetal aortic outgrowths expressed α1 and α2 integrins whereas cells near the aortic explants were reactive for α3 integrins [217]. Using immunoelectron microscopy, they were able to localize the α3 integrin to the membrane of periendothelial cells. Treatment with the synthetic peptide KDGEA, which competes with collagen for α2β1 integrin binding, significantly decreased cell migration. Zhang et al. found that aortic rings from α2 integrin deficient mice embedded in Matrigel produced the same number of vessels as control rings in response to VEGFA or VEGFC, but had a significantly enhanced angiogenic response to placental growth factor (PIGF) [238]. This paradoxical effect was attributed to the increased expression of the PIGF receptor VEGFR1 in the α2 null mice.

Trikha et al. reported that treatment of bFGF-stimulated collagen cultures of rat aorta with an antibody that bound all αv integrins reduced angiogenesis to ~30% of control values [225]. Similarly, Sassoli and coworkers obtained inhibition of angiogenesis in bFGF- and VEGF-stimulated collagen cultures with specific anti-αvβ3 and anti-αvβ5 antibodies [201]. These results suggest that bFGF and VEGF, which up-regulate expression of αvβ3 and αvβ5 integrins [51, 67], caused aortic microvessels to become dependent on these integrin subtypes [67].

Studies with isolated cells showed that the laminin integrins α3β1 and αv β1 promoted basement membrane assembly, pericyte association and tube stabilization [43] whereas the integrin α6β4 was involved in angiogenic sprouting [177]. These integrins closely associate with CD151, a tetraspanin family member that modulates endothelial cell spreading, migration and sprouting [93]. The angiogenic response of aortic rings from CD151 null mice in Matrigel was reduced by ~50%. These changes correlated with significant defects in endothelial cell migration, spreading, sprouting and signalling, particularly on a laminin substrate [222].

Paracrine regulation of angiogenesis: role of endogenous growth factors

My first experiments with the aortic ring model [170, 171] had shown that the aortic wall produced neovessels in response to injury. The presence of serum in the growth medium was however a confounding variable to the analysis of this phenomenon. The same consideration applied to the plasma clot which we were using as a substrate for angiogenic sprouting [170, 171]. We serendipitously overcame this limitation in a collaborative project with George Tuszynski, who was studying the cancer promoting properties of thrombospondin-1 (TSP-1), a platelet-derived protein [228]. Tuszynski asked us to test the angiogenic activity of TSP-1, but serum which contained abundant TSP-1, could not be used for this study. We therefore decided to culture the aortic explants without serum in gels of purified collagen or fibrin.

The serum-free modification of the aortic ring model was made possible by the work of Richard Ham and Gordon Sato who had separately developed methods to optimize the composition of culture media for the growth of mammalian cells [87, 91]. Following their pioneering studies, serum-free growth media had been used to culture a variety of isolated cell types but not to study angiogenesis. For the TSP-1 experiments we decided to use MCDB131, a growth medium optimized for microvascular endothelial cells [118] which had become commercially available under the trade name of endothelial basal medium. To our surprise, control cultures without serum or TSP-1, produced an angiogenic outgrowth that we could score by counting the number of microvessels. For the first time we were able to reproduce angiogenesis ex vivo under chemically defined culture conditions [168]. Similar observations were independently made by Kawasaki et al. [112]. Using the serum-free aortic ring assay we demonstrated that TSP-1 had the capacity to stimulate angiogenesis when bound to the ECM, and that this effect was mediated by myofibroblasts [173].

The discovery that the injured aortic wall produced vessels in the absence exogenous growth factors set us on a quest for the endogenous regulators of this process. In 1987 Gospodarowicz’ group had reported that endothelial cells expressed bFGF, a potent inducer of endothelial cell proliferation and angiogenesis, and proposed that endothelial bFGF could function as an autocrine stimulator of angiogenesis [203]. Arterial associated bFGF was also found in smooth muscle cells and macrophages [137, 218]. Because injury was believed to be the main mechanism of bFGF release [41], we suggested that bFGF was one of the autocrine/paracrine regulators of angiogenesis in our model. Immunohistochemical studies showed that the aortic wall expressed bFGF and rapidly released it following the injury of the dissection.
procedure [230]. bFGF-containing aorta conditioned medium and purified bFGF increased both the number and length of microvessels sprouting from the explants (Fig. 3). The bFGF stimulatory effect was most pronounced during the second week of culture when release of bFGF was minimal. The angiogenic response of aortic rings was reduced by 40% with a neutralizing anti-bFGF antibody [230]. Inhibition of angiogenesis was later obtained also with platelet factor-4 (PF-4) which blocks bFGF binding to endothelial cells [84].

While we were studying mechanisms of angiogenesis in the aortic ring model, three groups independently identified a novel endothelial growth factor in both neoplastic and normal cells [40, 131, 204]. This molecule originally described as a vascular permeability factor by Harold Dvorak and collaborators was later found by Napoleone Ferrara’s group to be a specific angiogenic regulator and termed vascular endothelial growth factor (VEGF) [33, 58, 59]. In our laboratory we found that aortic rings produced the alternatively spliced VEGF isoforms 164 and 188 and expressed the VEGF receptor flk-1 (VEGFR2). Rakic et al. later reported that the angiogenic outgrowths produced primarily VEGF120 whereas aortic explants express VEGF 164 and 120 and to a less extent VEGF188 [189]. Protein levels of VEGF in aorta conditioned medium decreased during the second week of culture when the explants became quiescent and microvessels stopped growing [165]. Exogenous VEGF potently stimulated angiogenesis in aortic cultures (Fig. 3) [165, 167], whereas an anti-VEGF blocking antibody inhibited angiogenesis by 70% [165]. These experiments established VEGF as a key regulator of angiogenesis in the aortic ring model.

