Chapter 2

Role of Notch, SDF-1 and Mononuclear Cells Recruitment in Angiogenesis

Ivanka Dimova and Valentin Djonov

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66761

Abstract

Intussusceptive angiogenesis (IA) known also as splitting angiogenesis is a recently described mechanism of vascular growth alternative to sprouting. It plays an essential role in the vascular remodeling and adaptation of vessels during normal and pathological angiogenesis. It is an "escape" mechanism during and after irradiation and anti-VEGF therapy, both inducing angiogenic switch from sprouting to IA by formation of multiple transluminal tissue pillars. Our recently published data revealed the significant induction of IA after inhibition of Notch signaling associated with an increased capillary density by more than 50%. The induced IA was accompanied by detachment of pericytes from basement membrane, increased vessel permeability and recruitment of mononuclear cells toward the pillars; the process was dramatically enhanced after injection of bone marrow-derived mononuclear cells. The extravasation of mononuclear cells with eventual bone marrow origin was associated with upregulation of chemotaxis factors SDF-1 and CXCR4. In addition, SDF-1 expression was upregulated in the endothelium of liver sinusoids in Notch1 knockout mouse, together with vascular remodeling by intussusception. In this chapter, we discuss this important mechanism of angiogenesis, as well as the role of Notch signaling, SDF-1 signaling and mononuclear cells in the complex process of angiogenesis.

Keywords: intussusceptive angiogenesis, Notch signaling, mononuclear cells, SDF-1/CXCR4 signaling

1. Introduction: intussusceptive angiogenesis

Angiogenesis is essential for normal embryonic development and reproductive cycle and plays a key role in pathological conditions such as tumor growth and ischemic cardiovascular diseases. This is a complex process involving essential signaling pathways for instance VEGF, bFGF, and Notch, etc., in vasculature, as well as additional players such as bone marrow-

derived endothelial progenitor cells. Better understanding the role of the different pathways and the crosstalk between different cells during angiogenesis is a crucial factor for developing more effective proangiogenic and antiangiogenic anticancer therapy.

Angiogenesis involves the formation of new blood vessels from a preexisting vascular plexus, and based on morphological characteristics two main distinct processes have been identified, sprouting and intussusceptive angiogenesis (IA) [1–3]. Sprouting angiogenesis has been well described since more than 150 years. Recent publications indicated that sprouting involves tip/stalk cell differentiation and crosstalk process which is tightly controlled by the VEGF and Notch/Dll4 signaling pathway [4, 5]. Intussusceptive angiogenesis (IA) is a particular form of vascular growth and remodeling in which endothelial cells make invagination intraluminally instead of extraluminally like it is in the sprouting. The cells form protrusions toward the vessel lumen resulting in the appearance of transluminal endothelial pillars–the hallmarks of intussusception. The arising pillars afterward are successively reshaped and fused and lead to "splitting" of the preexisting vessel in two segments, thus doing remodeling and organization of the vasculature. The effect is the formation of hierarchically organized vessels with supplying and draining function, pruning of arteries, and veins, and finally development of the primitive capillary plexuses into the functional vascular system. IA is a process with several consecutive steps, including intussusceptive microvascular growth, intussusceptive arborization, and intussusceptive remodeling [2, 3, 6, 7]. These processes end in the formation of mature vascular networks. In comparison to sprouting angiogenesis, intussusception is a quite fast process, enabling the vascular system to swift adaptation in unfavorable conditions. IA has been identified as the leading mode of vascular growth in animal models of liver regeneration and in alveolar angiogenesis following pneumonectomy. Intussusception appears to be predominant above sprouting in extra-embryonic vasculatures including the vitelline circulation as well [8-10]. The intensive work of our group in the past few decades clearly documented the morphological features of this specific angiogenic mode and demonstrated its definite presence during development and tumorigenesis as a complementary to sprouting vessel growth. Surprisingly, the cellular and molecular regulation of intussusception is less well known but recent evidence suggests that it might involve a component regulated by blood flow and Notch signaling [7, 9]. We provided with evidence showing that Notch regulates intussusception involving interaction with circulating mononuclear cells in developing vascular networks [11].

