Review

Morphofunctional basis of the different types of angiogenesis and formation of postnatal angiogenesis-related secondary structures

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Summary. We review the morpho-functional basis of the different types of angiogenesis and report our observations, including the formation of angiogenesis-related secondary structures. First of all, we consider the following issues: a) conceptual differences between angiogenesis and vasculogenesis, b) incidence of angiogenesis in pre- and postnatal life, c) regions of vascular tree with angiogenic capacity, d) cells (endothelial cells, pericytes, CD34+ adventitial stromal cells of the microvasculature and inflammatory cells) and extracellular matrix components involved in angiogenesis, e) events associated with angiogenesis, f) different types of angiogenesis, including sprouting and intussusceptive angiogenesis, and other angiogenic or vascularization forms arising from endothelial precursor cells (postnatal vasculogenesis), vasculogenesis mimicry, vessel co-option and piecemeal angiogenesis. Subsequently, we consider the specific morpho-functional characteristics of each type of angiogenesis. In sprouting angiogenesis, we grouped the events in three phases: a) activation phase, which includes vasodilation and increased permeability, EC, pericyte and CD34+ adventitial stromal cell activation, and recruitment and activation of inflammatory cells, b) sprouting phase, encompassing EC migration (concept and characteristics of endothelial tip cells, tip cell selection, lateral inhibition, localized filopodia formation, basal lamina degradation and extracellular changes facilitating EC migration), EC proliferation (concept of endothelial stalk cells), pericyte mobilization, proliferation, recruitment and changes in CD34+ adventitial stromal cells and inflammatory cells, tubulogenesis, formation of a new basal lamina, and vascular anastomosis with capillary loop formation, and c) vascular remodelling and stabilization phase (concept of phalanx cells). Subsequently, the concept, incidence, events and mechanisms are considered in the other forms of angiogenesis. Finally, we contribute the formation of postnatal angiogenesis-related secondary structures: a) intravascular structures through piecemeal angiogenesis, including intravascular papillae in vessel tumours and pseudotumours (intravascular papillary endothelioma or Dabska tumour, retiform hemangi-endothelioma, hemangiosarcoma and lymphangiosarcoma), vascular septa in hemorrhoidal veins and intravascular projections in some tumours; b) arterial intimal thickening; c) intravascular tumours and pseudotumours (e.g. intravenous pyogenic granulomas and intravascular myopericytoma); d) vascular glomeruloid proliferations; and e) pseudopalisading necrosis in glioblastoma multiforme.

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Angiogenesis is a process characterized by the formation of new vessels from an established microvasculature and is therefore responsible for vascular network expansion and remodelling (Folkman, 2003).

Conventionally, when the term angiogenesis is not specified, it generally refers to blood vessels (blood vessel angiogenesis). In lymphatic vessels, the more precise term of lymphangiogenesis is used.

### 1. Definition

Angiogenesis is indispensable in embryonic and foetal development, as well as in a large number of normal and pathological processes during postnatal life. Therefore, the area involved in the study of angiogenesis is far-reaching, covering numerous fields and many disciplines.

During embryonic and foetal development, new vessel formation or neovascularization from pre-existing microvasculature is an important event and has been studied at morphologic (Fig. 1A) and molecular levels (for review: Betz et al., 2016).

Angiogenesis is generally a quiescent process in the adult organism. Nevertheless, it may occur rapidly in several normal circumstances. For example, the need for additional vasculature is imposed on the cyclic evolution of transient structures in the female reproductive system (for review, see Rizov et al., 2017), including the sequential maturation of ovarian pre-ovulatory follicles (Kim et al., 2017) and subsequent development of the corpora lutea (Berisha et al., 2016; Woad and Robinson, 2016), cyclic extensions and repair of the endometrium (Zhang et al., 2016), decidual transformation, implantation and placentation (Okada et al., 2014; Chen et al., 2017), and mammary gland changes during pregnancy, lactation and involution (Andres and Djonov, 2010; VanKlompenberg et al., 2016).

Furthermore, angiogenesis is an important component of many pathological processes, such as chronic inflammation, regeneration, wound healing, organization of thrombi, collateral circulation development and neoplasias. Moreover, angiogenesis occurs in several conditions in which the term ‘angiogenic disease’ has been proposed, since an abnormality of capillary growth is their principal pathological feature (Folkman, 1989). Angiogenic diseases include hemangiomas, psoriasis (Heidenreich et al., 2009; Capkin et al., 2017), scleroderma (Mulligan-Kehoe and Simons, 2008; Manetti et al., 2016), arthritis (Szekanecz et al., 2010; Elshabrawy et al., 2015), atherosclerotic plaque (Camaré et al., 2017), diabetic retinopathy (Capitão and Soares, 2016), neovascular glaucoma (Kim et al., 2015) and ischaemic diseases. For example, the newly formed capillaries invade the joint and cartilage in arthritis, and the vitreous in diabetes, with secondary effects such as cartilage destruction and bleeding, respectively. The role of angiogenesis in neoplasias is of great interest, especially in the progressive growth and metastases of solid tumours (Folkman, 1985), and has resulted in numerous descriptive studies on angiogenesis, as well as on the possible mechanisms involved in this process (for review: Hillen and Griffioen, 2007; Makrilia et al., 2009; Krishna Priya et al., 2016; Ronca et al., 2017).

### 2. Conceptual differences between angiogenesis and vasculogenesis

Vasculogenesis and angiogenesis are two principle types of mechanisms that intervene in vessel development and growth. Classically, the concept of vasculogenesis was restricted to the embryonic development of certain vessels, such as the dorsal aortae and the posterior cardinal veins (Sabin, 1920), and is defined as capillary development (primary capillary plexus) from differentiating endothelial cells (ECs) in situ (Risau and Flamme, 1995; Auerbach et al., 1997; Jin and Patterson, 2009). Currently, the concept of vasculogenesis is broader and is considered as occurring in pre- and postnatal life, with neo-vessel formation from endothelial progenitor cells (EPCs). In the embryo, vasculogenesis takes place in blood islands, which originate from the splanchopleuric mesoderm. Thus, after segregation from the mesoderm, some mesenchymal cells transform into nests of isolated cell cords of hemangioblasts, which are the precursors of both ECs and blood cells. The peripheral cells of the angioblastic masses differentiate into ECs, while the blood precursor cells are found in the centre of the blood islands, where a lumen develops (Axnick and Lammert, 2012). The newly formed lumens soon coalesce (Risau and Flamme, 1995). Finally, smooth muscle cells and pericytes are aggregated from undifferentiated mesenchyme.

In the current concept of vasculogenesis, new blood vessels may develop from circulating and/or bone marrow-derived CD34+ EPCs (C-EPCs and BM-EPCs, respectively) (Asahara et al., 1997; Gehling et al., 2000; Asahara and Isner, 2002; Grant et al., 2002; Pelosi et al., 2002), from CD34+, CD110+ cord blood cells (Aoki et al., 2004), and from stem cells in a vasculogenic zone (between vessel adventitia and the media layer) (Zengin et al., 2006). Therefore, the recruitment, integration, migration and maturation of circulating EPCs with vascular development is considered to be a type of angiogenesis (see section Postnatal angiogenesis with endothelial precursor cell participation. Postnatal vasculogenesis by EPCs).

### 3. Incidence of angiogenesis in pre- and postnatal life

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Angiogenesis is generally a quiescent process in the adult organism. Nevertheless, it may occur rapidly in several normal circumstances. For example, the need for additional vasculature is imposed on the cyclic evolution of transient structures in the female reproductive system (for review, see Rizov et al., 2017), including the sequential maturation of ovarian pre-ovulatory follicles (Kim et al., 2017) and subsequent development of the corpora lutea (Berisha et al., 2016; Woad and Robinson, 2016), cyclic extensions and repair of the endometrium (Zhang et al., 2016), decidual transformation, implantation and placentation (Okada et al., 2014; Chen et al., 2017), and mammary gland changes during pregnancy, lactation and involution (Andres and Djonov, 2010; VanKlompenberg et al., 2016).

Furthermore, angiogenesis is an important component of many pathological processes, such as chronic inflammation, regeneration, wound healing, organization of thrombi, collateral circulation development and neoplasias. Moreover, angiogenesis occurs in several conditions in which the term ‘angiogenic disease’ has been proposed, since an abnormality of capillary growth is their principal pathological feature (Folkman, 1989). Angiogenic diseases include hemangiomas, psoriasis (Heidenreich et al., 2009; Capkin et al., 2017), scleroderma (Mulligan-Kehoe and Simons, 2008; Manetti et al., 2016), arthritis (Szekanecz et al., 2010; Elshabrawy et al., 2015), atherosclerotic plaque (Camaré et al., 2017), diabetic retinopathy (Capitão and Soares, 2016), neovascular glaucoma (Kim et al., 2015) and ischaemic diseases. For example, the newly formed capillaries invade the joint and cartilage in arthritis, and the vitreous in diabetes, with secondary effects such as cartilage destruction and bleeding, respectively. The role of angiogenesis in neoplasias is of great interest, especially in the progressive growth and metastases of solid tumours (Folkman, 1985), and has resulted in numerous descriptive studies on angiogenesis, as well as on the possible mechanisms involved in this process (for review: Hillen and Griffioen, 2007; Makrilia et al., 2009; Krishna Priya et al., 2016; Ronca et al., 2017).
Fig. 1. Angiogenesis in pre-(A) and postnatal (B and C) life. A. An embryonic sprouting vessel (arrow) originating from a vasculogenic vessel (vv). B. A sprouting vessel (arrow) originating from a postcapillary venule (pv) in the adipose tissue. C. A sprouting vessel (arrow) originating from the rat femoral vein (fv) induced by peri-venous administration of PGE2 and glycerol. D. Size of the rat femoral artery (black asterisk) and vein (white asterisk) (scale in mm). Scale bars: A, 20 µm; B, 15 µm; C, 10 µm.
4. Regions of vascular tree with angiogenic capacity (mainly venous side of circulation)

The regions with angiogenic capacity are the most ubiquitous and have a common characteristic: the presence, within or near, of an active preexisting microvasculature, where angiogenesis begins as a result of neighbouring angiogenic stimuli. Thus, the mother vessel microvasculature forms part of a general angiogenic-inflammatory-repair system in which the vessels not only intervene in new capillary formation but also in the recruitment of inflammatory cells and in the contribution of matrix-forming cells (fibroblasts-myofibroblasts, osteoblasts, chondroblasts), and contractile and adipose cells. Interestingly, sprouting angiogenesis mainly occurs in the venous side of the circulation, above all in the postcapillary venules (Fig. 1B) (Auszprunk and Folkman, 1977; Díaz-Flores et al., 1992, 1994a) with a negative pressure, an important fact in initial stages of this process, in which sprouts are immature (Díaz-Flores et al., 1992; 1994a) (see below). Although some authors restrict the origin of newly formed vessels, pointing out that they only arise from the microvessels of the venous side of the circulation, vessels of greater calibre, such as the rat femoral vein, with a discontinuous internal elastic lamina and smooth muscle cells in their media layer, are also capable of contributing to angiogenesis (Fig. 1C,D) (Díaz-Flores et al., 1994a,b; Madrid et al., 1998).

In avascular tissues (e.g. cartilage and cornea) and in fibrin deposits (e.g. wounds), vessels can only originate from neighbouring vascularized tissues (Díaz-Flores et al., 2012a). In this case, the intensity of vascular penetration into an avascular tissue depends on the properties of the latter (e.g. inhibitory action of antiangiogenic substances, as occurs in cartilage).