Subsequent studies showed that VEGF production in cells of the vessel wall was stimulated by nitric oxide donors and suppressed by nitric oxide synthase (NOS) inhibitors [49]. Aortic rings harvested from endothelial NOS (eNOS) deficient mice and cultured in Matrigel exhibited a 44% reduction in angiogenesis and a 68% reduction in DNA synthesis compared to control explants [125]. Expression of VEGF was found to be regulated also by prostaglandin E2 (PGE2) [20] which is angiogenic in vivo [65]. As a result, aortic rings from mice deficient in EP2, one of PGE2 receptors, produced fewer vessels than control aortas [110].

Aortic cultures also produced PDGF, a potent mural cell mitogen and chemotactic factor [159, 197] which Risau and collaborators had found to be angiogenic as well [195]. In the aortic ring model endogenous PDGF was required for pericyte recruitment (see below) but not for endothelial sprouting [57, 159]. Exogenous PDGF molecules however dose dependently potentiated the angiogenic response of the aortic rings. PDGF-BB was as effective as VEGF and more potent than PDGF-AA, as reported earlier by Risau and collaborators [167, 195]. Unlike VEGF which was stimulatory since the beginning of the experiment, PDGF molecules promoted angiogenesis during the second week of culture when vessels were regressing in unstimulated cultures. PDGF-stimulated cultures contained many fibroblast-like cells which increased progressively in number over time. These findings suggested that the pro-angiogenic effect of PDGF was indirectly mediated by endothelial growth factors produced by PDGF-stimulated
connective tissue cells. Consistent with this interpretation we found that fibroblasts and smooth muscle cells had the capacity to promote angiogenesis in cultures of isolated aortic endothelial cells or from aortic rings [173–175, 231]. More recently Li et al. demonstrated that the angiogenic response of the aortic rings was stimulated by PDGF-CC. They also showed that the angiogenic effect of PDGF-CC was suppressed by concurrent treatment with an anti-VEGF antibody [135].

**Paracrine regulation of angiogenesis: role of resident macrophages and inflammatory cytokines and chemokines**

In 2004, the Rat genome Sequencing Project Consortium completed a decade long study aimed at deciphering the rat genome [74]. Meanwhile microarray analysis of cDNA had become a powerful method to detect genes dysregulated in physiological or pathological conditions [202]. To further define genetic events occurring in the aortic wall prior to angiogenic sprouting, we applied the microarray analysis method to rat aortic rings treated with angiogenic factors. For this study, aortic explants were made quiescent by a 14-day pre-incubation step in serum-free medium prior to collagen embedding. This procedure abrogated the spontaneous angiogenic activity of the rings while preserving their capacity to sprout in response to angiogenic factors or re-injury of the vessel wall. This approach produced angiogenic ‘on’ and ‘off’ conditions that enabled us to identify transcriptional events induced by angiogenic factors prior to endothelial sprouting (Fig. 4). We stimulated quiescent aortic rings with VEGF or angiopoietin-1 (Ang-1). Ang-1 had been identified by Yancopoulos’ group as the ligand of the tyrosine kinase receptor Tie2 and a key regulator of angiogenic sprouting, vessel branching and vessel maturation [44, 119, 219, 220]. Microarray analysis of quiescent aortic rings treated with VEGF or Ang-1 were confirmed by real time polymerase chain reaction and ELISA for selected genes.

Of the more than 31,200 transcripts analysed, ~3.5% were dysregulated by VEGF and Ang-1 [12]. Approximately one fourth of these transcripts corresponded to known genes, and the largest group of these was related to the immune system. Up to 10 cytokines and chemokines were up-regulated by VEGF and/or Ang-1. Five of these – GRO-1, interleukin (IL)-1β, MIP-1α, MIP-2 and tumour necrosis factor (TNF)-α – were stimulated by both factors. A cocktail of these VEGF- and Ang-1 induced cytokines and chemokines stimulated the angiogenic response of freshly cut aortic rings and was synergistic with a low dose of VEGF (5 ng/ml) which was otherwise minimally angiogenic when used by itself. In addition, abrogation of GRO-1 and MIP-2 signalling through pharmacological inhibition or genetic disruption of the G-coupled receptor CXCR2 significantly reduced the spontaneous angiogenic response of the aortic rings [70]. The pattern of immune gene up-regulation in VEGF- and Ang-1-treated aortic cultures mimicked activation of Toll-like receptors (TLRs) which cells of the innate immune system use to respond to microbial pathogens and endogenous molecules released by injured cells [221, 226]. In addition VEGF induced expression of TLR2 whereas Ang-1 stimulated expression of CD14, a TLR4 coreceptor [12]. The angiogenic response of the aortic rings and the endogenous production of VEGF were stimulated by treating the cultures with TLR ligands (unpublished observations). In a separate study concurrent treatment of aortic cultures with VEGF and IL-8, the human equivalent of GRO-1, produced an additive angiogenic effect [153]. Synergistic stimulation of angiogenesis *in vivo* was attained by combining a low non-angiogenic dose of VEGF with stromal derived factor-1 (SDF-1), a VEGF-inducible chemotactic factor for leucocytes and pro-angiogenic accessory cells of bone marrow origin [79, 122, 200]. SDF-1 also promoted the spontaneous angiogenic response of aortic rings in collagen gel culture [200].