Intussusceptive angiogenesis (IA) is a well-documented and widely spread mode of angiogenesis, occurring during both normal development and in pathological conditions. In contrast to sprouting angiogenesis, whereby abluminal sprouts outgrow and subsequently merge with the existing capillaries, intussusceptive angiogenesis is elaborated by intraluminal growth of endothelial cell processes. The last protrude from the opposing sides of the vessel wall and form transluminal tissue pillar, representing endothelial bilayer, which is afterward perforated and stabilized from outside by collagen bindles. Repetitive formation of pillars and their subsequent fusion leads to the splitting of vessels and vascular expansion. Another way of intussusceptive angiogenesis is to increase the size of the pillar and form meshes, thus splitting the vessel. Intussusceptive angiogenesis is a process linked to both blood vessel replication and remodeling in development. It is present within the regions of increased vascular density in

alveolar angiogenesis during compensatory growth after pneumonectomy in a murine model of postpneumonectomy lung growth [8]. The remodeling of the retiform meshworks in the avian lung was essentially accomplished by intussusceptive angiogenesis as well [12].

In addition to its developmental role, intussusceptive angiogenesis is well documented as a mechanism of vascular adaptation in response to different environmental stimuli. In the adult mouse retina, it was reported as a main adaptive mechanism to chronic systemic hypoxia [13]. These investigations contribute to our understanding of hypoxia-induced angiogenesis and microvascular remodeling. The process of intraluminal division participates in the inflammation-induced neovascularization associated with chemically induced murine colitis [14]. *Scanning electron microscopy* (SEM) of vascular corrosion casts demonstrated replication of the mucosal plexus without significant evidence of sprouting angiogenesis, whereas pillar formation and septation were present within days of the onset of inflammation. The authors conclude that intussusceptive angiogenesis is a fundamental mechanism of microvascular adaptation to prolonged inflammation. It is also a mechanism of compensation for vascular growth. In a capillary regression model of inflamed murine corneas, the abrupt termination of capillary sprouting is followed by an intussusceptive response [15]. The capillary repair during kidney recovery in Thy1.1 nephritis was done by intussusceptive angiogenesis [16]. Inhibitors of angiogenesis and radiation induce compensatory changes in the tumor vasculature both during and after cessation of treatment. There is a switch from sprouting to intussusceptive angiogenesis, which may be an adaptive response of tumor vasculature to cancer therapy that allows the vasculature to maintain its functional properties [17–19]. Potential candidates for molecular targeting of this angioadaptive mechanism are yet to be elucidated in order to improve the currently poor efficacy of contemporary antiangiogenic therapies. Important is the involvement of intussusceptive angiogenesis in pathological conditions. Vascular remodeling of the hepatic sinusoidal microvasculature in the course of liver nodular hyperplasia is a result of intussusceptive growth [9]. This angiogenic mode is widely involved in tumor development. By using electron and confocal microscopy, Paku et al. [20] observed intraluminal nascent pillars that contain a collagen bundle covered by endothelial cells (ECs) in the vasculature of experimental tumors. Tumor angiogenesis in liver metastasis from colon carcinoma is a controversial subject. Ceauşu et al. [21] concluded that in liver metastasis principal mechanism of neovascularization formation is based on intussusception. In metastatic tumors of the brain there was intussusceptive angiogenesis, whereby the fibrosarcoma cells were attached to the vessel, filled the developing pillars, and caused lumen splitting [22]. Branching angiogenesis was not observed either in the tumors or in control cerebral wounds. These data suggest that sprouting angiogenesis is not needed for the incipient growth of cerebral metastases and that tumor growth in this model is a result of incorporation of host vessels. Prolactin was found to directly stimulate angiogenesis in breast cancer progression, enhancing vessel density and the tortuosity of the vasculature by pillar formation, which are hallmarks of intussusceptive angiogenesis [23]. It is a preferred mode of angiogenesis in oral squamous cell carcinoma [24] and in hepatocellular carcinoma [25, 26].

Despite this variety of intussusceptive angiogenic roles, most of the current research is focused on the mechanism of sprouting angiogenesis because this mechanism was first