5. Cells and extracellular matrix involved in angiogenesis

Cells involved in angiogenesis include those of the vessel wall, circulating precursor cells, inflammatory cells and cancer cells. The cells in the vessel wall are ECs (intimal layer), pericytes/smooth muscle cells (mural cells in the media layer) and CD34+ fibroblasts/stromal cells (adventitial or external layer) (Fig. 2A-C). The participation of ECs and pericytes/smooth muscle cells in angiogenesis has received great attention in the literature (for review: Díaz-Flores et al., 2009a; Ribatti et al., 2011); not so the CD34+ adventitial fibroblasts/stromal cells. Here we consider the coordination and interactions between pericytes and ECs, and the role of CD34+ adventitial stromal cells.

The coordination and interactions between pericytes and ECs may be as follows: a) direct contact, which includes interdigitations (peg and socket) (Fig. 2D), gap junctions, and adherent and occluding plaques, leading to intercellular communications, passage of molecules and transmission of mechanical forces (Tillet et al., 2005; von Tell et al., 2006; Díaz-Flores et al., 2011a), b) release of vesicles (Fig. 2D), such as multivesicular bodies, shed vesicles and exosomes, which contain several molecules including microRNA (Dellett et al., 2017; Todorova et al., 2017), and c) paracrine regulators, including vascular endothelial growth factor A (VEGF), sphingosine-1-phosphate, angiopoietin 1 and 2, transforming growth factor beta (TGF beta), semaphoring 3-A and several cytokines (Caporal et al., 2017). These cells have the ability to modulate cellular interactions during angiogenesis and vessel stabilization (Díaz-Flores et al., 1991; 2009a).

The major fibroblastic cell components of adventitial vessel walls are CD34+, CD31-, CD146- and CD45-, and are considered MSC progenitors (Corselli et al., 2012; Lin and Lue, 2013; Díaz-Flores et al., 2014; 2015a,b; 2016a,b). These cells have received numerous names, including fibroblasts, fibrocytes, dendrocytes, keratocytes, telocytes and stromal, dendritic, adventitial, supraadventitial, perivascular, paravascular and delimiting cells (for review: CD34+ stromal cells/fibroblasts/fibrocytes/telocytes, Díaz-Flores et al., 2014). Some authors point out that the microcirculation contains no CD34+ adventitial stromal cells. However, these cells extend continuously through most of the vascular system. Therefore, a very thin rudimentary adventitia is found in several microvessels (Fig. 2A), except for some small vessels in certain locations. Examples of microvessels with CD34+ adventitial stromal cells are those of the reticular dermis and submucosa, muscular propria, and serosa of the intestinal wall and gallbladder (Fig. 2A-C). Examples of microvessels without CD34+ fibroblasts are those of the papillary dermis, and the intestinal and gallbladder mucosa (Fig. 2E) (Díaz-Flores et al., 2014). The presence of these ‘delimiting fibroblasts’ around small vessels is easily demonstrated by electron microscopy (Fig. 2C). In the adventitia of large vessels, CD34+ fibroblasts/stromal cells appear in several layers, which decrease in number as vessel size reduces and, when present in the capillaries, are arranged in a single layer. Although CD34 is also intensely expressed in ECs, both types of cells are easily distinguished by this different response to CD31 (anti-CD31 stains ECs but not CD34+ adventitial stromal cells), and by their characteristics and respective locations in the vessel wall. Pericytes and SMCs are positive for anti-αSMA and negative for anti-CD34. Since CD34 is expressed in stromal cells of the adventitia and in ECs of the tunica intima, the CD34-stained vessels show a double-ring appearance (stained endothelium and adventitia), owing to two concentric circles sandwiching the unstained media layer (smooth muscle or pericytic layer) (Fig. 2A) (Lin et al, 2010; Maumus et al., 2011; Díaz-Flores et al., 2014). A similar image is obtained with double stained (CD34/brown and αSMA/red) vessels, although with red staining in the media layer (Fig. 2B). Some CD34+ fibroblasts/stromal cells in the vascular wall may extend their processes into
Fig. 2. Vessel wall cells involved in angiogenesis. 

A. CD34 expression in a microvessel with a double ring appearance (CD34 stained endothelial - ec- and stromal adventitial cells -sc- sandwiching the unstained mural cells of the media layer). 

B. A double stained vessel with anti-CD34 (staining ECs -ec-and stromal adventitial cells -sc - brown) and anti-αSMA (staining mural cells -mc- red). Note the double ring appearance of CD34 stained cells sandwiching αSMA mural cells of the media layer. 

C. Electron photomicrograph showing projections of adventitial stromal cells (sc), a pericyte (p) and endothelial cells (ec). 

D. Interdigitations (peg and socket) (arrow) between the process of a pericyte (p) and an endothelial cell (ec). A multivesicular body in formation is also observed in this interdigitation. 

E. In the mucosae of the bladder, double staining with anti-CD34 (brown) and anti-αSMA (red) show capillaries devoid of CD34+ stromal cells. Only one vessel presents αSMA+ perivascular mural cells (asterisk). Scale bars: A, 10 µm; B, 15 µm; C, 2 µm; D, 1 µm; E, 35 µm.
surrounding tissues and vice versa. Frequently, these extensions associate with other tissue cells (native or recruited).

The other cells involved in angiogenesis will be considered in other sections, thus circulating precursor cells in “Postnatal angiogenesis with endothelial precursor cell participation”, the inflammatory cells in “Sprouting angiogenesis” and the cancer cells in “Vasulogenic mimicry”.

The pre-existing or incorporated components in the extracellular matrix comprise collagens (including collagen I, III, IV, V, VIII, XV, XVIII – mainly collagen IV), elastin, laminins, perlecans, hyaluronan, fibronectin, entactin/nidogen, TsP, SPARC, and plasminogen. In the basement membrane of quiescent microvasculature there are several proteins, including laminins, type IV collagens, perlecans, nidogens, SPARC, fibulins, trombospondins, and collagen types VIII, XV and XVIII, (for review: Seano and Primo, 2015; Randles et al., 2017). During angiogenesis, these molecules may undergo local modifications (matricryptic sites of Davis) (Davis et al., 2000) by the action of proteases, such as MMPs, and their extracellular matrix fragments originate matrikines (Bellón et al., 2004), which are pro- or anti-angiogenic factors (see below).

6. Events associated with angiogenesis

Angiogenesis is a complex biological process, which may be associated with coagulation, inflammation, immunity, tissue repair and remodelling. Despite the continuous and orderly nature of the process, these findings occur in several overlapping phases, according to predominant mechanisms. Therefore, several phenomena, such as vascular dilation and increased vascular permeability, coagulation, activation of numerous cell types, including vascular and inflammatory cells, as well as modifications of the extracellular matrix, take place during new-vessel formation.

7. Types of angiogenesis

Several types of angiogenesis have been described, including sprouting and intussusceptive angiogenesis, and other forms of vascularization by means of circulating endothelial precursor cells (EPCs) (postnatal vasculogenesis), vasulogenic mimicry and vascular co-option. Following, we describe these types of angiogenesis, and we also consider a recently reported form: piecemeal angiogenesis, which intervenes in the formation of angiogenesis-related secondary structures.

7.1. Sprouting angiogenesis

7.1.1. Definition

Sprouting angiogenesis is the process by which vessel sprouts grow out of a pre-existing vasculature.

7.1.2. Events of new blood vessel formation in sprouting angiogenesis

Sprouting angiogenesis is a complex multistep process, in which the following overlapping events have been described: a) activation of vascular cells, b) EC migration, c) proteolytic destruction of extracellular matrix, including basement membrane degradation, d) EC proliferation, e) pericyte mobilization, proliferation and recruitment, f) vascular lumen formation, g) changes in extracellular matrix with development of a new basement membrane, and h) vascular maturation and remodelling, with capillary network formation, eventual organization of larger microvessels, and involution of most newly formed vessels. Vasodilation and an inflammatory response may precede and accompany the process. Furthermore, during angiogenesis and vascular involution, other phenomena may occur in the interstitium, mainly the participation of precursor stromal cells, which may originate matrix-forming cells (fibroblast-myofibroblasts, osteoblasts, chondroblasts), contractile cells (SMCs, myofibroblasts and myointimal cells) and adipose cells.

In this review, we group these events in three phases: Activation phase, Sprouting phase and Stabilization phase.

7.1.3. Activation phase

Vascular dilation, increased vascular permeability, activation of vascular cells and diapedesis of leukocytes form part of the activation phase. Below, we outline these findings, which precede and accompany sprouting angiogenesis.

7.1.3.1. Vasodilation and increased permeability

Dilation and increased permeability of the blood venules and capillaries occur rapidly in the initial phase of angiogenesis (Fig. 3A), in which the opening of interendothelial cell junctions is observed (Fig. 3B). Indeed, in response to proangiogenic factors, such as VEGF (see below), capillaries and postcapillary venules show vasodilation and increased vascular permeability. For example, in corneal vascularization induced by silver nitrate, dilation takes place within an hour (McCracken et al., 1979) and intensifies progressively. The changes in vascular permeability during angiogenesis were studied using the intravenous injection of different markers, such as colloidal carbon or Monastral blue to label leaky vessels (Fig. 3C) (Garbett and Gibbins, 1987; Diaz-Flores et al., 1992).

Increased vascular permeability is also demonstrated by the presence of extravascular fibrin and dilated lymphatics. Extravascular fibrin deposits, which are found during inflammation, wound healing (Madri and Pratt, 1988) and in the periphery of tumours (Nagy et al., 1989), are important during angiogenesis. Thus, neovascularization is induced by fibrin gels in vitro and
Fig. 3. Phenomena in the activation phase of angiogenesis. 

A. A dilated and congestive capillary between oedematous spaces. Note perivascular activated CD34+ adventitial stromal cells (arrows).

B. Ultrastructural image of the opening of the interendothelial junctions (arrow).

C. Demonstration of vascular permeability during angiogenesis. Under electron microscopy, Monastral Blue particles (asterisk) are observed between endothelial cells (ec) and pericytes (p) in a leaky vessel.

D. A dilated vessel showing early migration of leukocytes (L) between an opened interendothelial cell junction (arrow). Note an activated CD34+ adventitial stromal cell (sc) with a nucleus increased in size and with a prominent nucleolus.

E. Portion of an activated mast cell with degranulation between a process of a stromal cell (sc) and pericytes (p). Endothelial cell: ec. Scale bars: A, 25 μm; B, D, E, 1 μm; C, 2 μm.
in vivo (Dvorak et al., 1987). Also, it has been demonstrated that cultured ECs synthesize fibronectin and that growing capillaries produce fibronectin in situ (Clark et al., 1982a).

### 7.1.3.2. Activation of ECs

In pre-existing vasculature (parent vessels), the earliest morphological changes in the normally quiescent ECs consist of hypertrophy with bulging in the vascular lumen, nuclear enlargement, nucleolar prominence, dispersal of the ribosomes into their free form, and an increase in the number of organelles and projections formation (Auspink and Folkman 1977; McCracken et al., 1979; Díaz-Flores et al., 1992, 1994a). The ECs projecting towards the interstitium have been described as filopodia (Kurz et al., 1996; Ruhberg et al., 2002). Increased endothelial DNA-synthesis occurs at the onset of angiogenesis (Burger et al., 1983; Díaz-Flores et al., 1992) and an increased proliferative index is demonstrated with Ki67. In addition, modifications of the tight junctions (organized by claudins, occludins and junctional adhesion molecules) and adherens junctions (organized by catenins and cadherins), as well as extracellular and intracellular associations play an important role in EC activation (Dejana et al., 2008; Dejana and Orsenigo, 2013; Dejana and Lampugnani, 2014). All these phenomena facilitate the recruitment of inflammatory cells (Sprague and Khalil, 2009) and lead to local deposition of serum proteins, originating a fibrin-rich provisional matrix (Mehta and Malik, 2006).