We observed that quiescent aortic rings produced neovessels in response to VEGF but lost their ability to respond to the cytokine/chemokine cocktail that was instead angiogenic in cultures of freshly cut explants [70]. This change in angiogenic behaviour correlated with depletion of adventitial macrophages, and near complete loss of endogenous VEGF production in response to the cytokines/chemokines [70]. To investigate the angiogenic role of resident macrophages, we treated prior to the assay freshly cut aortic rings with liposomal clodronate, a compound that is toxic to phagocytic cells [229]. Selective killing of macrophages markedly impaired the angiogenic response of the aortic rings and their ability to produce VEGF. A comparable effect was obtained by ablating macrophages with diphtheria toxin in aortic cultures from CD11bDTR transgenic mice [31]. Angiogenic sprouting and VEGF production were restored by co-culturing macrophage-depleted rings with exogenous macrophages of bone marrow origin [70].

Further studies with immunosuppressive compounds corroborated the hypothesis that endogenous stimulation of angiogenesis in the aortic ring model was regulated by the immune system. Aortic angiogenesis was inhibited with hydrocortisone [168], a commonly used anti-inflammatory drug, TGFβ1 [155, 167], an inhibitor of the immune system [37, 237] and deactivator of macrophage function [227], and rmob-1, the rat homologue of the human chemokine interferon γ inducible protein 10, IP-10, which inhibits bone marrow colony formation and angiogenesis [1, 9, 215]. Angiogenic sprouting in aortic cultures was also blocked with IL-4 and IL-13 [70], both of which inhibit the pro-inflammatory response of macrophages [88] and with adenoviral vectors carrying the IL-4 or IL-13 genes [82, 83]. Pre-treating the aortic explants with macrophage colony stimulating factor (M-CSF/CSF-1) prior to preparation of the rings promoted angiogenesis whereas treatment with anti-CSF1 antibody significantly impaired the angiogenic response [70]. More recently we found that granulocyte macrophage colony stimulating factor (GM-CSF) stimulates the angiogenic response of the aortic rings (unpublished observations). Others reported that MCP-1, a potent monocyte/ macrophage chemokine, promoted aortic angiogenesis while an anti-MCP1 antibody or a synthetic peptide that...
blocked MCP1 binding to its receptor CCR2 abrogated the MCP-1 effect [115].

Recently McGonigle and coworkers reported that osteoprotegerin (OPG), a soluble TNF receptor family molecule involved in skeletal and immune system function and development [17, 107], stimulated aortic angiogenesis in collagen culture, and found that this effect was blocked by pre-incubating OPG with its ligands receptor activator of NF-κB ligand (RANKL) and TNF-related apoptosis inducing ligand (TRAIL). They also observed that RANKL but not TRAIL potently inhibited basal and VEGF-stimulated angiogenesis. OPG promoted endothelial proliferation whereas RANKL had antiproliferative and pro-apoptotic effects [146].

Expression and function of neural related genes

Studies conducted during the past decade showed that developing nerves and blood vessels share molecular mechanisms of cellular path finding and morphogenesis [154, 205]. Several axonal guidance ligand/receptor systems were found to regulate angiogenesis: Ephrin/Eph, Neuropilin/Semaphorins, Notch/Delta, Netrin/UNC5, Neogenin and DCC (deleted in colorectal cancer) and Slits/Roundabouts.

Our microarray studies showed that aortic rings up-regulate numerous neural related genes prior to angiogenesis [12]. Michael Klagsbrun’s group found that semaphorin 3A, also known
as collapsin-1, competed with VEGF for binding to neuropilin-1 (NRPI) and VEGFR2 coreceptor, and acting through NRPI inhibited the motility of endothelial cells, as previously reported for neuronal axons. Using the aortic ring assay they demonstrated that semaphorin 3A blocked endothelial sprouting by 80–90% [147]. Studies from other laboratories implicated the Slit/Roundabout (Robo) system in angiogenesis [68, 126]. Slits are large secreted proteins that bind to Robo transmembrane receptors inducing a repulsive signal during neuronal migration. One of these ligands, Slit2, was shown to either inhibit or stimulate endothelial cell migration by different investigators [185, 206, 233]. An endothelial specific Robo protein termed Robo4 was specifically localized to areas of angiogenesis [101]. Robo4-Fc, a soluble chimeric receptor, potently inhibited the angiogenic response of aortic rings. This effect correlated with the ability of Robo4-Fc to inhibit endothelial cell migration and proliferation in vitro and angiogenesis in vivo [216].

Studies with genetically modified mice showed that Ephrins and their Eph receptors regulated angiogenesis in different systems [3, 71, 234]. Ephrins are cell membrane-bound ligands which signal through Eph receptors at sites of cell–cell interactions. Ephrins and Eph receptors are divided into two classes, A and B. Ephrins A bind to Eph A receptors while Ephrins B bind to Eph B receptors, but there is significant promiscuity of receptor-ligand interactions within each class [69]. Blockade of Ephrin A signalling with soluble EphA2 receptor (EphA2-Fc) dose dependently inhibited angiogenesis in aortic cultures [48]. Simultaneous treatment with EphA2-Fc and the VEGFR2 inhibitor CEP-5214 had additive effects resulting in 90–95% inhibition of angiogenesis. Conversely treatment with EphrinB1-Fc or Ephrin B2-Fc promoted the angiogenic response of the aortic rings [120, 141].

Neural development is regulated by netrins, bifunctional proteins capable of attracting or repelling axons. The netrin effects are mediated by receptors of the DCC and uncoordinated 5 (UNC5) families. DCC receptors include DCC and Neogenin whereas the UNC5 family comprises four members UNC5A to UNC5D [2, 130, 132]. DCC receptors mediate attractive signals whereas repulsive signals are regulated by UNC5-DCC receptor heterodimers or UNC5 dimers [56, 92, 113]. Using transgenic mice expressing LacZ under the control of the UNC5b promoter, Lerrivee and coworkers identified expression of UNC5b in sprouting microvessels. When they subjected collagen cultures of mouse aorta to gradients of netrin-1 they observed filopodial retraction in endothelial sprouts from UNC5b<sup>+/+</sup> and UNC5b<sup>–/–</sup> aortas but not in sprouts from UNC5b<sup>+/–</sup> aortas [124]. Conversely Nguyen and Cai reported that aortic discs embedded in fibrin produced more vessels when treated with netrin-1 and that this effect was abrogated with an anti-DCC antibody [157]. Although it remains unclear if netrin-1 is pro- or anti-angiogenic, these studies suggest that netrins and their receptors are potential regulators of aortic angiogenesis.