described and most existing experimental models are related to sprouting angiogenesis. Consequently, the mechanism of intussusceptive angiogenesis is often overlooked in angiogenesis research [27]. Intussusception is an alternative to the sprouting mode of angiogenesis. The advantage of this mechanism of vascular growth is that blood vessels are generated more rapidly and the capillaries thereby formed are less leaky [1]. The regulation of intussusceptive angiogenesis is still to be elucidated. There are some hypotheses about the possible drivers of intussusception. In the sprouting type of angiogenesis related to hypoxia, there is no blood flow in the rising capillary sprout. In contrast, it has been shown that an increase of wall shear stress initiates the splitting type of angiogenesis in skeletal muscle [7]. Inflammation-associated intussusceptive angiogenesis in adult mice was associated with vessel angle remodeling and the morphometry of the vessel angles suggests the influence of blood flow on the location and orientation of remodeled vessels [28]. Regarding molecular regulation, very little is known for the molecular factors with potential significance. Application of the essential angiogenic factors VEGF and bFGF in an arteriovenous loop model demonstrated advanced neovascularization in the phase of remodeling by a higher incidence of intussusception, compared to control without these factors [29]. It was shown in Ewing sarcomas and rhabdomyosarcomas that treatment suppressing IGF-1 signaling decreases intussusceptive angiogenesis [30].

The main factors for maturation and hierarchical organization of vessels, especially arterial ones, are Notch, angiopoietin, and ephrin. In addition, it was shown that SDF-1 (CXCL12) is a crucial maturation factor in coronary arterial vasculature, since its mutants have immature capillary plexus and selective failure in arterial maturation, particularly with the onset of coronary perfusion [31].

Our preliminary results suggest that intussusception is most probably synchronized by chemokine factors since intussusceptive growth was associated with the recruitment of mononuclear cells [11]. After injection of bone marrow-derived mononuclear cells, we observed robust induction of intussusception in Notch inhibited samples. Notably, the chemotactic factors SDF-1/CXCR4 were upregulated only due to the Notch inhibition. Our hypothesis is that Notch inhibition disturbed vessel stability and led to pericyte detachment followed by extravasation of mononuclear cells due to the activation of the SDF-1/CXCR4 axis. The stromal cell-derived factor SDF-1 is binding to its receptor CXCR4 and directs migration of progenitor cells into the appropriate sites. The mononuclear cells contributed to the formation of transluminal pillars with sustained IA resulting in a dense vascular plexus.

2. Notch signaling and intussusceptive angiogenesis

The crucial role for Notch/DLL4 signaling in regulating vascular development was established based on findings from the analysis of targeted mouse and zebrafish mutants in Notch pathway components [32–35]. The common characteristics of the most of these mutants were the absence of angiogenic vascular remodeling, lack of arterial markers, and arteriovenous malformations. Mouse embryos deficient for Notch-ligand Jagged1 (Jag1), Notch1, Notch1/Notch4,

or the presenilins, die between E9.5 and 10.5 and have severely disorganized vasculature [36]. Transgenic mice with inappropriate activation of Notch4 also display similar defects and die, which suggests that the appropriate Notch expression pattern (in levels, sites, and time) is critical for embryonic vascular development [37]. It was found out that Notch1, Notch2, and Notch4 are expressed predominantly in endothelial cells of aorta and arteries, whereas Notch3 was in VSMCs of arteries [38].

Recently, we have established that Notch inhibition disturbs vessel stability and induces intussusceptive neo-angiogenesis, triggering in this way the augmentation of the capillary plexus but without the accompanying vascular maturation and remodeling. It was associated with extravasation of mononuclear cells of bone marrow origin possibly by upregulation SDF-1/CXCR4 chemotactic factors.

Using the chick area vasculosa (and inhibiting Notch signaling by the γ-secretase inhibitor–GSI) and a mouse model of Notch inhibition (MxCre Notch1lox/lox mice), we have demonstrated that in already existing vascular beds disruption of Notch-signaling triggers rapid augmentation of the vasculature predominantly by intussusceptive angiogenesis [11]. The process is initiated by pericyte detachment followed by extravasation of mononuclear cells (**Figure 1**). The latter cells contributed to formation of transluminal pillars [11]. The sustained IA results in a very dense vascular plexus but without the usual concomitant vascular remodeling or maturation.