Tissue hypoxia activates ECs and angiogenesis-promoting signals, mainly VEGF, through stimulation of VEGFR2. VEGF acts not only in this EC activation, but also in other phases of vessel sprouting (see below).

### 7.1.3.3. Activation of pericytes

During the initial phase of angiogenesis, activated pericytes in pre-existing vasculature bulge, shorten their processes and increase their somatic volume, adopting an angiogenic phenotype (Díaz-Flores et al., 1992, 1994a). Their nuclei display some folding and the nucleoli become prominent. The cytoplasm acquires numerous ribosomes, either singly or in aggregates, and multiple profiles of rough endoplasmic reticulum are observed. Micropinocytic vesicles are widely distributed along the cell membrane. Moreover, pericytes may show increased DNA synthesis, and a sudden and intense pericyte proliferation occurs (Díaz-Flores et al., 1992). Proliferation markers, such as Ki-67, also demonstrate a higher proliferative index in pericytes. In the parent vessels, the pericyte basement membrane is disrupted and fragmented, sometimes away from the pericyte cell surface. Many pericytes project into the perivascular space and appear detached from the vessel walls (Díaz-Flores et al., 1992).

VEGF is a negative regulator of pericyte function and vessel maturation, and facilitates the loss of pericyte coverage, leading to vessel destabilization (Greenberg et al., 2008). In addition, VEGF induces overexpression of Ang2 in ECs, which also facilitates pericyte dissociation, and vessel destabilization and regression (Zhang et al., 2003; Cao et al., 2007). Pericyte dissociation may also be facilitated by migrating macrophages.

### 7.1.3.4. Activation of CD34+ adventitial stromal cells

In this phase, CD34+ adventitial stromal cells separate from the vascular wall, increase in size and appear as large, plump, stellate or ovoid cells in oedematous spaces (Fig. 3A). The nuclei also increase in size and show one or two prominent nucleoli (Fig. 3D) (Díaz-Flores et al., 2014, 2015a,b, 2016a,c). These cells secrete VEGF, bFGF, PDGF-α and IL-8, which contribute to the induction of an angiogenic phenotype in ECs, and promote angiogenesis in vivo and in vitro (Hartlapp et al., 2001; Miranville et al., 2004) (e.g. adipose derived stromal cells support postnatal neovascularization - Miranville et al., 2004).

### 7.1.3.5. Recruitment and activation of inflammatory cells

Inflammation may play an important role in angiogenesis, with vascular and leukocyte communication, and inflammation-induced angiogenesis (for review, Szade et al., 2015; Kreuger and Phillipson, 2016). Intravascular accumulation of platelets and polymorphonuclear leukocytes (PMNs) occurs within a few hours, with early diapedesis of leukocytes outside the vessel lumen. Thus, before and during vascular sprouting, inflammatory cells are observed adhering to the endothelium of the parent vessels, as well as passing through the endothelial junctions and the pericyte-endothelial space. Between 1 and 6 h after angiogenic stimuli, the PMNs predominate. Thereafter, the number of monocytes/macrophages increases, while the number of PMNs decreases dramatically. Frequently, the monocytes/macrophages, either individually or in small clusters of two or three, simultaneously appear trapped between the ECs and the pericytes, within the basement membrane (Díaz-Flores et al., 2009b).

VEGF, CSF-1 (colony stimulating factor 1), PIGF (placental growth factor) and chemokines participate in leukocyte recruitment. For example, in monocytes, CXC, CC, C and CX3C chemokines act through transmembrane G protein-coupled receptors, mainly CCL2 (C-C chemokine ligand 2)/CCR2 (Ding et al., 2012). The new vessels may arise following the margination and diapedesis of the leukocytes, which do not seem to be essential for the initiation and continuation of angiogenesis. For example, corneal vascularization has been produced in the absence of leukocytes in rats and rabbits (Sholley et al., 1978). Nevertheless, leukocytes may have a facilitatory or augmentative role in vascularization, by the secretion of cytokines, chemokines, growth factors and matrix-
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degradation enzymes (see inflammatory cells in sprouting phase).

Activated mast cells (Fig. 3E) play an important role in angiogenesis by producing and releasing pro-angiogenic factors (for review: Maltby et al., 2009; Ribatti and Crivellato, 2012a; Cimpean et al., 2017). Thus, mast cells may release VEGF stored in their granules or in a degranulation-independent form, enhancing EP2 receptor by PGE2 (Boesiger et al., 1998; Abdel-Majid and Marshall, 2004). Other angiogenic mediators stored in their granules, such as histamine, heparin, IL-8, TNF-alpha, TGF-beta, NGF and FGF-2 may be released by degranulation (Kanbe et al., 2000). Finally, mast cell granules contain tryptase and chymase, which participate in the degradation of extracellular matrix components. In particular, tryptase stimulates the proliferation of endothelial stalk cells and facilitates tubulogenesis, activation of MMPs, and VEGF and FGF-2 release (Blair et al., 1997).

7.1.4. Sprouting phase

This phase includes EC migration, extracellular matrix changes with basement membrane degradation facilitating EC migration, EC proliferation, pericyte and CD34+ stromal cell mobilization, proliferation, differentiation and recruitment, inflammatory cell participation, tubulogenesis (vascular lumen formation), formation of a new basement membrane and sprouting connection. During the process in which the ECs protrude and the vascular basement membrane is degraded, scant microscopic bleeding may occur.

7.1.4.1. EC migration

Traditionally, it was considered that blood vessels grew through EC movement (His, 1868). This concept of EC migration is maintained as an important step during angiogenesis (Auspriun and Folkman, 1977). Most researchers agree that these changes precede endothelial replication, and that migration and mitosis are independent phenomena (Sholley et al., 1977a,b; Wall et al., 1978). Therefore, angiogenic stimuli may operate through chemotaxis, and EC mitosis may be a secondary event (Sholley et al., 1977a,b).

The current concept of endothelial tip, stalk and phalanx cells is important to understand these findings (Gerhardt et al., 2003; Ribatti and Crivellato, 2012b) (see below). In this phase, endothelial tip cells are predominantly involved in EC migration, whereas stalk cells are involved in proliferation and have the capacity to acquire a lumen-forming cell phenotype. Tip cells were previously known as migrating, leader and projecting ECs. The term filopodia was also applied (Kurz et al., 1996; Ruhrberg et al., 2002).

7.1.4.1.1. Concept and characteristics of endothelial tip cells. Currently, the term tip cells is used for specialized cells at the tips of a wide variety of developing structures, predominantly of tube or branched formations (Gerhardt et al., 2003; Weavers and Skaer, 2014). In angiogenic sprouts, endothelial tip cells are therefore leader migrating (invading) cells (Fig. 4A,B) that sense the environment, and precede and guide endothelial stalk cells. In the initial phase of neo-vascularization, tip cells degrade cell-cell junctions (VE-cadherin and tight junction protein-1), and contribute to extracellular matrix proteolysis (Arroyo and Iruela-Arispe, 2010) and degradation of the vascular basement membrane of the parent vessel. Subsequently, they protrude through the vessel wall and begin to migrate in a polarized form into the interstitial space towards the angiogenic stimulus.

Tip cells show an EC transient modified phenotype (De Smet et al., 2009), with numerous free polyribosomes and abundant intermediate filaments (Fig. 4A), as well as filopodia that sense attractant or repellent signals and guide polarized migration (Ruhrberg et al., 2002; Gerhardt et al., 2003; Lu et al., 2004; De Smet et al., 2009; Geudens and Gerhardt, 2011). They proliferate minimally and express the tyrosine-kinase receptor VEGFR2, VEGFR3/FIT-4, Delta-like 4 (DLL4), PDGF beta, EC specific molecule I ECM-I1, homolog b (netrin receptor UNC5b) and angiopoietin. Notch-signalling activity is low (Gerhardt et al., 2003; Claxton and Fruttiger, 2004; Lu et al., 2004; Siekmann and Lawson, 2007; Suchting et al., 2007; Hellstrom et al., 2007a; Tammela et al., 2008; Zheng et al., 2014a). In early migration, ECs show extracellular-matrix-degrading podosomes on their surface. These specialized cell-matrix contacts contain adhesive (e.g. stalin, vinculin, pixelin) and proteolytic (e.g. MT1-MMP) molecules, surrounding a core rich in actin (Moreau et al., 2006; Osiak et al., 2005). Individual podosomes and podosome rosettes are induced by VEGF-A, which up-regulates integrin αβ1 (Primo et al., 2010; Seano et al., 2014), and participate in the degradation of the basement membrane and in the control of blood vessel branching (Seano et al., 2014).

Attractant signals, mainly VEGF-A, and repulsive signals, mainly semaphoring 3A, guide sprout migration (see below). The chemotactic behaviour of ECs at the tips of growing vessels is facilitated by the secretion of plasminogen activator and collagensases (Moscatelli et al., 1981). EC migration, in response to the extracellular matrix, depends on the integrin family of cell adhesion receptors (Leavesley et al., 1993). Indeed, EC attachment, spreading and migration are mediated by integrins α2β1 and α4β3 (Leavesley et al., 1993). EC migration on collagen and vitronectin is mediated by α4β3 and occurs in a calcium-dependent mechanism, while collagen recognition by α2β1 promotes EC migration in the absence of calcium (Leavesley et al., 1993).

Important findings during EC migration are tip cell selection with lateral inhibition, localized filopodia formation and sprouting guidance.