Neuropeptide Y (NPY), a sympathetic vasconstrictor molecule released during nerve activation and ischemia, stimulated endothelial migration, proliferation and angiogenesis by activating receptors Y1, Y2 and Y5 [145, 247]. When tested in the aortic ring model NPY markedly stimulated endothelial sprouting from mouse aortic rings cultured in Matrigel. The pro-angiogenic effect of NPY was significantly reduced in aortic cultures from aged animals probably due to reduced expression of the receptor Y2 and the NPY converting enzyme [116].

### Signal transduction pathways

Our studies implicated bFGF and VEGF as key regulators of angiogenesis in aortic cultures [165, 230]. Because bFGF can induce secretion of VEGF [19], its rapid release from pre-existing aortic depots after injury [230] may be one of the earliest events responsible for the induction of VEGF production and angiogenesis in aortic cultures. Support to this hypothesis was provided by a study on the angiogenic role of Net (Elk-3/Sap-2/ERP), a transcription factor that is converted from a negative to a positive regulator through phosphorylation by Ras, Src and MAPK signalling. bFGF-induced endothelial sprouting was severely impaired in aortic cultures from Net deficient mice. This defect was corrected with exogenous VEGF, suggesting that impaired production of VEGF was responsible for the decreased sprouting [239]. Zhu et al. found that bFGF and VEGF production could be induced by H-2g, a glucose analogue of blood group antigen H [240], through the NF-κB pathway. H-2g-mediated stimulation of aortic angiogenesis in Matrigel cultures was inhibited by AG490, a JAK2 inhibitor and LY294002, a PI3 kinase inhibitor. Similar effects were obtained with antisense oligonucleotides (ODN) against these signalling molecules.

Studies aimed at developing synthetic inhibitors of VEGFR tyrosine kinases for therapeutic applications further implicated VEGF in the angiogenic response of the aortic rings. Wood and coworkers obtained dose-dependent inhibition of angiogenic factors in fibrin gel culture with the VEGFR2 inhibitor PTK787/ZK 222584 [235]. Ruggeri and collaborators observed that the VEGF-R2 signalling inhibitor CEP-5214 (20 nm) significantly reduced the sprouting of aortic rings in collagen gels [198]. They also found that simultaneous inhibition of both VEGF and EphA2 pathways caused near complete inhibition of angiogenesis [48]. Anti-angiogenic effects were obtained with JNU-17029259, a tyrosine kinase inhibitor that blocked VEGFR2 phosphorylation and MAPK signalling [53]. Furthermore transduction of aortic rings with an adenovirus carrying HCTPA, a tyrosine phosphatase that binds to VEGFR2, caused inhibition of angiogenesis due to impairment of VEGF-mediated VEGFR2 autophosphorylation and MAPK activation [100]. Aortic angiogenesis was also blocked by AZM475271 a src kinase inhibitor required for the phosphorylation of Net [239] and the transduction of VEGF signals [52, 104, 186].

In our laboratory we found that intracellular signalling downstream of the VEGF/VEGFR system was characterized by biphasic phosphorylation of p44/p42 MAPK and Akt, with peaks at 15 min. and 4–24 hrs. Both pathways were important for VEGFR signalling
because the spontaneous angiogenic response of aortic rings was blocked with specific inhibitors of p44/42 MAPK (U0126) and Akt/PI3-kinase (LY294002) [244]. p44/42 MAPK and Akt were also activated by Ang-1, but only transiently at 15 min. Ang-1 promoted angiogenesis in collagen gel cultures of freshly cut aortic rings, but unlike VEGF did not significantly induce sprouting from quiescent rings. This suggested that sustained phosphorylation of p44/42 MAPK (U0126) and Akt/PI3-kinase were needed to induce aortic angiogenesis. Huang and coworkers observed that d-duction of aortic rings with an adenovirus carrying the PTEN gene, a phosphatase that inhibits PI3-kinase signalling, caused shortening of vascular sprouts in Matrigel culture whereas the dominant negative form of PTEN promoted the formation of longer vessels [99]. VEGF and Ang-1 also activated p38 MAPK, but inhibitors of this pathway interfered with pericyte recruitment without significantly affecting endothelial sprouting [242] (see below).

Takeshita et al. reported that VEGF signalling downstream of PI3 kinase/Akt involved activation of γ-secretase activity and cleavage of Notch-1, a transmembrane receptor implicated in the regulation of embryonal angiogenesis [121, 136]. Endothelial sprouting was reduced by 80% in cultures of aortic rings from haploinsufficient Notch 1+/−/−mice [223]. In a separate study Paris et al. found that the γ-secretase inhibitor DAPT dose-dependently inhibited angiogenic sprouting in aortic cultures. The same authors observed that aortic angiogenesis was also blocked by β-secretase inhibitors [184]. Both γ- and β-secretase are involved in the production of the β amyloid component of plaques in Alzheimer’s patients. In addition to Notch and the Notch ligands Delta and jagged, γ-secretase also mediates the cleavage of E-cadherin and N-cadherin [184].