The genetic approach in mice substantiated by pharmacological studies for developing vascular networks in chicken embryo enabled us to show that Notch is critical for intussusceptive angiogenesis. In both models we demonstrated considerable changes in vascular morphogenesis, resulting in massive induction of intussusception. Inhibition of DLL4/Notch signaling by novel therapeutic antibody against DLL4, performed in a recently published study [39], was associated with threefold increase in vessel density and stimulation of vessel formation. At the same time, marked reduction in the number of smooth muscle actin (SMA)-positive mural cells was noted. Two-dimensional appearance of the blood vessels in the described phenotype highly resembles the data in our chicken and mouse models. Similar phenotypes were observed after Notch inhibition during developmental angiogenesis, in skeletal muscle and in tumor models, showing increased vessel number and increased vascular permeability [40–43]. The terminology for the resultant vascular pattern, used by authors in these studies, was "abnormal vessels" or "excessive, nonproductive angiogenesis." They focused mainly on the front of sprouting invasion after blocking Notch signaling, thus describing only newly developing, nonperfused vasculature. Along with the observed significantly increased vessel density under Notch inhibition, there was evidently demonstrated reduced mural cell coverage. The authors reported positivity for endothelial markers in the endothelial protrusions toward the vessel lumen and in the intraluminal vessel occlusions [44], but they did not attribute this phenomenon to the induced intussusceptive angiogenesis. The detailed investigation of this vascular pattern behind the sprouting mode of vessels invasion demonstrated that Notch inhibition led to IA in already perfused vascular bed and this is a complementary mechanism of angiogenesis. In fact, the authors above mentioned here nicely described the characteristic features of intussusceptive microvascular growth even though they did not use the terminology.