7.1.4.1.2. Tip cell selection and lateral inhibition. Not all activated ECs acquire the tip cell phenotype, since the ECs that participate in migration are selected by a
Fig. 4. EC migration in sprouting phase. A. An endothelial tip cell (tc), devoid of basement membrane, is observed in an angiogenic vessel. Note numerous free polyribosomes and abundant filaments. In the insert, an image under light microscopy of a migrating CD34-stained EC. B. Migrating CD34+ ECs with projections in their tips (arrows). C. Double stained microscopic section with anti-CD34 (staining endothelial cells - brown) and anti-αSMA (staining pericytes - red). Note the absence of pericytes around the migrating and neighbouring ECs (arrow). Scale bars: A, 1 µm; B, C, 14 µm.
process in which EC stimulated migration and lateral inhibition occur. VEGF, Dll4 (delta-like 4)/Notch signalling and the ligand Jagged I participate in this process of tip cell phenotype specification and lateral inhibition with stalk cell phenotype acquisition (Hellström et al., 2007b; Leslie et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007; Bentley et al., 2008; Benedito et al., 2009; De Smet et al., 2009; Potente et al., 2011). VEGF activates VEGFR2 (De Smet et al., 2009) and is an important inducer of tip cell selection and formation. Thus, only ECs exposed to the highest VEGF levels become tip cells (VEGF activation of VEGFR2 predominates in this mechanism). VEGFR2 signalling induces Dll4 expression, which binds to NOTCH receptor on adjacent ECs and activates NOTCH signalling. The latter inhibits VEGFR2 and VEGFR3 in these adjacent ECs, preventing tip cell formation and promoting a stalk cell phenotype in immediately neighbouring ECs (Williams et al., 2006a; Hellstrom et al., 2007b; Leslie et al., 2007; Lobov et al., 2007; Suchting et al., 2007). The ligand Jagged I antagonizes Dll4 and controls the tip cell number (Benedito et al., 2009). Determination of stalk cell phenotype is also facilitated by bone morphogenetic protein signalling pathway (Moya et al., 2012). Taking into consideration the above, the mechanism that facilitates the selection of the tip cells may be a small imbalance in local VEGF concentration. A time-based formulation of EC behaviour (temporal adaptation) has been hypothesized for the cell decisions (Bentley and Chakravartula, 2017). Therefore, this competitive cellular mechanism limits the number of sprouts (excessive branching) (Hellström et al., 2007b; Lobov et al., 2007; Suchting et al., 2007; Jakobsson et al., 2010; Geudens and Gerhardt, 2011) and facilitates sprout guidance and the change of tip cells for other cells within the stalk region (relative levels of VEGFR1 and VEGFR2) (see below). FGF might also control endothelial tip cell migration (Vitorino and Meyer, 2008). Moreover, several inflammatory signals that act in vascular permeability also participate in EC activation and in extracellular matrix proteolysis by endothelial tip cells. These factors include TNF alpha, CCL2/MCP-1, bradiquin, nitric oxide, PGE2 and sphingosine-1-phosphate (SIP) (Langlois et al., 2004; Galvez et al., 2005; Lee et al., 2006; Petrovic et al., 2007; Alfranca et al., 2008; Sainson et al., 2008). Likewise, the action of some inflammatory cells (e.g. monocyte/macrophage MMPs) induces tip cell phenotype, guides tip cells and cooperates with neovascularization (Anghelina et al., 2006; Chung et al., 2009).

7.1.4.1.3. Localized filopodia formation in tip cells. Filopodia are slender, polarized, highly dynamic plasma-membrane protrusions (microspikes) (Fig. 4B), which contain actin filaments, establish adhesions with components of the extracellular matrix, sense their environment (like antennae) and participate in cell migration. Remodelling of the cytoskeleton, mainly down-regulation of myosin II contraction, determined by extracellular matrix properties, facilitates filopodia formation (Gerhardt et al., 2003; Fisher et al., 2009; Myers et al., 2011). Heparin-binding VEGF isoforms, ephrin B2 and a small GTPasa of the Rho family (CDC42) mediate internalization of VEGFR2, and participate in the directionality and extension of filopodia, as well as in actin remodelling (Ruhberg et al., 2002; Etienne-Manneville, 2004; Sawamipahk et al., 2010; Fantin et al., 2015). The non-tyrosine kinase transmembrane protein, neuropilin 1(NRP1) is essential for extracellular matrix-induced CDC42 activation (Fantin et al., 2015). Pericytes may or may not be present near the tip cells (Fig. 4C).

7.1.4.1.4. Basement membrane degradation and extracellular changes facilitating EC migration. During angiogenesis, local proteolysis of the basement membrane of the parent vessels is observed, which is a necessary step for EC migration (Ausprunk and Folkman, 1977). Complete disintegration occurs on the side closest to the angiogenic stimulus, coinciding with those areas in which the ECs start to grow outwards (Fig. 4A), while subtle alterations appear around the whole circumference of the parent vessel. Therefore, EC sprouts have no basement membrane, but a homogeneous provisional substratum with altered proteoglycans (Clark et al., 1982b). Proteases act in this process (Moscatelli and Rifkin, 1988; Ghajar et al., 2008; van Hinsbergh and Koolwijk, 2008), and matrix metalloproteinases (MMPs) and ADAM family proteins are the main proteases of the metzin superfamily that control capillary morphogenesis. MMPs involved in these functions include membrane-type matrix metalloproteinases (MT1-MMP, MT2-MMP/MMP-15, MT3-MMP), and MMP-2, MMP-3, MMP-7, MMP-9 and MMP-13. Among these MMPs, the MT1-MMP is the main pericellular fibrinolisin and collagenase involved in EC sprouting (Hiraoka et al., 1998; Zhou et al., 2000; Yana et al., 2007), and crosstalk between neo-vessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells (Yana et al., 2007). The extracellular-matrix-degrading podosomes (see above) participate in the degradation of the basement membrane (Primo et al., 2010; Seano et al., 2014), since individual podosomes and podosome rosettes, induced by VEGF-A, which up-regulate integrin α6β1, contain proteolytic molecules, mainly MT1-MMP (Primo et al., 2010; Seano et al., 2014).

Likewise, the components of the microvascular extracellular matrix, such as laminins and collagens undergo dramatic changes (Nicosia and Madri, 1987). The peptides released by partial proteolysis of extracellular matrix macromolecules (matrikines, see above) (Maquart et al., 2004) may have pro- and/or antiangiogenic activity, promote protease and cytokine production, and/or facilitate neutrophil migration and monocyte recruitment (Bellon et al., 2004; Adair-Kirk
7.1.4.2. EC proliferation

Mature ECs, normally in a resting state, have an extremely slow turnover rate (2 months or more) (Folkman and Cotran, 1976; Chen et al., 1995). Therefore, angiogenesis is generally a quiescent process in the healthy adult organism. Nevertheless, ECs can quickly convert to a proliferative state during angiogenesis in several processes, such as endothelium repopulation in organ transplants, repair of large vessel defects and thrombi rechanneling (Cavallo et al., 1973; Folkman, 1984). However, EC proliferation is not absolutely essential, since angiogenesis has been shown to take place even in the absence of EC replication (Sholley et al., 1984). Thus, initial sprouts may progress without cell division, although proliferation is required for sustained sprouting (Auspunk and Folkman, 1977). As mentioned above, endothelial DNA synthesis occurs in parent vessels before sprouting and, according to some authors, as early as 6 to 8 hours after an angiogenic stimulus is applied (Cavallo et al., 1973). EC mitoses appear in both the parent and newly formed vessels. Classically, it was pointed out that mitoses occur at the tip of the sprouts (Clark and Clark, 1939, Hadfield, 1951), but it is now accepted that when capillary sprout budding begins, endothelial proliferation takes place in cells that follow the endothelial tip cells. In other words, the zone of replication is closer to the parent vessel.

7.1.4.2.1. Concept of endothelial stalk cells. Cells with capacity to proliferate, and to form tubes and branches are currently named endothelial stalk cells (see above) (Gerhardt and Betsholtz, 2005). Thus, these cells proliferate facilitating sprout extension, establish intercellular junctions, develop basement membrane and are coated by pericytes (Fig. 5A-C) (Eiken and Adams, 2010; Geudens and Gerhardt, 2011; Wacker and Gerhardt, 2011).

Stalk cell proliferation depends on VEGF concentration, while tip cell migration depends on a VEGF gradient (Ruhberg et al., 2002; Gerhardt et al., 2003). VEGF induces filopodia formation and the expression of DI4 in tip cells, and bridge formation with filopodia of other tip cells during vessel sprout connection (Bentley et al., 2009). The ability of angiogenic stimuli to induce replication in confluent ECs is associated with disruption of cell-cell contacts (Bavisotto et al., 1990). Likewise, the replicative state and its ability to respond to endogenous mitogens may depend on cytoskeletal organization, such as microtubule destabilization or changes in the cell shape (Liaw and Schwartz, 1993). Finally, collagen in the interstitium may influence EC proliferation (Madri and Stenn, 1982; Schor et al., 1983).

The orientation of stalk cell divisions is important and is also regulated by VEGF gradient (Zeng et al., 2007). During sprout extension in absence of blood flow, the plane of division is perpendicular to the axis of the vessel sprout (Zeng et al., 2007). Conversely, in perfused vessels, through CD42 activation, VEGFR2, PECAM1 and VE-cadherin sense the blood flow, and the plane of EC division is determined by the shear-stress direction (Tzima et al. 2003, 2005; Gomes et al., 2005).

Therefore, when an entire endothelial tip cell migrates into the interstitium, it is followed by another stalk cell, and EC sprouts are formed in the perivascular stroma. Endothelial stalk cells align with others to create either a solid sprout or one with an intercellular slit-like lumina. The proliferation, elongation and rearrangement of endothelial stalk cells progressively lengthen the sprout (the sprout extension and tubulogenesis being interlinked processes) (Aydogan et al., 2015). The presence of abundant contractile intermediate filaments in the endothelium of the sprouts might be important for the extension and migration of these sprouts (Sauteur et al., 2014).

7.1.4.3. Pericyte mobilization, proliferation and recruitment in the sprouting phase of angiogenesis

Pericyte mobilization, proliferation and association with EC sprouts occur during the sprouting phase of angiogenesis (Rhodin and Fujita, 1989; Schlingemann et al., 1990, 1991, 1996; Díaz-Flores et al., 1991, 1992, 1994a; Nehls et al., 1992; Wesseling et al., 1995; Morikawa et al., 2002; Gerhardt and Betsholtz, 2003; McDonald and Choyke, 2003). In addition, pericyte recruitment and EC investment include alignment, contacts and phenotype changes. Although EC sprouts may initially form without pericyte involvement (representing a plasticity window, allowing ECs to remodel), pericytes are also among the first cells to invade newly vascularized tissues (Díaz-Flores et al., 1991; Nehls et al., 1992; Reynolds et al., 2000). Most of the authors are of the opinion that the investment of sprouts by pericytes occurs around trailing trunk ECs (stalk cells). Therefore, endothelial tube formation is followed by investment of pericytes, which use EC sprouts as migration clues. In the association of pericytes with the endothelium, cytoplasmic processes of pericytes and ECs have been observed penetrating each other (peg and socket) (Fig. 2D) (Diaz-Flores et al., 2011a).

Pericytes are recruited in the vascular sprouts (Fig. 5A-C) in response to growth factors, such as PDGF and TGF beta, and angiopoietin1 and 1 phosphatase (Nicosia and Villaschi, 1995; Abramsson et al., 2002; Hashizume and Ushiki, 2002; Bergers and Song, 2005; Hoffmann et al., 2005). Nitric oxide also mediates pericyte recruitment (Kashiwagi et al., 2005). This association between pericytes and ECs is key to vascular maturation during remodelling and to vascular patterning, diameter regulation, vessel stabilization and endothelial cell survival (Hellström et al., 2001; Gerhardt and Betsholtz, 2003; Betsholtz et al., 2005; Gerhardt and Semb 2008). Although the mere presence of pericytes appears not to protect vessels from undergoing aggression after inhibition of VEGF (Morikawa et al., 2002; Inai et al., 2004), pericyte
Angiogenesis and related secondary structures

Fig. 5. Endothelial stalk cells in sprouting phase of angiogenesis. A, B. Vascular sprouts (arrows) are observed in double stained sections with anti-CD34 (staining endothelial stalk cells - brown) and anti-αSMA (staining pericytes - red). Note in A that sprouts originate from one side of the parent vessels, probably leading to angiogenic stimulation. C. Ultrastructural image of endothelial stalk cells (ec) around a narrow or virtual lumen (L). Interendothelial cell junctions (arrows) and a mitotic endothelial stalk cell are observed. Also note developing basement membrane, a perivascular cell (p) and projections of perivascular cells. Observe the contacts between the perivascular cell and endothelial stalk cells. Insert 1: Detail of the interendothelial cell junctions (arrows). Insert 2: Pericytes (stained in red with anti-αSMA) and endothelial stalk cells (stained in brown with anti-CD34) under light microscopy. Scale bars: A, 20 µm; B, 30 µm; C, 2 µm.
An example of transitional cell forms occurs in...
Fig. 6. Perivascular CD34+ stromal cell and macrophage behaviour, and tubulogenesis in sprouting phase of angiogenesis. **A.** One CD34+ perivascular stromal cell in mitosis (arrow) around a microvessel (asterisk). **B.** Double staining with anti-ki67 and anti-CD34 reveals ki-67 expression in nuclei of CD34+ perivascular stromal cells (arrows). **C.** Double staining with anti-CD34 (brown) and anti-αSMA (red) shows stromal cells expressing either CD34 or αSMA. **D.** Double staining with anti-CD68 (staining macrophages – red) and anti-CD34 (staining stromal cells – brown) reveal association between macrophages and stromal cells. **E.** Ultrastructural image of vascular lumen formation (tubulogenesis) (asterisks) between endothelial stalk cells (ec). Pericyte: p. Scale bars: A, 10 µm; B, 15 µm; C, 25 µm; D, 20 µm; E, 1 µm.
tug-macrophage towing a ship-perivascular cell-myofibroblast (Díaz-Flores et al., 2009b).