Bos and coworkers reported that the angiogenic response of aortic rings from mice deficient in Rap1b, a GTPase that modulates cell migration, differentiation and growth, was significant reduced compared to that of normal control rings, and was not restored by exogenous VEGF or bFGF [27]. These findings correlated with decreased in vivo neovascularization in Rap1b-deficient mice and suggested that VEGF and bFGF signalling in the aortic ring model was regulated by Rap1b [38].

Pericyte origin and recruitment mechanisms

An intriguing feature of the aortic outgrowths was the presence of pericytes, which are characteristically found in the microcirculation. Where did these pericytes originate from? Using cultures of everted aortic tubes we found that in microvessels sprouted directly from the intimal layer were enveloped by pericytes [164]. Mural cells isolated from the intimal aspect of the aorta exhibited directly from the intimal layer were enveloped by pericytes [164]. Upon exposure to serum, PPC attached to the culture dish, lost expression of CD34, and acquired smooth muscle cell markers such as α-SMA, calponin and desmin. PPC isolated from suspension cultures responded to PDGF BB and generated pericytes when co-cultured with rat aorta outgrowths or capillary networks formed by isolated endothelial cells [98].

The possibility offered by the aortic ring model to directly visualize pericytes in the living cultures prompted us to study the molecular mechanisms of pericyte recruitment (Fig. 5). We were able to reduce the number of pericytes with a neutralizing antibody against PDGF-B, which mediates pericyte recruitment during embryonal and postnatal angiogenesis [21, 72], by neutralizing angiopoietin function with Tie2-Fc [106], by blocking the p38 MAPK pathway [242] and by treating the cultures with heparin [174]. Conversely, we observed increased pericyte numbers in cultures treated with Ang-1 or Ang-2 [106] or with an adenoviral vector carrying the MMK6 gene, an upstream activator of p38 MAPK [242]. We observed an increased recruitment of pericytes also in aortic cultures treated with MCP-1 [10] an Ang-1-induced chemokine [12]. Paik et al. observed that siRNA-mediated knock down of N-cadherin, a cell adhesion molecule involved in endothelial-pericyte interactions markedly reduced the pericyte coating of microvessels in aortic cultures and suppressed the mural cell stimulatory effect of sphingosine-1-phosphate, a platelet-derived mediator of mural cell recruitment [183].

Role of proteolytic enzymes in angiogenesis and vascular regression

Aortic ring cultures produce a broad spectrum of matrix proteases and inhibitors of proteolysis including plasminogen activators (PA), MMPs, PA inhibitor (PAI-1) and tissue inhibitors of MMPs (TIMPs) [16, 30, 89, 241]. When we first cultured aortic rings in fibrin gels under serum-free conditions, we observed that endothelial sprouting was precluded by rapid (overnight) and extensive lysis of the fibrin. Blocking fibrinolysis with the serine protease inhibitor e-aminocaproic acid preserved the fibrin scaffold and allowed endothelial cells to sprout freely into the fibrin gel [168]. Consistent with this observation, Devy and collaborators observed that aortic rings from plasminogen deficient mice maintained their capacity to sprout, though angiogenesis in these cultures was delayed [39, 46]. Likewise single deficiency of tissue type PA activator (IPA), uPA or uPAR, as well as combined deficiencies of uPA and IPA, did not significantly affect the angiogenic
response of aortic explants [46, 95]. However, up-regulated expression of uPA through adenoviral transduction markedly inhibited microvessel growth [143]. Defective angiogenesis was also observed in cultures of aortic rings from mice lacking PA inhibitor-1 (PAI-1). This angiogenic defect could be reversed by adding recombinant PAI-1 to the culture medium or by transducing the explants with an adeno virus carrying the PAI-1 gene. Exogenous PAI-1 added to cultures of PAI-1 deficient aortic rings was stimulatory at low concentrations (<100 ng/ml) and inhibitory when used at higher dose (>1000 ng/ml). Transduction studies with specific mutants showed that PAI-1 promotes angiogenesis through its anti-proteolytic activity and not through its ability to interact with vitronectin [46].

Aortic rings cultured in collagen gel lysed the matrix over time. Using antibodies against a specific fragment of cleaved type I collagen or denatured collagen, Stephen Weiss' group identified collagen degradation products in the ECM of neovessel sprouts [39]. Studies from our laboratory and by others demonstrated that collagen lysis in aortic cultures was mediated by MMPs. The most abundant MMPs found in aortic cultures were MMP-2 and MMP-9. MMP-2 was abundantly secreted in the culture medium whereas MMP-9 was predominantly found in the gel and specifically in sprouting microvessels [89, 142, 241]. Aortic cultures also produced MMP-3, MMP-10, MMP-11, and MT1-MMP (MMP-14) [30]. Treatment with the broad spectrum MMP inhibitors BB-94 (batimastat), BB-2516 (matimastat) or GM6001 blocked the angiogenic response of the aortic rings in collagen gel cultures [39, 241]. However, the angiogenic response of aortic rings was not affected by isolated or combined genetic disruption of MMP-2 or MMP-9 [13, 39, 142]. Similarly, aortic explants from mice deficient in CD44, an MMP-9 cell surface partner, retained their ability to sprout [96].