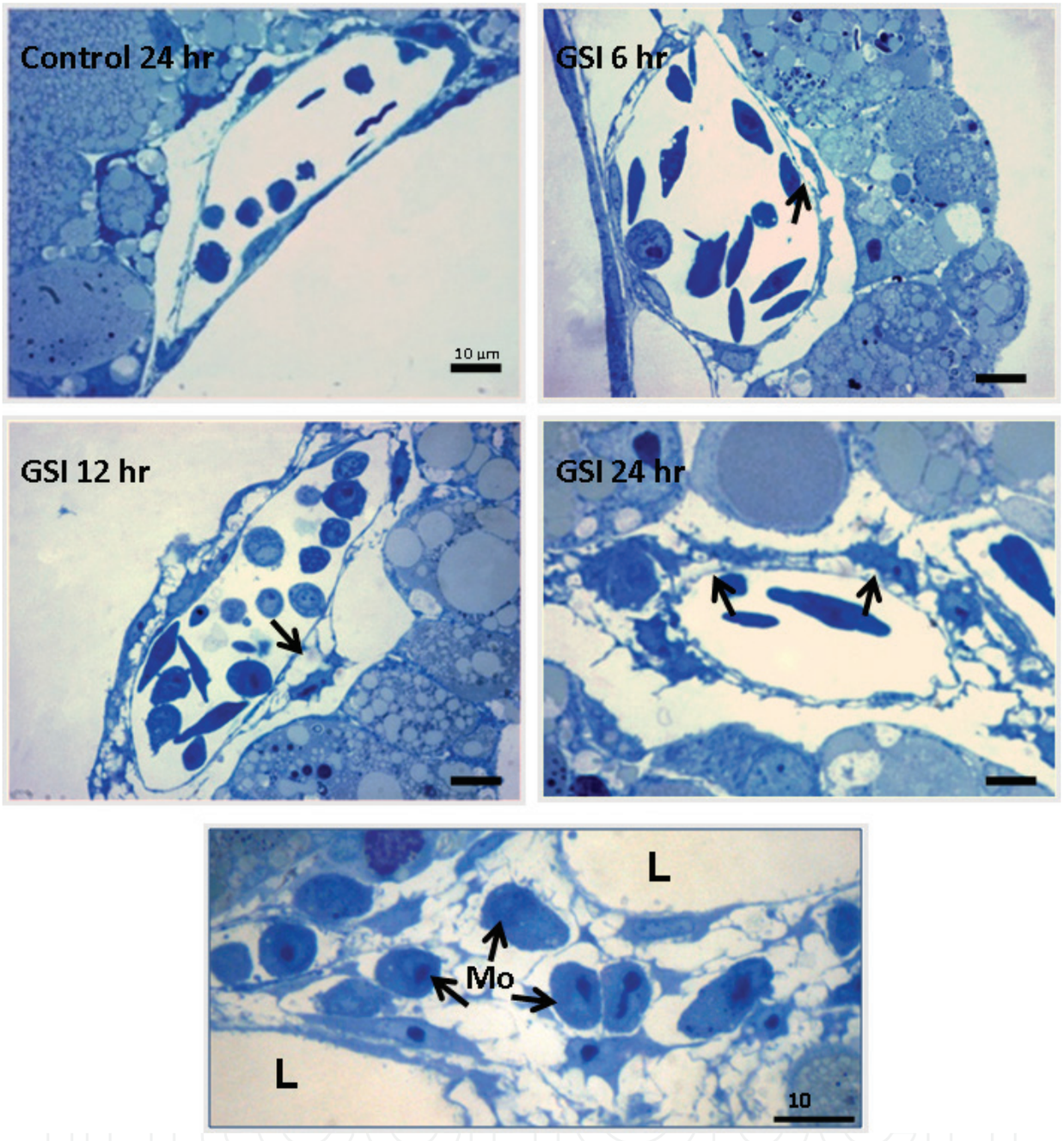

Figure 1. Inhibition of Notch signaling led to detachment of pericytes from endothelium (indicated by arrows), as it is shown at different time points after the application of GSI; it is followed by the extravasation of mononuclear cells (Mo); L–vessel lumen.

3. Role of mononuclear cells in angiogenesis

To test the role of bone marrow cells in the process of intussusception, in our previous study we isolated bone marrow mononuclear cells (BMMNC) from E14 chicken embryos and/or 4-week-old mice, labeled them with a fluorescent cell tracker (TAMRA) and injected them into the Notch inhibited (by GSI) and control samples 3 hours prior to time point 24 hours at which

time the samples were visualized by FITC injection [11]. About 3–4 hours after BMMNCs injection, we observed a significant induction of intussusception in the GSI-treated area as we detected high increase in the pillar number (4.2-fold) in inhibited samples as compared to controls. Injection of BMMNCs in the area vasculosa after Notch inhibition dramatically induced increase in microvascular density by onset of IA. Microvascular area density increases significantly by 80% after injection of BMD cells in Notch-inhibited samples in comparison with injection of BMD cells in PBS. Pillar density demonstrated dramatic augmentation by 63% compared to the Notch inhibition alone and more than 400% as compared to PBS.

We have largely expanded our knowledge about the role of bone marrow-derived cells in stimulating angiogenesis after their discovery in 1997 [45] and now their capability to promote vessel formation is intensively investigated. There are large clinical perspectives for their use in many diseases, connected to angiogenesis.

With the tendency of aging, the elderly will account for a great part of population world wide. This aging will be accompanied by chronic vascular dementia, due to chronic cerebral hypoperfusion. Cellular therapy is an emerging investigational approach for cerebral ischemia. The most attractive source for such therapy is bone marrow-derived mononuclear cells (BMMNC), since they consist of different types of stem cells. Several independent studies report the significant effects of BMMNC in ischemic repair after acute and chronic ischemic disorders. The intravenous infusion of BMMNC into rat brain ischemic model reduced neurologic impairments, increased angiogenesis and cognitive function in rodent [46]. Mononuclear cells from blood have therapeutic potential as well. The neuroprotective potential of CD34+ human cord blood cells was demonstrated in regard to Parkinson's disease [47]. These cells did not differentiate into neural phenotypes, but they rather exerted their effect by stimulating the production of new neuroblasts and angiogenesis. CD34+ stem cell therapy was enrolled in 2011 for 37 patients with longstanding dilated cardiomyopathy (DCM) by cell mobilization with colony stimulating factor (G-SCF) and apheresis collection [48]. Clinical response and stem cells retention were evaluated. About half of the patients (51%) were responders to the stem cells therapy, whereby the clinical response was predefined as an increase in left ventricle ejection fraction (LVEF) of >5% in 3 months. Looking for biomarkers, which can be instrumental in prediction which patients will be responders, the authors suggested some baseline factors, positively associated with both clinical response and retention, such as G-CSF, SDF-1, LIF, MCP-1, and MCP-3. The most recent study described the significant effect of human cord blood mononuclear cells (CB-MNCs) injection for cardiac repair in ischemic heart disease, mainly by promotion of angiogenesis in the infarcted region [49].

The mechanisms of action for mononuclear cells in angiogenesis have been intensively studied. The domain comes to be multifaceted and contradictory data were sometimes arising.