7.1.4.6. Tubulogenesis (vascular lumen formation)

In the formation of the lumen during sprouting angiogenesis, ECs form tubular channels (Fig. 6E) capable of carrying blood. This process requires a complex mechanism and the participation of numerous molecules (for review: Iruela-Arispe and Davis, 2009). Current and classical studies, and according to the five potential morphogenetic processes described for different lumen and tubule formation (Lubarsky and Krasnow, 2003), two mechanisms have been considered in vascular sprout lumenogenesis: cell hollowing or intracellular vacuolization and budding or cord hollowing. In the cell hollowing or intracellular vacuolization, contiguous endothelial stalk cells originate exocytotic vacuoles/vesicles, which interconnect and lead to a luminal space (Folkman and Haudenschild, 1980; Furusato et al., 1984). In the budding or cord hollowing mechanism, initial intercellular channelling occurs by curvature of the trailing trunk ECs (stalk ECs) (Wakui, 1988; Jin et al., 2005; Axnick and Lammert, 2012; Yu et al., 2015). In general, it is accepted that the lumen of the capillary sprout is formed between the adjacent endothelial processes, which bud off the wall of the parent vessels and conserve their cellular polarity (Wakui, 1988). Thus, the capillary sprout lumen may be an elongation of the parent vessel lumen (Wakui et al., 1988). Other authors are of the opinion that the new vessel lumen appears first in the sprout, merging later with the mother vessel (Folkman, 1984).

An important finding, which has received little attention in vascular tubulogenesis in vivo, is the origin of sprouts from the venous side of circulation with negative blood pressure (see above). In our view, although ECs have cell-cell interactions in early lumenogenesis, they are devoid of sufficient support. Therefore, low physical forces facilitate structural preservation of the developing endothelial tubes. When tip cells join to form capillary loops and contact with the arterial side of circulation, the gradual impact of positive blood pressure occurs in the newly formed vessels and contributes to remodelling them (Díaz-Flores et al., 2009b).

7.1.4.7. Changes in extracellular matrix. Formation of a new basement membrane

Initially, active ECs secrete matrix-degrading enzymes, such as plasminogen activator and collagenase, causing fragmentation of the basement membrane (see above). Thus, in the initial stages of the developing microvessels, fibronectin is a predominant component of the provisional matrix, making up a delicate fibrillary network. Fibrils of type IV and V collagen, patchy amorphous deposits of laminin and rare to absent fibrils of type I and III collagen have also been described in these initial stages (Nicosia and Madri, 1987). Progressively, the deposits of fibronectin decrease, becoming discontinuous, while laminin and type IV collagen increase, accumulating and forming a continuous feltwork in the subendothelial space. In the late stages of angiogenesis, increased amounts of types I and III collagen in the perivascular space are observed. In some conditions, greatly thickened and/or multilayered basement membrane is present in mature vessels, probably by repeated episodes of EC death and regrowth (Díaz-Flores et al., 1994a). Pericyte and endothelial cells participate in the formation of mature basement membrane. Thus, pericytes stimulate vascular basal membrane formation by specific induction of fibronectin, nidogen-1, perlecan and laminin isoforms (Stratman et al., 2009). Likewise, connective tissue growth factor (CCN2) is essential for basement membrane formation and pericyte adhesion (Hall-Glenn et al., 2012).

7.1.4.8. Vascular anastomosis and capillary loop formation

Vascular anastomosis occurs as a result of the formation of stable contacts between ECs of two angiogenic sprouts and/or between sprouts and functional blood vessels (Fig. 7A). The connection between sprouts is initiated by contacting tip cell filopodia and is followed by the formation of adherent junctions, in which cDh5/VE-cadherin plays an important role (Lenard et al., 2013). When the fusion is with a functional vessel, tip cell filopodia in angiogenic sprout and ECs of the target vessels form adhesive sites (apical membrane initiation sites) (Betz et al., 2016). Macrophages may act as bridging cells between tip cells for vascular anastomosis downstream of VEGF-mediated tip cell induction (Maden et al., 2009).

When vascular anastomosis occurs, capillary loops are formed. Other capillary sprouts then appear from these loops to form a plexus. It has been suggested that pericytic processes, which seem to bridge the gap between the leading edges of opposing endothelial sprouts, may serve as guiding structures for loop formation (Nehls et al., 1992).

Vessels originating from the venous side of the circulation form vascular networks, which connect then to the arterial side and become functional. Subsequently, remodelling of circulation occurs to adapt to tissue needs (see below).

7.1.4.2. Phase of vascular remodelling and stabilization

We have considered three main phases in angiogenesis for a better understanding of this process, although the phenomena in each phase overlap with the next. Thus, most vascular remodelling and stabilization phenomena initiate in the sprouting phase (see above). Therefore, in this section, we address only the most outstanding phenomena in this final phase of
Fig. 7. Sprouting angiogenesis: vascular anastomosis, remodelling and stabilization. A. Double staining showing CD34+ endothelial cells (brown) anastomosing to (arrow) microvessels (v) with periendothelial αSMA+ mural cells (red). B, C. Ultrastructural images in which regressing vessels with EC degenerative phenomena (B) (arrow) and with intravascular accumulations of platelets (C) are observed. D. Orderly arrangement of phalanx endothelial cells (ec), which show a sessile morphology. Mural cells: mc. Scale bars: A, 15 µm; B-D, 2 µm.
angiogenesis.

7.1.4.2.1. Vascular remodelling. During angiogenesis, a dense network of neovessels is generally created and is followed by a process of remodelling, in which many vessels undergo involution and branching remodelling. In this mechanism, pruning of excessive blood vessels is essential. Two different types of pruning have been described, depending on whether vessels are lumenized or not (Kochhan et al., 2013; Franco et al., 2015; Lenard et al., 2015; Betz et al., 2016). When vessels are lumenized, pruning involves cell-self fusion (Lenard et al., 2015) and formation of a unicellular tube, in which the transcellular lumen collapses (vessel constriction and occlusion), followed by apical membrane retraction and reduction in cell-cell contacts, with EC apoptosis and/or migration. This process is similar to intussusceptive angiogenesis (see below), in which intravascular pillars may form in vessels originated by sprouting angiogenesis (Djonov et al., 2001; Makanya et al., 2005; Hlushchuk et al., 2011). In non-lumenized vessel pruning, cell rearrangements occur with formation of a unicellular bridge, followed by apical compartment separation, a reduction in cell-cell contacts, EC apoptosis and/or migration and detachment of the vessel. In both types of pruning, residual ECs may migrate and return to neighbouring vessels by a reverse sprouting mechanism (Chen et al., 2012; Udan et al., 2013). Elevated levels of oxygen, low VEGF levels, angiopoietin II signals and vessel flow changes play an important role in vascular pruning (Claxton and Fruttiger, 2005; Lange et al., 2009; Hlushchuk et al., 2011). Indeed, pruning involves EC apoptosis (Fig. 7B) (high oxygen level—Lange et al., 2009—and macrophage through WNT7b—Lobov et al., 2005—induced apoptosis), endothelial migration-dependent regression (apoptosis-independent vascular regression) (Dimmeler and Zeiher, 2000; Wietecha et al., 2013) and ‘reverse intussusception’ (intussusceptive vascular pruning) (Hlushchuk et al., 2011) (for review: Ricard and Simons, 2015). Intraluminal flow changes (flow level, orientation and periodicity) also regulate cellular rearrangement during blood vessel pruning and branching remodelling, in which the axial polarity between the Golgi and nucleus of ECs determines polarized EC migration (Le Noble et al., 2004; Lee et al., 2011; Chen et al., 2012; Tirziu et al., 2012; Kochhan et al., 2013; Udan et al., 2013; García and Larina, 2014; Lenard et al., 2015; Franco et al., 2015; Ricard and Simons, 2015) Thus, it has been proposed that EC migration and incorporation into high-flow segments depend on Notch pathway signalling (Phng et al., 2009) and low blood flow, resulting in a smaller total vessel area and an increase in blood flow. Alternatively, EC apoptosis and intussusceptive regression are triggered by low VEGF levels, resulting in lower blood flow and a smaller total vessel area (Ricard and Simons, 2015; Lenard et al., 2015; Franco et al., 2015).

Involutive vessels may also show marked intravascular accumulation of platelets (platelet thrombi) (Fig. 7C). We have hypothesized that this accumulation of factor-releasing platelets during the massive regression of neocapillaries may convert highly vascularized zones (as occurs in granulation tissue) into a ‘paracrine transitional organ’, facilitating fibroblast/myofibroblast proliferation (Díaz-Flores et al., 2009b). Subsequently, homogenized platelets, endothelial cell debris and basement membrane residues are observed in the regions affected by vascular remodelling. This massive regression of capillaries originates capillary-free zones and marked reduction in overall vascular density (Benjamin et al., 1998).

7.1.4.2.2. Vascular maturation and stabilization. Maturation and stabilization of persistent vessels involves maturation regulating molecules, recruitment of pericytes (see above), extracellular matrix formation, and vessel type (arteries, arterioles, capillaries, venules and veins) and organ-specific differentiation. Maturation regulating molecules include angiopoietins and PDGFs (Potente et al., 2011). Pericytes act in vessel stabilization by the release of stabilizing factors including angiopoietin-1 and TMP3 (Suri et al., 1996). Angiopoietin-1 induces DLL4 expression through TIE2-receptor. DLL4 activates Notch signalling which down-regulates VEGF2, thereby preventing further sprouting and inducing the expression of NRARP, which enhances WNT signalling, increasing tight junction stabilization. In addition, pericytes stimulate vascular basal membrane formation (Stratman et al., 2009) (see above). Likewise, pericytes prevent regression by releasing inhibitors of metalloproteinases (e.g., tissue inhibitor of metalloproteinase 3). However, there are apparently conflicting data in the role of pericytes in vessel stabilization (von Tell et al., 2006). Although pericyte coating may prevent vessel regression (Benjamin et al., 1998), pericytes have been observed in immature, mature and involutive microvessels (Díaz-Flores et al., 1991; Inai et al., 2004). Moreover, pericyte-like cells persist in some involutive vessels presenting EC apoptosis.

Concept of phalanx cells

During vessel maturation and stabilization, ECs may adopt a thickly apposed, orderly cobblestone-like appearance, acquiring a more sessile cell fate (Fig. 7D) and acquiescent phenotype, as well as the capacity to sense and regulate perfusion, and to inhibit metastasis in tumours (De Bock et al., 2009; Mazzone et al., 2009). In this stage, these cells are known as endothelial phalanx cells, since their alignment and characteristics resemble those of ancient Greek soldiers forming military phalanxes.