Weiss and coworkers demonstrated that cellular invasion of collagen matrices required membrane type (MT) MMPs, which focus lytic activity to the invadopodia of migrating cells [236], and not soluble MMPs [96]. Disruption of the MT1-MMP gene caused complete abrogation of aortic angiogenesis in collagen [39]. Reduction of angiogenesis was also obtained by treating normal aortic rings with TIMP-2, which inhibited MT1-MMP, but not with TIMP-1, a weak inhibitor of MT1-MMP [75]. A similar effect was obtained by transducing aortic rings with a retrovirus carrying the TIMP-2 gene [86]. The MT1-MMP requirement was matrix dependent because MT1-MMP deficient rings sprouted normally in fibrin gel [39]. However, angiogenesis in fibrin was inhibited by TIMP-2 and TIMP-3, which can block other MT-MMPs besides MT1-MMP [97]. In addition aortic rings from TIMP-3 deficient mice had a significantly enhanced angiogenic response in fibrin gels compared with normal aortic rings [108]. One possible explanation for these results is that the expression and requirement of proteolytic enzymes by aortic outgrowths is influenced by the external matrix and/or growth factor milieu in which vessels sprout. Burbridge et al. observed that collagen induced overexpression by aortic rings of MT1-MMP, MMP-13, MMP-10 and MMP-9 whereas fibrin up-regulated MMP-2 and MMP-3 [30]. In addition, bFGF stimulated the production of MMP-2, MMP-3, MMP-9, MMP-10, MMP-11 and MMP-13 whereas VEGF induced
overexpression of MMP-2 only. Furthermore sprouting of MMP-2 deficient aortas in Matrigel following stimulation with bFGF was reduced to 40% of control whereas no differences were observed when cultures were treated with serum instead of bFGF [111].

When we first analysed the role of MMPs in aortic angiogenesis we noticed that the few microvessels formed in the presence of synthetic MMP inhibitors survived longer than those of untreated controls. MMP inhibitors added to the growth medium after the growth phase potently inhibited collagen lysis and vascular regression, and promoted vascular survival (Fig. 6) [241]. We recently found that TIMP-2, TIMP-3 and TIMP-4, which are anti-angiogenic, stabilized neovessels if added to the culture medium after the growth phase. TIMP-1, which is not anti-angiogenic, similarly promoted vessel survival. Conversely aortic rings from TIMP-1 or TIMP-2 deficient mice regressed at a faster rate than normal control rings [13].

Influence of genetic background and aging on angiogenic response

When we first studied the angiogenic behaviour of the mouse aorta we noticed significant interassay variability. Unlike rat aorta cultures which were prepared from Fisher 344 rats only, mouse aorta cultures had different genetic backgrounds. To determine if this variable could account for the inconsistent angiogenic response of mouse aortic rings, we compared four different strains of mice for their ability to sprout in response to bFGF or VEGF [243]. Aortic rings from 129/SVJ mice produced a much stronger and sustained angiogenic response to bFGF than those from C57BL/6 or BALB/c mice which were in turn more angiogenic than rings from FVB mice. C57BL/6 mouse aortic rings produced more microvessels in response to VEGF compared to rings

Fig. 6 Vascular regression in the aortic ring model. Serum-free cultures of rat aorta can be used to study mechanisms of vascular regression following angiogenesis. Aorta-derived microvessels spontaneously regress after the first week of growth. Vascular regression is characterized by cell detachment from the ECM, vessel fragmentation and retraction of the remaining stumps. Molecules that interfere with cell attachment to the ECM such as RGD peptides and antibodies against β1 integrin or β3 integrins have the capacity to accelerate vascular regression. The anti-β1 integrin antibody induces vascular regression in collagen. The anti-β3 antibody has no effect in collagen but it synergizes with the anti-β1 integrin antibody to promote vascular regression in fibrin. Vessel survival can be extended by supplementing gels of interstitial collagen with basement membrane molecules and by blocking MMP activity with synthetic inhibitors, TIMPs or anti-MT1-MMP antibody. Vessel survival is also promoted by bFGF or PDGF. Photomicrographs show a control culture (upper panel) in an advanced stage of collagen lysis and vessel regression (residual short vessel stumps marked by arrowheads) and a parallel culture (lower panel) treated with the MMP inhibitor Marimastat to block collagen lysis and promote vascular survival (vessels marked by arrows). Magnification bar = 800 μm.
from 129/SVJ, BALB/c or FVB mice, which were capable of only a limited response. In a separate study Rohan et al. made comparable observations and identified the 129 mice as the strongest responders to bFGF [196].

Aortic sprouting in collagen was significantly influenced by the age of the animals. Aortic rings from young mice stimulated with bFGF or VEGF produced microvessels faster, more uniformly and in greater number than aortic rings from older mice. As a result the angiogenic response of aortic rings from 10-month-old mice to VEGF was ~22% of that from 1-month-old animals [243]. Comparable results were obtained by Shimada and collaborators who observed reduced angiogenesis and eNOS release in Matrigel culture by aortic rings from genetically modified mice with disrupted angiogenic responses [214]. Facchetti et al. found that aortic rings from 24-month-old rats produced 90% fewer vessels than rings from 3.5-month-old animals in collagen gel culture. Interestingly, fasting dietary regimens boosted the angiogenic response of rings from old animals by 45–63% [55].

### Adaptation of the aortic ring model to different species and vessel types

Originally developed to study the angiogenic response of the rat aorta, the aortic ring model was later applied to other species and vessel types (Table 1). The adaptation of this model to the mouse aorta enabled investigators to study the angiogenic role of proteins whose expression had been silenced or up-regulated through genetic manipulation. Human vessels were used to test the activity of pro-angiogenic and anti-angiogenic regulators prior to clinical trials.