First, it was proposed and evidence was provided that myeloid cells can turn into endothelial cells in hypoxic tissue demand. Asahara et al. reported that purified CD34+ hematopoietic progenitor cells in adults can differentiate *ex vivo* to an endothelial phenotype [45]. The cells were at the same time positive for VEGFR2, a specific endothelial marker and they were named endothelial progenitor cells (EPC). Thus, EPC expresses both hematopoietic stem cell and endothelial cell markers on their surface [50]. The intensive studies in the past few years

allowed distinguishing subpopulations of mononuclear cells existing in the adult bone marrow and circulating in peripheral blood which support angiogenesis without incorporating permanently into the newly formed vessel–circulating angiogenic cells (CAC) [51]. Currently, bone marrow-derived (BMD) cellular populations with angiogenic properties are classified according to their phenotypic markers in the following groups: (i) EPC, which express VEGFR2, Tie2, CXCR4, CD31, CD34, CD133 (for immature progenitor cells) and they are negative for CD14; (ii) monocytes, which express CD14 and have different subclasses such as positive for Tie2, CXCR4, VEGFR2, or VEGFR1; and (iii) macrophages, mostly positive for CXCR4 and VEGFR1 [52].

Several clinical studies have shown a correlation between a high number of tumor-associated macrophages and increased microvessel density, suggesting that these cells might promote tumor angiogenesis, particularly due to production of proangiogenic and angiogenesis modulating factors [53]. A number of functional *in vitro* and *in vivo* studies demonstrate that tumors stimulate neutrophils to promote angiogenesis and immunosuppression, as well as migration, invasion, and metastasis of the tumor cells [54]. In inflammation, the SDF-1/CXCR4 signaling pathway plays an important role in the modulation of neutrophil activity, not only by promoting chemotaxis but also by suppressing cell death [55]. Although limited, there is evidence to suggest that tumor-infiltrating eosinophils can influence angiogenesis [53]. Freshly isolated human blood eosinophils or supernatants from cultured eosinophils induce endothelial cell proliferation *in vitro* and angiogenesis in the rat aortic ring assay, suggesting that eosinophils can directly influence angiogenesis. The high number of mast cells (MC) has been observed in various tumors where increased MC density positively correlates with increased microvessel density [53]. Dendritic cells (DC) promote tumor angiogenesis both by their secretion of proangiogenic cytokines (vascular endothelial growth factor (VEGF), interleukin (IL)-8, tumor necrosis factor (TNF)-alpha) and their ability to serve as a local supply of endothelial progenitors [56]. Natural killer (NK) cells control both local tumor growth and metastasis and participate in cancer elimination by inhibiting cellular proliferation and angiogenesis [57]. T helper (Th) cell-mediated immunity has traditionally been viewed as favoring tumor growth, both by promoting angiogenesis and by inhibiting cell-mediated immunity and subsequent tumor cell killing, there are also many studies demonstrating the antitumor activity of CD4+ Th2 cells, particularly in their collaboration with tumor-infiltrating eosinophils or due to direct antiangiogenic effects of IL-4 [58]. T regulatory cells (Tregs) are potent immunosuppressive cells that promote progression of cancer through their ability to limit antitumor immunity and promote angiogenesis. The accumulation of Tregs in tumors correlates with biomarkers of accelerated angiogenesis such as VEGF overexpression and increased microvessel density, providing clinical cues for an association between Tregs and angiogenesis [59].

Mononuclear cells, derived from bone marrow or umbilical cord, yielded in culture two types of cells with angiogenic properties, distinguished by morphology–late endothelial progenitor cells (EPC) and mesenchymal stem cells (MSC). Quantitative PCR analyses revealed high expression levels of Ang-1, FGF-2, SDF1α, and VEGF-A in the MSC, whereas late EPC had higher expression of PDGF-B, PlGF, KDR, CD31, VE-cadherin, and Ang-2 [60]. After transplantation of EPC and MSC in the ischemic hearts, mRNA levels of Ang-1, FGF2, SDF1α, and IGF-1 were significantly increased in tissues collected from the peri-infarct zones; notably

the upregulated factors were the same in both cell types transplanted. The data demonstrate that these cells upregulate a number of paracrine factors connected to angiogenesis and cell survival during the critical period of heart repair.

Although the role of bone marrow and peripheral blood mononuclear cells in neovascularization has been convincingly shown, the question remains: how do these cells improve neovascularization? The discovery that mononuclear cells can home to sites of hypoxia and enhance neoangiogenesis has faced the possibility for using the isolated hematopoietic stem cells or EPC for therapeutic vasculogenesis [61]. Remarkably, infusion of terminally differentiated mature endothelial cells did not improve neovascularization [62, 63] suggesting that a not-yet-defined functional characteristic (e.g., chemokine or integrin receptors mediating homing) is essential for EPC-mediated vascular augmentation after ischemia [64]. Monocytic cells may play a crucial role also in collateral growth by adherence to the vascular wall during both arteriogenesis and tumor angiogenesis [53]. These data suggest that monocytic cells are necessary for arteriogenesis and possibly neovascularization. For therapeutic application, the local enhancement of monocyte recruitment might be better suited than systemic infusion of monocytic cells, which only leads to a relatively minor increase in the number of circulating monocytes. During endothelial repair after vascular injury and during tumor angiogenesis BMMNC do not seem to be involved in reendothelialization stressing rather their supportive role over trans-differentiation [65, 66].