7.2. Intussusceptive angiogenesis

7.2.1. Definition and nomenclature

Intussusceptive angiogenesis is the process by which pre-existing vessels split or remodel through the
formation of transluminal tissue pillars, leading to expansion (intussusceptive microvascular growth), arborisation (intussusceptive arborisation) or branching remodelling (intussusceptive branching remodelling) of the pre-existing vasculature (Caduff et al., 1986; Burri and Tarek, 1990; Patan et al., 1996, 2001b; Augustin, 2001; Djonov et al., 2003; Burri et al., 2004; Makanya et al., 2009; Paku et al., 2011).

Other terms used to describe intussusceptive angiogenesis are intussusception/intussusceptual angiogenesis (Caduff et al., 1986), non-sprouting angiogenesis, splitting angiogenesis, inverse sprouting angiogenesis (Paku et al., 2011), intraluminal or internal splitting angiogenesis (Zhou et al., 1998; Williams et al., 2006b) and longitudinal splitting or longitudinal division angiogenesis (Egginton, 2009). In addition, and depending on the final results of the process, the aforementioned terms, intussusceptive microvascular growth, intussusceptive arborisation and intussusceptive branching, are also specifically used for the three main forms of intussusceptive angiogenesis.

7.2.2. Incidence

Intussusceptive angiogenesis may occur in developing tissues and in several pathologic processes, including tumours. Thus, intussusceptive angiogenesis has been described during development of lung, kidney, ovary, retina, bone and skeletal muscle, among other tissues and organs (Macchiarelli et al., 2006; Andres and Djonov, 2010; De Spieghelaere et al., 2010, 2012), and experimentally in hypoxic and inflammatory processes (Konerding et al., 2010), as well as in liver cirrhosis (Van Steenkiste et al., 2010). Several tumours also show intussusceptive angiogenesis, including colon, renal and mammary carcinomas, gliomas and lymphomas (Patan et al., 1996; Djonov et al., 2001; Nico et al., 2010; Ribatti and Djonov, 2012).

7.2.3. Events in intussusceptive angiogenesis

The events in intussusceptive angiogenesis mainly refer to pillar formation, in which four successive phases have been described (Burri and Tarek, 1990; Patan et al., 1997; Burri and Djonov, 2002; Djonov et al., 2000, 2003; Egginton et al., 2009; Styp-Rekowska et al., 2011; Paku et al., 2011): 1) intraluminal endothelial pillar formation (trans-capillary inter-endothelial bridge) from endothelial cells of the opposite sites of a vessel wall (contact between opposite capillary walls) (Fig. 8A), 2) reorganization of the junctions between the pillar endothelial cells, which increase in size, flatten and adopt a bilayer arrangement with a central virtual core, 3) central core perforation and invasion by pericyte and myofibroblast extensions with extracellular matrix formation, including collagen fibres (Fig. 8B), and 4) increased size of the pillar, fusion with other pillars and splitting of the vessel lumen. Throughout this process, vessel permeability and the basement membrane remain unmodified. Intussusceptive angiogenesis phenomena may occur together with sprouting angiogenesis, predominantly in the later stages of growth and remodelling (Makanya et al., 2005; Macchiarelli et al., 2006).

Paku et al. (2011) have followed the mechanisms for pillar formation during tumour-induced intussusceptive angiogenesis, observing suction and subsequent transport of perivascular collagen bundles into the vessel lumen. For this mechanism, the authors coined the term “inverse sprouting”.

Taking into account the three main forms of intussusceptive angiogenesis (intussusceptive microvascular growth, intussusceptive arborisation and intussusceptive branching remodelling), in intussusceptive microvascular growth, expansion of the microvasculature occurs through the development of numerous pillars, which fuse and split the pre-existing vessels (Makanya et al., 2009; Mentzer and Konerding, 2014). In intussusceptive arborisation, series of pillars participate in vascular branch angles (Ackermann et al., 2014). Finally, in intussusceptive branching remodelling, two forms may optimize the vascularization: branching pattern or branching pruning (see above). Changes in blood flow and in metabolic needs occur in all types of intussusceptive angiogenesis (Djonov et al., 2003).

Experimentally, we demonstrated that perivenous application of PGE2 and glycerol originates intense vascularization of the wall of the rat femoral vein from its intimal endothelial cells (Díaz-Flores et al., 1994b; 2011a; 2017). In these conditions, the intraparietal microvessels interconnect, encircle and separate components of the vein wall, giving rise to intravenous projections, in which the EC cover is formed from outgrowing ECs and the core by the incarcerated components of the vein wall, including pericyte-like cells (Díaz-Flores et al., 1994b, 2011a, 2017). The results resemble the pillars/folds formed by intussusceptive angiogenesis in the vein of the ovarian pedicle after ovariectomy or tumour implantation (Patan, 2000, 2001a,b).

7.2.4. Mechanisms

Several molecules and blood flow participate in the regulation of intussusceptive angiogenesis. Variable expression of VEGF and VEGF receptors (VEGFR1 and VEGFR2) may condition sprouting or intussusceptive angiogenesis (Makanya et al., 2005; De Spieghelaere et al., 2010; Hlushchuk et al., 2011; Gianni-Barrera et al., 2013). Hypoxia-inducible factor 2 alpha, angiopoietin 1 and 2, FGF (fibroblast growth factor 2), and platelet-derived growth factor beta (PDGF beta) also act in intussusceptive angiogenesis regulation (Patan, 1998; Augustin et al., 2001; Kurz et al., 2003; Makanya et al., 2005, 2007; Taylor et al., 2010). Blood flow changes (e.g. shear stress and/or cyclic stretch) play an important role in vascular plexus remodelling and differentiation of venules and arterioles (Djonov et al., 2002; Le Noble et
Fig. 8. Intussusceptive, mimicry and co-option forms of vascularization. A. In double staining with anti-CD34 (staining ECs: brown) and anti-αSMA (staining pericytes: red), an endothelial pillar (transcapillary interendothelial bridge) is observed (arrow). B, insert. Ultrastructural images of cross-sectioned pillars (arrows), covered by endothelial cells (ec), and with central core formation (presence of collagen fibres (co)). C, channels containing red cells and lined with melanotic cells and some melanophages (vascular mimicry) in a melanoma. D, observe connection between endothelial-lined vasculature (elv) and a channel lined with melanotic cells (clm), and with red cells in its lumen. E, melanotic cells forming cuff around vessels (arrows) are observed in a melanoma (co-option form of vascularization). Scale bars: A, C-E, 25 µm; B, 1 µm.
Angiogenesis and related secondary structures

7.3. Postnatal angiogenesis with endothelial precursor cell participation (postnatal vasculogenesis by EC precursors – EPCs)

7.3.1. Concept and incidence

Angiogenesis with participation of endothelial precursor cells (EPCs) can be defined as the process by which these precursor cells enter the circulation from their niches (bone marrow, vessel wall ECs, cord blood), incorporate into the pre-existing vasculature, and differentiate into mature ECs, contributing to the newly formed vasculature.

The contribution of EPCs in angiogenesis is difficult to establish, and the intensity of their participation is controversial, mainly in tumours (Kim et al., 2003; Larrivee et al., 2005).

7.3.2. Characteristics of EPCs

As the name suggests, EPCs are precursor cells capable of differentiating into mature ECs and may act in vasculogenesis and angiogenesis (pre- and postnatal angiogenesis, also considered postnatal vasculogenesis). In successive works, the characteristics of EPCs were established mainly by the expression of specific markers (Asahara et al., 1997; Shi et al., 1998; Gehling et al., 2000; Peichev et al., 2000). Moreover, as these cells mature, they lose some markers and acquire others. Thus, expression in the cells of CD34+, AC133+ and VEGFR2+ has been considered representative of a highly proliferative EPC population, whereas CD34+, AC133-, VEGFR2+ cells represent more mature circulatory EPCs (Asahara et al., 1997, Shi et al., 1998; Gehling et al., 2000; Peichev et al., 2000). In more mature EPCs, other markers may be observed, including CD31, CD144 (VE-cadherin), CD62E (E-selectin) and CD184 (chemokine receptor CXCR-4).

7.3.3. Events and mechanisms in angiogenesis with EPC participation

The principal events in angiogenesis in which EPCs participate are as follows: a) EPC release from the bone marrow niche, and incorporation into the blood (circulating EPCs), b) recruitment and integration within angiogenic vasculature (incorporation into the vascular endothelium of EPCs) and c) differentiation into mature ECs.

7.3.3.1. Release of EPCs and incorporation into the blood

This phase includes detachment of c-kit positive cells from their niches, movement of EPCs to the vascular zone and incorporation in the circulation (Heissig et al., 2002). In general, several factors, including VEGF, participate in the release and mobilization of EPCs from their niches (Asahara et al., 1999). Factors other than the VEGF involved in release and mobilization of EPCs include SDF-1 (stromal derived-1) through CXR-4, FGF, osteopontin, PLGF (placental growth factor), angiopoietin-1 and macrophage colony stimulating-factor (Takahashi et al., 1999; Moore et al., 2001). Moreover, VEGF and SDF-1 promote MMP9/endothelial nitric oxidase synthase-mediated release of membrane-bound kid ligand (transformation to a soluble form).

7.3.3.2. Recruitment and integration of EPCs within angiogenic vasculature

When EPCs reach sites of injury, they participate in the restoration of vascular integrity and angiogenesis. Thus, it has been demonstrated that EPCs can integrate into blood vessels of neovascularized ischemic limbs in different experimental models (Asahara et al., Kalka et al., 2000). HMGB1 (high-mobility group box 1) activates integrin-dependent homing of EPCs (Chavakis et al., 2007), and P-selectin and E-selectin also act in the adhesion of EPCs (Vajkoczy et al., 2003). Using a parabiosis model, EPC location was demonstrated at sites of intussusceptive angiogenesis (Chamoto et al., 2013).

7.3.3.3. Differentiation of EPCs into mature EPCs

When EPCs in blood vessels lose their progenitor characteristics, they acquire those of mature ECs, including expression of EC markers, such as endothelial nitric oxide synthase, von Willebrand factor and VE-cadherin (Lin et al., 2000). VEGF participates in this differentiation of EPCs (Gehling et al., 2000).

7.4. Vasculogenic mimicry (vascular mimicry)

7.4.1. Concept

Vasculogenic mimicry or vascular mimicry is the generation of perfusable, non-endothelial-lined tube-like or channel structures, which are generally formed by: a) highly plastic migratory/invasive tumour cells that upregulate endothelial-selective markers, and b) remodelled PAS+ extracellular matrix on the inner wall of the tube-like structures (Maniotis et al., 1999; Paulis et al., 2010). The network formed by these structures is connected with or adjacent to blood vessels, transporting blood or fluid from connecting or adjacent leaky vessels, respectively (Maniotis et al., 1999; Paulis et al., 2010). Therefore, the networks of channels originated by vascular mimicry may form part of the complex vasculature in certain tumours, in which all the types of angiogenesis described here participate (sprouting and intussusceptive angiogenesis, postnatal vasculogenesis by precursor ECs, vessel co-option and vascular mimicry) (Döme et al., 2007; Hillen and Griffioen, 2007; Filipovic et al., 2009; Tsuda et al., 2009; Lee et al., 2010, 2011; Styp-Rekowska et al., 2011).
Carmeliet and Jain, 2011). This complex vasculature explains the difficulty of disrupting tumour growth using anti-angiogenic therapy (Dey et al., 2015), since the loss of one type of angiogenesis may be compensated by other types (e.g. resistance to anti-VEGF therapy in glioblastoma multiforme - Soda et al., 2013).