In our laboratory we observed that mouse aortic rings were unable to produce an angiogenic response in serum-free medium. We later found that this deficiency was due to the small size of the rings and the excessive dilution of the growth factors produced by the explants. Mouse aortic rings sprouted only if treated with exogenous bFGF, VEGF or 2.5% autologous serum [46, 243]. We overcame this limitation by reducing the volume of the medium from 400 to 150 μl and the size of the culture well from 17 mm (4- or 24-well culture dishes) to 6.4 mm (96-well culture plates), levels of endogenous growth factors including VEGF increased several fold and mouse aortic rings generated vessel sprouts without the need for exogenous growth factors or serum [11]. This approach also enhanced the spontaneous angiogenic response of rat aortic rings. Akimoto and coworkers observed that addition of serum, VEGF or bFGF to cultures of rat aortic rings from genetically modified mice with disrupted anti-aging klotho gene [208]. In a modified mouse aortic ring assay VEGF-induced angiogenic sprouting was reduced by 40% in aged animals compared to controls [190]. Recently Facchetti et al. found that aortic rings from 24-month-old rats produced 90% fewer vessels than rings from 3.5-month-old animals in collagen gel culture. Interestingly, fasting dietary regimens boosted the angiogenic response of rings from old animals by 45–63% [55].

| Vessel type          | Species       | Biomatrix     | References |
|----------------------|---------------|---------------|------------|
| Aorta                | Rat           | Collagen, fibrin, Matrigel | [168–170] |
| Aorta                | Mouse         | Collagen      | [46, 243]  |
| Aorta                | Chicken       | Matrigel      | [156]      |
| Aorta                | Rabbit        | Collagen      | [35]       |
| Aorta                | Cow, Dog      | Matrigel      | [214]      |
| Aorta                | Human         | Collagen      | [6]        |
| Carotid artery       | Pig, cow      | Matrigel      | [214]      |
| Carotid artery       | Rat           | Collagen      | Nicotia, unpublished observations |
| Saphenous vein       | Human         | Fibrin, Matrigel | [94] |
| Vena cava            | Rat           | Collagen      | [176]      |
| Thoracic duct        | Rat, mouse    | Collagen      | [29, 158]  |

Stiffey-Wilus et al. reported that square pieces of porcine carotid arteries embedded in Matrigel and cultured in growth medium supplemented with EGF, bovine hypothalamic extract and 2% serum generated an angiogenic response that was inhibited with the MMP inhibitor Batimastat, 2-methoxyestradiol or suramin. They also observed angiogenic sprouting in Matrigel cultures of aortic or carotid explants from cows or dogs, but porcine carotid arteries proved to be the most reliable material for this study. Porcine and bovine arteries obtained from an abattoir remained angiogenically viable after being kept on ice for 24 hrs [214]. Results comparable to the rat aortic ring model were obtained by culturing explants of rabbit thoracic aorta in collagen gel under serum free conditions. Normal aortic explants from control rabbits or atherosclerotic explants from hypercholesterolaemic animals produced angiogenic outgrowths whereas lesion-free explants from hypercholesterolaemic rabbits exhibited a markedly reduced angiogenic growth. Microvessels formed in cultures of atherosclerotic aortas were surrounded by foamy macrophages and became more numerous than control vessels over time. Angiogenesis in this system was stimulated by bFGF and blocked by anti-bFGF antibody or oxidized LDL [35].

Auerbach and collaborators adapted the aortic ring model to the chick embryo. This assay developed to test thalidomide, which had limited effects in rodents, used explants of aortic arches. Rings placed on Matrigel generated vessel-like structures within 24–48 hrs. Angiogenesis in this model was stimulated by bFGF and inhibited by endostatin [156].

Hiran and coworkers showed that fragments of human saphenous vein produce neovessel sprouts in Matrigel or fibrin. They used this assay to test the effect of the angiogenic inhibitor endostatin and to evaluate the role of the α5β1 integrin which was expressed in mature vessels and down regulated in sprouting...
endothelial cells [94]. Enzymatic removal of the intimal endothelium significantly impaired the angiogenic response of the saphenous vein [138].

Alessandri et al. used the aortic ring assay to study the angiogenic behavior of aortic explants from 11- to 12-week-old aborted human embryos. Aortic rings embedded in collagen gels produced branching capillary-like structures from immature endothelial precursor cells localized in the outer layer of the aortic wall [6]. Undifferentiated CD34+ and CD31+ mesenchymal cells isolated from the embryonal aortas and cultured on collagen-fibronectin, differentiated into CD31+ and vWF+ endothelial cells and formed networks of capillary-like structures on Matrigel. This study showed that the human embryonic aorta is a rich source of endothelial progenitor cells that have the capacity to form neovessels de novo through a vasculogenic process.

In our laboratory we found that explants of rat inferior vena cava cultured in collagen under serum-free conditions produced microvascular outgrowths which were dose-dependently stimulated by VEGF and bFGF. VEGF and the VEGF/bFGF combination also promoted pericyte recruitment. The spontaneous angiogenic response of the vena cava was significantly reduced by blocking endogenous VEGF with a neutralizing antibody. Neovessels of vena cava origin were longer and had fewer pericytes than aorta-derived vessels. Aortic and venous explants co-embedded in the same gel stimulated each other’s growth and formed anastomosing networks of microvessels [176].
Recently Agnes Noel’s group developed a quantifiable three-dimensional culture system for the study of mouse lymphangiogenesis \textit{ex vivo}. This study confirmed and expanded my earlier observation that rat thoracic ducts have the capacity to sprout and form lymphatic-like channels in plasma clot culture [158]. In their assay, Noel and collaborators demonstrated that mouse thoracic duct fragments embedded in a collagen gel formed lymphatic capillaries and that this process was stimulated by serum, low oxygen levels (5%), PDGF-BB and the lymphangiogenic factor VEGF-C. Interestingly, bFGF or PlGF had no direct stimulatory effects. Lymphangiogenesis was inhibited by blocking the VEGF receptor VEGFR3 but not with the bFGF inhibitor suramin [29].