The hypothesis for endothelial trans-differentiation of EPC and MSC was tested in the experiment with CM-Dil-labeled (red fluorescent dye) mononuclear cells and subsequent transplantation in infarcted hearts. Interestingly, both EPC and MSC were detected in the pericytic or perivascular areas with minimal and negligible endothelial trans-differentiation effects (<1%). It was suggested that these cells function mainly by paracrine action and vessel stabilization in the perivascular area. The efficiency of neovascularization therefore may not solely be attributable to the incorporation of these cells in newly formed vessels, but may also be influenced by the release of proangiogenic factors in a paracrine manner [67]. It was recently shown that secreting factors from peripheral blood mononuclear cells enhance neoangiogenesis [68]. The capacity of EPC to physically contribute to vessel-like structures may contribute to their potent capacity to improve neovascularization as well [69]. Further studies are in demand to be designed to elucidate the contribution of physical incorporation, paracrine effects and possible effects on vessel remodeling and facilitating vessel branching in EPC-mediated improvement of neovascularization. Likely, paracrine effects contribute in addition to the physical incorporation of EPC into newly formed capillaries. The influence of the incorporation of a rather small number of circulating cells on remodeling and vessel maturation has to be further elucidated.

Only recently the bone marrow-derived monocytes have been related to VEGF-independent angiogenesis [70]. An open question is what drives BMD and PBM cells recruitment to the sites of angiogenesis? Ischemia is believed to upregulate VEGF or SDF-1 [71], which in turn are released to the circulation and induce mobilization of progenitor cells from the bone marrow via a MMP-9–dependent mechanism [72, 73]. Indeed, SDF-1 has been proven to stimulate recruitment of progenitor cells to the ischemic tissue [74]. SDF-1 protein levels were

increased during the first day after induction of myocardial infarction [75]. Moreover, overexpression of SDF-1 augmented stem cell homing and incorporation into ischemic tissues [74, 75]. Interestingly, hematopoietic stem cells were shown to be exquisitely sensitive to SDF-1 and did not react to G-CSF or other chemokines (e.g., IL-8 and RANTES) [76]. The migratory capacity of EPC or bone marrow cells toward VEGF and SDF-1, respectively, determined the functional improvement of patients after stem cell therapy [77].

SDF-1/CXCR4 axis is crucial in the homing mechanisms of hematopoietic cells and the metastasis of solid tumors. In the past few years, numerous studies have focused on studying the role of this signaling in angiogenesis and proved its angiogenic activity in organ repair and tumor development. However, the precise mechanisms by which SDF-1 exerts its proangiogenic effects are not fully elucidated. Since it is supposed to be an angiogenic growth factor, it is a good candidate for pro- and antiangiogenic therapy. It was reported that transient disruption of the SDF-1/CXCR4 axis using CXCR4 blocking antibody blocked the recruitment of bone marrow-derived cells into the angiogenic sites of tumor tissue, and resulted in complete inhibition of accelerated tumor growth after chemotherapy in mouse [78]. SDF-1 is constitutively expressed in the bone marrow and various tissues, which enables it to regulate the trafficking, localization, and function of immature and mature leukocytes, including monocytes, neutrophils, dendritic cells, and T lymphocytes [79]. All these immune cells play an important role in tumor angiogenesis and vascularization. However, the precise role of SDF-1/CXCR4 axis on function of monocytes/macrophages, neutrophils, DC, T lymphocytes in the process of angiogenesis is still known and is worthy to be investigated.

4. SDF-1 as a key regulator of vessel development

Global mouse knockouts of SDF-1 (CXCL12) or of its receptor CXCR4 die shortly before birth with vascular deficiency in gut, kidney, and skin and with multiple hematopoietic and neural defects [80-82]. Disrupted CXCL12 signaling led to defective coronary vessel organization in mouse embryos. This signaling was connected to perfusion of the coronary arteries and respectively to embryo growth [31].