7.4.2. Incidence

Vascular mimicry formed by tumour cells has been described in several types of aggressive tumours. Likewise, non-endothelial or tumoral cells may also participate in vascular mimicry, including fetal cells of the trophoblastic lineage in the hemochorial maternal vascular spaces (Rai and Cross, 2014) and macrophages (Barnett et al., 2016) (see other forms of vascular mimicry). Most vascular mimicry studies have been carried out on highly invasive melanoma (Fig. 8C,D), in vivo and in vitro. In addition to melanoma, vascular mimicry has been observed in several neoplastic processes, such as carcinomas of the lung, breast, ovary, skin, kidney, prostate, liver and bladder, gliomas (above all in glioblastomas), and sarcomas, including synovial sarcoma, alveolar rhabdomyosarcoma, osteosarcoma, mesothelioma and Ewing tumour (Maniotis et al., 1999; Paulis et al., 2010).

7.4.3. Events, and characteristics of tumour cells and extracellular matrix forming vascular channels

The most important findings in vascular channels are those related to tumour cells (Fig. 8C) and extracellular matrix. Tumour cells involved in vascular mimicry exhibit high plasticity and down-regulation of the original phenotype markers, showing some characteristics of embryonic progenitors and ECs (Bittner et al., 2000; Seftor et al., 2002; Zhang et al., 2003). Moreover, these tumour cells have anti-coagulant properties and act in matrix remodelling, providing a perfusion pathway. Thus, in melanoma, the tumour cells that form vascular channels show down-regulation of genes related to a melanocytic phenotype (e.g. tyrosinase and melan A) (Demou and Hendrix, 2008), while expression of endothelium-associated genes occurs (e.g. CD34, CD105, neuropilin 1, E-selectin, VE-cadherin and tyrosine-kinase receptor 1). Extracellular matrix in the networks of tubular structures shows PAS positivity and presence of collagen IV and VI, laminin, heparan-sulphate proteoglycans and other components.

7.4.4. Mechanisms

Signalling pathways in vascular mimicry are highly complex (for revision: Seftor et al., 2012). Briefly, hypoxia participates in tumour cell plasticity, contributing to the phenotype switch of tumour cells (Mihic-Probst et al., 2012). VE-cadherin and EphA2, P13K (phosphoinositide 3 – kinase) and FAK (focal adhesion kinase) play an important role in vascular mimicry (Hendrix et al., 2001; Hess et al., 2001, 2003, 2006a,b).

7.4.5. Functionality

Vascular mimicry acts as a complementary perfusion pathway of the tumours, facilitates metastasis (Hendrix et al., 2003) and is important for the evaluation of clinical tumour outcome (poor clinical outcome) (Cao et al., 2013). As mentioned above, perfusion in vascular mimicry may occur through interconnecting channel structures and endothelial-lined vasculature (Fig. 8D) or through the transportation of fluid from adjacent leaking vessels.

7.4.6. Other forms of vascular mimicry by non-tumour cells

In both tumours and angiogenesis in in vivo models, it has been demonstrated that macrophages may form primitive non-endothelial, functionally connected channels (Barnett et al., 2016). Hypoxia inducible factors play an important role in the formation of these channels (Barnett et al., 2016).

7.5. Vessel co-option

7.5.1. Concept

Vessel co-option is the process by which tumour cells grow using pre-existing vessels (tumour cells co-opt host vessels). Therefore, this process is not a true angiogenic mechanism, since the neoplastic cells grow over and along vessels (tumour cells parasitize on the pre-existing vasculature).

7.5.2. Incidence

Tumours in well-vascularized organs, such as the lung and brain, can be a substrate for this type of growth (Wesseling et al., 1994; Pezzella et al., 1997), mainly during initial tumour phases. This process has been described in glioblastoma, melanoma, Kaposi sarcoma, and in a model of neuroblastoma, non-small cell carcinoma, Lewis lung carcinoma and murine ovarian cancer (Pezzella et al., 1997; Holash et al., 1999; Döme et al., 2002; Kim et al., 2002; Zhang et al., 2003). A similar mechanism has been suggested for myofibroblasts during wound healing (Kilarski et al., 2009), although this finding may be the expression of differentiating precursor adventitial cells (Díaz-Flores et al., 2014). We have observed vessel co-option in melanoma implanted in mice, particularly following prior radiation of the tumour bed before implantation, and in human glioblastoma multiforme. In the latter, the growing tumour cells emit numerous processes around the pre-existing vessels, which follow an undulating route. Subsequently, vessel regression is observed. In this case, cell projections form what is described as palisade necrosis (see below).
7.5.3. Events and mechanisms in vessel co-option

Tumours with vessel co-option show viable tumour cells forming cuffs around the co-opted vessels (Fig. 8E). In some of these tumours, perivascular cuffs appear between necrotic areas. An important finding is the involution of the pre-existing co-opted vessels (Holash et al., 1999; Kim et al., 2002; Zhang et al., 2003), in which loss of pericytes and smooth muscle cells occurs with regression of the ECs. The balance of angiopoietin II and VEGF is involved in the regression of the co-opted vessels. Angiopoietin II and VEGF expression facilitate vessel sprouting, while angiopoietin II expression in the absence or marked decrease of VEGF induces vessel regression (Holash et al., 1999; Kim et al., 2002). These regressive phenomena have been considered as a host defence mechanism (Hillen and Griffioen, 2007) and may lead to the angiogenic formation of secondary structures (see below).

7.6. Piecemeal angiogenesis

7.6.1. Concept

Piecemeal angiogenesis is a mechanism by which a variable number of intravascular papillary structures form in tumours, pseudo-tumours, reactive processes, and malformations of the blood and lymphatic vessels, and in the intravascular incorporation of different tissue components, including neoplastic cells. Through this type of angiogenic mechanism, ECs grow from a pre-existing vessel, and encircle and split up portions of the wall of the vessel itself, perivascular tissues and/or fibrin components, giving rise to the intravascular papillae partially connected to the vessel wall (Díaz-Flores et al., 2016d, 2017). In these intravascular papillae, ECs form the cover and encircled components the core.

This piecemeal angiogenic mechanism has also been demonstrated experimentally and is of interest to explain several forms of angiogenic-related intravascular structures. In this section, we consider the experimental studies, while the angiogenic-related structures originated by this process will be outlined in the section “Participation of angiogenesis in formation of secondary structures during postnatal life’’.

7.6.2. Events and mechanisms in experimental piecemeal angiogenesis

Experimentally, a large number of vessel sprouts and intravascular papillae have been demonstrated in the rat femoral vein wall after PGE2 and glycerol injection into the soft tissue surrounding the vein (Díaz-Flores et al., 1994b, 2011a, 2017). Vascular sprouts emerge from exuberant EC growth in the intima of the vein, forming conspicuous microvessel networks in the media layer and papillae with the following sequence: a) activation of vein ECs, b) migration of intimal ECs through gaps in the discontinuous internal elastic lamina, originating numerous vascular sprouts in the media layer, where they are surrounded by modified SMCs, which adopt pericytic characteristics, c) interconnection between vascular sprouts (anastomosing channels) in the vein wall forming microvessel networks, in which their slit-like or more apparent lumens connect to the vein lumen, d) presence of papillary structures (Fig. 9A), whose endothelial cover is formed from one side of the pairs of migrating ECs in the innermost networks of the vein wall, and whose cores are formed from stromal wall components of the original vessel (extracellular matrix, modified SMCs and/or other microvessels).

Taking into account the above, the anastomosing vascular channels that penetrate the vessel wall and perivascular structures remain connected to the vessel lumen, form networks encircling components of the vein wall or perivascular structures and give rise to apparently free papillae. Therefore, in the papillae, the EC cover is formed of ECs from these anastomosing vascular channels, and the core is formed by the incarcerated components of the vein wall and perivascular structures. These components may vary depending on the depth of microvesSEL penetration in the vessel wall (penetration is scant when papillae with little stromal support are formed). Proliferating endothelial cells may even fold onto themselves, originating tuft-like structures, or onto thrombotic fibrin components. This pathogenic mechanism of vascular network and papillae formation combines the sprouting and intussusceptive mode of angiogenesis (Díaz-Flores et al., 1994b; Patan, 2000; Patan et al., 2001a; Burri et al., 2004; Ackermann et al., 2014) with development of loops in the vein wall (which may occur on the venous side in vessels of all sizes).

8. Participation of angiogenesis in formation of secondary structures during postnatal life

Angiogenesis may participate in the formation of postnatal angiogenesis-related secondary structures, including: a) intravascular structures through piecemeal angiogenesis, such as intravascular papillae in vessel tumours and pseudotumours, vascular septa in hemorrhoidal veins and intravascular projections in some tumours, b) arterial intimal thickening, c) intravascular tumours and pseudotumours, d) vascular glomeruloid proliferations, and e) pseudo-palisading necrosis. In this review we do not include the participation of angiogenesis in postnatal formation of granulation tissue (a provisional tissue during repair) and mature mesenchymal tissues (connective, bone, cartilage and adipose), nor the widely studied pericyte and CD34+ stromal cell plasticity.

8.1. Angiogenesis in the formation of intravascular structures through piecemeal angiogenesis

A piecemeal form of angiogenesis (see above) plays an important role in the formation of several intravascular structures, including intravascular papillae in vessel tumours and pseudotumours, vascular septa in
Fig. 9. Piecemeal angiogenesis in experimental conditions and in the formation of angiogenesis-related secondary structures (papillae) in vessel tumours and pseudotumours. A, semithin section showing intravascular papillae (arrows) after PGE2 and glycerol administration around the rat femoral vein. B, intravascular papillary endothelial hyperplasia. Numerous papillae are observed in a section stained with anti-CD34 (staining ECs: brown) and anti-αSMA (staining mural cells: red). C, numerous papillae are also observed in a cavernous hemangioma. Note the wall of a vessel with endothelial (brown) and mural (red) cells (arrow), as well as the papillae in the lumen. D-F, Papillae in a cavernous lymphangioma. Sections stained with podoplanin show a linear arrangement of papillae, some of which are joined by ECs (F, arrow). Scale bars: A, 25 µm; B, C, 15 µm; D, 35 µm; E, F, 7 µm.
hemorrhoidal veins and intravascular projections of some tumours (e.g. in minimally invasive follicular carcinoma of thyroid).

8.1.1. Intravascular papillae in vessel tumours and pseudotumours as a piecemeal form of angiogenesis

A piecemeal form of angiogenesis that originates intravascular papillae occurs in several types of vessel tumours and pseudotumours, and has been described in experimental conditions (Díaz-Flores et al., 1994b, 2011a, 2016d, 2017). By this procedure, growing ECs encircle and separate components of the vessel wall, perivascular tissue and/or fibrin, giving rise to intravascular structures, partially connected to the vessel wall (Díaz-Flores et al., 1994; 2011a, 2016d, 2017).