\textbf{Limitations of the aortic ring assay}

When compared with \textit{in vivo} models of angiogenesis, the aortic ring assay has no blood flow, a limitation shared with all other \textit{in vitro} and \textit{ex vivo} models of angiogenesis. As a result, the role in angiogenesis of haemodynamic factors and mechanochemical forces cannot be assessed in this system [210]. Aortic explants contain macrophages and dendritic cells, but lack other immune cell types, which can however be added to the cultures. The same consideration applies to platelets which are major carriers of both angiogenic and anti-angiogenic factors [28, 105]. Manual counts of microvessels is the most rapid method to measure the angiogenic response in the aortic cultures [11, 168]. This approach allows the observer to follow the angiogenic response over time and to generate curves of microvascular growth and regression. Proper training and knowledge of scoring criteria reduces intra- and inter-observer variability to negligible levels. However, when angiogenic stimulation with exogenous growth factors results in very high numbers, the margin of error becomes high, and the counting process becomes tedious and time consuming. Counting vessels manually is also not practical when the assay is being used for high-throughput screening of drugs. This limitation can be overcome by computer aided analysis of digital images. A number of approaches have been developed to perform image analysis of aortic cultures and to specifically measure vascular area, vessel length and branching patterns. Some of these methods allow automated batch processing of multiple digital images per hour [23–25, 178]. The angiogenic response of the aortic rings to agonists or antagonists represents the net result of complex paracrine interactions between endothelial cells, macrophages, pericytes, dendritic cells and fibroblasts. While the presence of different cell types renders the aortic ring model a more physiological system than other in vitro models with isolated endothelial cells, it also adds complexity to the interpretation of data and poses additional challenges to the investigator. Finally, the arterial nature of the aortic explants represents a potential limitation because neovessels \textit{in vivo} characteristically originate from the venous side of the vascular bed. Although results obtained with the aortic ring assay consistently correlate with \textit{in vivo} observations, more studies are needed to comparatively evaluate the angiogenic response of arterial and venous vessels. The recent adaptation of the aortic ring assay to veins [94, 138, 176] provides the opportunity to bridge this gap and evaluate in a chemically defined culture environment the angiogenic response of arteries and veins to the same angiogenic regulators.

\textbf{Summary and conclusion}

More than a quarter century after we first reported that explants of rat aorta have the capacity to generate vessels \textit{ex vivo} [170] the aortic ring model has become one the most commonly used assays of angiogenesis. Many factors have contributed to its popularity including reproducibility, cost effectiveness, ease of use and good correlation with \textit{in vivo} studies. An important milestone in the establishment of this model was the discovery that endothelial sprouting from the aortic explants does not require serum or exogenous growth factors and is driven by endogenous mechanisms triggered by the injury of the dissection procedure [112, 168]. This fundamental observation has enabled investigators to analyse mechanisms of angiogenesis in a chemically defined culture environment in which native macrophages, dendritic cells, fibroblasts, pericytes and endothelial cells interact through paracrine and juxtacrine mechanisms comparable to those operating in the live animal. The relative ease with which the soluble and solid phases of the culture system can be modified has facilitated the molecular analysis of specific steps of the angiogenic process from the induction of endothelial sprouting to the recruitment of pericytes and the reabsorption (regression) of the newly formed vasculature. These studies have helped define the role in angiogenesis of many growth factors, inflammatory cytokines and chemokines, axonal guidance molecules, tyrosine kinase receptors, integrin receptors, ECM molecules, MMPs, as well signal transduction pathways and transcription factors (Fig. 7). Co-cultures of aortic rings with different cell types have provided novel insights on the angiogenic role of fibroblasts, smooth muscle cells, macrophages, platelets and cancer cells [28, 70, 168, 174, 231, 232]. The self-limited nature of the angiogenic process and the induction of angiogenic quiescence have facilitated the analysis of the angiogenic cascade from its earliest inductive events to its final resolution through vascular regression. Studies with aortic rings from young and old animals and different animal strains have demonstrated that the angiogenic process is greatly influenced by age and genetic background. The adaptation of the aortic ring model to different species and vessel types has broadened its use to genetically modified mice and human beings. Finally the development of methods to virally transduce the aortic rings has facilitated the study of genes involved in the regulation of the angiogenic process.

In 2004, more than 30 years after Judah Folkman proposed anti-angiogenic therapy as a cancer treatment [62], bevacizumab (Avastin), an anti-VEGF monoclonal antibody, was approved by the FDA as adjuvant therapy for metastatic colorectal cancer [60].
Two years later anti-VEGF therapy with ranibizumab (Lucentis) became the treatment of choice for wet age-related macular degeneration, an angiogenesis-dependent cause of vision loss in the elderly [187]. These successful clinical applications of angiogenic research have proven that angiogenesis-dependent disorders in human beings can be halted or even reversed by blocking the activity of a single angiogenic factor. Implementation of anti-angiogenic therapy has however revealed pitfalls and new challenges. For example existing anti-VEGF therapies have caused side effects such as hypertension, thrombotic microangiopathy and proteinuria due to inhibition of the non-angiogenic functions of VEGF [54, 109]. In addition this type of treatment has slowed down the progression of cancer without inducing tumour dormancy as originally suggested. It has also become clear that experimental tumours may develop resistance to anti-VEGF therapy due to production of other angiogenic factors [211]. Furthermore anti-VEGF therapy has recently been shown to elicit tumour adaptation and progression to greater invasiveness and metastatic behaviour following an initial anti-tumour growth effect [50, 182]. These observations have revealed gaps in our understanding of the angiogenic process, underscoring the need for additional studies aimed at better characterizing the molecular mechanisms of angiogenesis. To that end the aortic ring model represents a powerful tool for the dissection of the angiogenic cascade and the identification of novel molecular targets for therapeutic intervention in cancer and other angiogenesis-dependent disorders.

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