Examples of vascular pseudotumours, and benign and malignant vascular tumours with intravascular papillae include intravascular papillary endothelial hyperplasia (IPEH), vascular transformation of the sinuses in lymph nodes, papillary intralymphatic angioendothelioma (PILA/Dabska tumour), retiform hemangioendothelioma, hemangiosarcoma and lymphangiosarcoma (Clearkin and Enzinger, 1976; Kuo et al., 1976; Hashimoto et al., 1983; Sanz-Trelles et al., 1997; Bhatia et al., 2006; Neves et al., 2011; Li et al., 2013; Mota et al., 2013; Kugler et al., 2016; Liu et al., 2015; Emberger et al., 2009; Kuo et al., 2015; Díaz-Flores et al., 2016d). In IPEH (Díaz-Flores et al., 2016d) and in other lesions, papillae, which may be numerous (myriad of papillae), show a cover formed by ECs and a connective or fibrinous core (Fig. 9B-F), as occurs in experimental conditions (perivenous administration of PGE2 and glycerol) (Díaz-Flores et al., 1994b, 2011a, 2017) (see above). However, there are differences in the number of papillae, as well as variations in papillary size, distribution, arrangement, EC cover and core components (Fig. 9B-F). The resulting papillae are similar to folds and pillars described in the ovarian pedicle after ovariectomy or tumour implantation (Patan, 2001a,b), and in florid intussusceptive-like microvascular disangiogenesis of bronchopulmonary dysplasia (De Paepe et al., 2017).

Therefore, these intravascular projections in vessel tumours and pseudotumours (papillae for pathologists), and in intussusceptive angiogenesis (folds and pillars), present a similar structure, supporting the hypothesis of a piecemeal angiogenic mechanism in papillary formation in the experimental and pathological conditions, with association of sprouting and intussusceptive types of angiogenesis (Díaz-Flores et al., 2016d, 2017).

8.1.2. Compartmentalization of hemorrhoidal veins

Closely associated with intravascular papillae is the compartmentalization of hemorrhoidal veins. In the latter, successive series of secondary papillae may be arranged in linear fashion, forming several septa with compartmentalization of the vein lumen (Fig. 10A,B) (unpublished observations).

8.1.3. Piecemeal angiogenesis in vascular invasion/pseudo-invasion.

In some tumours, angioinvasion/pseudo-angioinvasion may be based on a piecemeal mechanism of angiogenesis. For example, in minimally invasive follicular thyroid carcinoma (angioinvasive encapsulated carcinoma), the invasion of small to medium vessels within or immediately adjacent to the tumour capsule may also be related to the formation of intravascular structures. Indeed, ECs grow out of the vessel walls and encircle tumour glands following a piecemeal mechanism (Fig. 10C) (see above). In these conditions, the glands remain in the core of the newly formed peculiar papillae and therefore isolated from the circulation. This mechanism may explain how vascular invasion evolves with infrequent metastasis and requires further studies.

8.2. Arterial intimal thickening

In several forms of intimal thickening (e.g. arterial segments isolated between ligatures), an intense intraarterial neovascularization originates from the vasa-vasorum, predominantly in the early phase of intimal thickening development (Díaz-Flores and Domínguez, 1985). We have demonstrated this penetration of microvessels through the arterial wall by injecting contrast, and subsequent tissue clearance and observations in HE stained sections (Fig. 11A,B). Indeed, in occluded arterial segments, the following occurs: a) early capillary ingrowth through the adventitia and tunica media, b) development of numerous microvessels in the occluded arterial lumen, originating a granulation-like tissue, c) coalescence of the lumen of microvessels, forming an axial arterial neolumen, d) involution of most microvessels and persistence of interstitial cells, especially those with a pericytic aspect, which acquire characteristics of intimal cells, and e) presence of intimal thickening and a patent lumen in the occluded segment connected to some perforating vessels (Díaz-Flores and Domínguez et al., 1985, Díaz-Flores et al., 1990).

8.3. Formation of intravascular tumours and pseudotumours

Intravascular tumours and pseudotumours may be related to angiogenesis. For example, in intravenous pyogenic granuloma and intravascular myopericytoma. In intravenous pyogenic granuloma, numerous microvessels with prominent ECs and pericytes form the intravascular lesion (Fig. 11C,D). In myopericytoma, as
Fig. 10. Piecemeal angiogenesis in compartmentalization of hemorrhoidal veins and in vascular invasion/pseudo-invasion. A, B. Images of hemorrhoidal veins in which successive series of secondary papillae, arranged in a linear fashion (arrow) (A), form several septa (arrows) with compartmentalization of the vein lumen (B). C. Minimally invasive follicular thyroid carcinoma. Detail of an intravascular tumour gland (asterisk), encircled by CD34+ endothelium, as occurs in papillary structures. Note a gland (double asterisk) partially encircled by CD34+ endothelial cells, which originate from the vessel wall. A and B: double staining with anti-CD34 (brown) and anti-αSMA (red). C: staining with CD34 (brown). Scale bars: A, 15 µm; B, 40 µm; C, 12 µm.
Fig. 11. Angiogenesis in intimal thickening formation and in intravascular tumours. 

A, B. In early phases of intimal thickening development in occluded arteries, microvessel ingrowth in the arterial lumen from the vasa vasorum is demonstrated by injecting contrast after tissue clearance (A) and in an HE stained section (B). Observe in B microvessels crossing the artery wall (aw), reaching the intimal thickening (it).

C, D. Intravascular pyogenic granuloma with numerous microvessels similar to those of granulation tissue. Double staining with anti-CD34 (staining ECs - brown) and anti-αSMA (staining mural cells/pericytes - red).

Scale bars: A, 0.5 mm; B, 40 µm; C, 0.1 mm; D, 35 µm.
occurs with arterial intimal thickening, intravascular neovascularization plays an important role, and these lesions are therefore related (Diaz-Flores et al., 2011c, 2012b). This hypothesis is supported by the following: a) frequent participation of intimal thickening as a myopericytoma component, b) phenotypic similarities between the myoid cells (myopericytes) of the tumour and the neointimal (myointimal) cells of the concomitant intimal thickening, and c) possibility of a common cell origin for both lesions and of an angiogenic pattern in their initial stages (Diaz-Flores et al., 2011c, 2012b).

8.4. Glomeruloid vascular proliferations

8.4.1. Definition

Glomeruloid vascular proliferations or vascular glomeruloid bodies are structures that resemble the renal glomeruli and are formed by closely packed anastomosing capillaries, which show irregular and tortuous narrow lumina, prominent ECs and pericytes.

8.4.2. Incidence

Vascular glomeruloid bodies have been described in vascular tumours (frequently associated with POEMS syndrome – Forman et al., 2007; Yuri et al., 2008; Gonzalez-Guerra et al., 2009) and malformations of the skin, in glioblastoma multiforme (Fig. 12A,B) (Rojiani and Dorovini-Zis, 1996), and in other tumours, including breast, lung, endometrium, prostate and meningeal tumours (Ohtani 1992; Bläker et al., 1999; Straume et al., 2002; Goffin et al., 2003; Kandemir et al., 2014), as well as in experimental conditions (e.g. after VPF/VEGF-164 gene delivery) (Sundberg et al., 2001).

8.4.3. Characteristics

In glomeruloid bodies, florid microvascular proliferation, with packed anastomosing capillaries, shows prominent ECs, which express CD31 and CD34 (Fig. 12A). These ECs present weak VEGF reactivity and a Ki-67 proliferation index above 10% (Kandemir et al., 2014). In addition, the pericyte population, with αSMA expression, is relatively important in vessel walls of glomeruloid structures (Fig. 12B).

8.4.4. Mechanisms and sequence of formation and prognostic importance

Experimentally, it has been demonstrated that VPF-VEGF-164 is sufficient for induction of glomeruloid bodies. Likewise, VPF-VEGF-164 is also necessary for maintaining these vascular bodies (Sundberg et al., 2001). The sequence of glomeruloid body formation is as follows: a) vessel dilation and focal accumulation in the vessel EC layer of plump primitive cells, which show EC markers, increased expression of VGFR-2 and lack of pericyte markers; b) the cells in the accumulations proliferate and extend toward the vessel lumen and the extravascular tissue; c) incorporation of proliferating pericytes, which intermingle with the ECs; d) formation of a multilayered basement membrane between ECs and pericytes; e) partial occlusion of the mother vessel lumen and formation of small channels in the developing glomeruloid structures and f) development of microvessels with erythrocytes containing lumens, originating characteristic glomeruloid bodies. In glioblastoma multiforme, we have observed (non-published observations) that several glomeruloid microvascular structures may appear along a pre-existing vessel, adopting an arciform distribution. The presence of glomeruloid microvascular proliferation indicates an aggressive angiogenic phenotype in human cancers and is therefore an unfavourable biological marker (Straume et al., 2002). However, in some conditions, glomeruloid structures have been considered as reactive vascular proliferations of undetermined biological importance (Kandemir et al., 2014).

8.5. Angiogenesis in the formation of pseudopalisading necrosis

Two important structures in glioblastomas are related to angiogenesis: pseudopalisading necrosis and glomeruloid vascular structures described above.

In glioblastoma, two main types of necrosis can be observed: pseudopalisading necrosis and large necrosis. Pseudopalisading necrosis in glioblastoma is formed by a garland-like arrangement of tumour cells (rim of cells) around a central degenerative region (Fig. 12C). The resulting pseudopalisades can be long and undulating or small and rosette-like. This pseudopalisading configuration of astrocytic cells is essentially unique for glioblastoma. The hypothesis of a mixed population of neoplastic and inflammatory cells to explain the pseudopalisades is not sustainable, since practically all cells in this location are neoplastic astrocytic cells. Currently, several authors are of the opinion that pseudopalisades could represent an astrocytic cell population that rapidly migrates away from a dysfunctional vasculature (from a hypoxic zone) (Brat et al., 2004; Rong et al., 2006). In pseudopalisades, we have observed that neoplastic astrocytic cells emit numerous processes toward an involutive vessel in the axis of the degenerative central zone. These processes are positive for anti-gliofibrillar acid protein (Fig. 12D and E) and therefore form most of the degenerative central zone, in which debris of CD34+ ECs of the involutive vessel are also observed (Fig. 12F). Loss of pericytes or vascular smooth muscle cells occurs in these vessels. Normally, astrocyte processes envelop synapses, make contact with nodes of Ranvier, form gap junctions between distal processes of other astrocytes and establish interactions with blood vessels, where they form astrocytic endfeet. Our results (obtained in conjunction with Spreafico MA†, nonpublished observations) agree with the concept that pseudopalisades are formed by a mechanism in which neoplastic astrocytes grow over and along a vessel, which
Fig. 12. Angiogenesis in glomeruloid vascular proliferations and in pseudopalisading necrosis formation. A, B. Glomeruloid bodies with florid microvascular proliferation, showing prominent CD34+ ECs (A) and an abundant pericyte population (B). C-F. Pseudopalisading necrosis in glioblastoma formed by a garland-like arrangement of tumour cells (rim of cells) around a central degenerative zone (cz), observed in HE (C), anti-protein gliofibrillar acid (D, E) and in anti-CD34 stained sections. Note that the central degenerative region is formed by the gliofibrillar acid + projections of the neoplastic astrocytic cells (D, E), which converge in a central vessel (E, arrow). F. Degenerative phenomena (residual expression of CD34) (arrow) in a central vessel of the pseudopalisading necrosis. Scale bars: A, 20 µm; B, 15 µm; C-E, 50 µm; F, 40 µm.
shows involutive phenomena. Therefore, our interpretation of the findings is that mumified or partially disintegrated processes of the astrocytes form the degenerative central zone around vessels (Fig. 12D,E). This concept may also justify the absence of these specific pseudopilisades in tumours other than glioblastomas. The explanation of the involution of the central vessel may be similar to that in vessel co-option (see above).

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