SDF-1–positive endothelium was found lining the newly formed intraluminal vessels in lobular capillary hemangiomas [83], possibly these were sites of pillar formation. Wrag et al. demonstrated that transplantation of rat bone marrow-derived progenitor cells, positive for VEGFR1, and CXCR4, in ischemic hind limbs increased capillary density by an SDF-1–dependent manner, but did not differentiate into vascular structures like endothelial cells or smooth muscle cells [84]. In our previous study, we observed upregulation of SDF-1 and CXCR4 after Notch inhibition being in association with intussusceptive angiogenesis. These chemokine factors are most probably essential for the recruitment of mononuclear cells, participating in the formation of pillars.

It is well known that blocking of SDF-1/CXCR4 axis results in prevention or delay of tumor recurrence after irradiation by inhibiting the recruitment of CD11b+ monocytes/macrophages that participate in tumor revascularization [85]. It was shown that CXCR4 is expressed on

eosinophils [86] and concentrations of SDF-1 correlates with eosinophil recruitment [87]. It is known that SDF-1/CXCR4 signaling has pivotal role in mast cell (MC) recruitment in tumor tissue [88] and that MC produce proangiogenic chemokines in response to SDF-1 [89]. CXCR4+ dendritic cells (DC) promote angiogenesis during embryo implantation in mice [90] and CXCR4 is known as a critical chemokine receptor for migration of plasmacytoid DC [91]. CXCR4 is expressed on both NK and NKT cells and regulates their migration in inflamed and tumor tissues in response to SDF-1 as well [92, 93]. SDF-1/CXCR4 signaling is important for migration and activation of T cells [94]. However, the role of SDF-1/CXCR4 signaling in T cell–mediated angiogenesis is unknown. B cells promote tumor progression through STAT-3 regulated angiogenesis [95] and SDF-1/CXCR4 axis is essential for B-lymphocyte production [96] and maintenance of B-cell homeostasis [97].

SDF-1 is the key regulator for homing of stem and progenitor cells to the ischemic injury and its gradient is the major determinant of these cells' recruitment. It has been shown that SDF-1 expression levels are increased in ischemic cardiomyopathy and this was accompanied by the improvement of cardiac function. In addition, SDF-1 high circulation levels were detected in patients with heart failure. Using the ELISA method, Liu et al. [98] proved significantly higher circulating SDF-1 levels in HF patients (5101 ± 1977 pg/ml) compared to controls (1879 ± 1417 pg/ml). Platelet-bound SDF-1 was correlated with acute coronary syndrome and congestive heart failure as well. It was associated with the number of circulating CD34+ progenitor cells. CD34 is coexpressed with CXCR4, which is the ultimate SDF-1 receptor, in progenitor cells, originated from bone marrow, cord blood, and fetal liver. SDF-1 levels were measured in 3359 Framingham Heart Study participants and was suggested as a biomarker of heart failure and all-cause mortality risk. In this study, CD34+ cell frequency was inversely related to SDF-1, in contrast to above-mentioned direct associations. The study has several limitations as the SDF-1 measurement at one time point. There is evidence for modulation of SDF-1 levels in humans and its effect is rather cumulative and chronic than acute.

The crucial role of SDF-1 for cardiac repair in chronic ischemic heart failure was the reason for conducting clinical trial using SDF-1 nonviral gene therapy via its endomyocardial administration [99]. This blinded placebo-controlled STOP-HF trial demonstrated improvement of clinical status based on composite endpoint of six MWD (6-min walk distance) and MLWHFQ (Minnesota Living with Heart Failure Quality of life Questionnaire). Another clinical trial–MARVEL was announced in 2015 to advance into US FDA Phase 3 clinical evaluation of regenerative/cellular therapy of chronic heart failure, planned to be combined with SDF-1.

What trigger the SDF-1 upregulation is still elusive. Some authors postulate it is induced by HIF1α in response to hypoxia. However, other mechanisms of induction are also possible such as inflammation and subsequent release of mediators like interleukin-1 or tumor necrosis factor-α into circulation. It is evident by its induction not only in ischemic, but also in nonischemic cardiomyopathy. SDF-1 circulating levels were not influenced by the local heart hypoxia, but showed positive correlation with CRP, which is a marker for inflammation.

Recently, we have demonstrated the endothelial expression of SDF-1 in liver of Notch1 knockout mouse, whereby it was associated with intussusception (**Figure 2**).

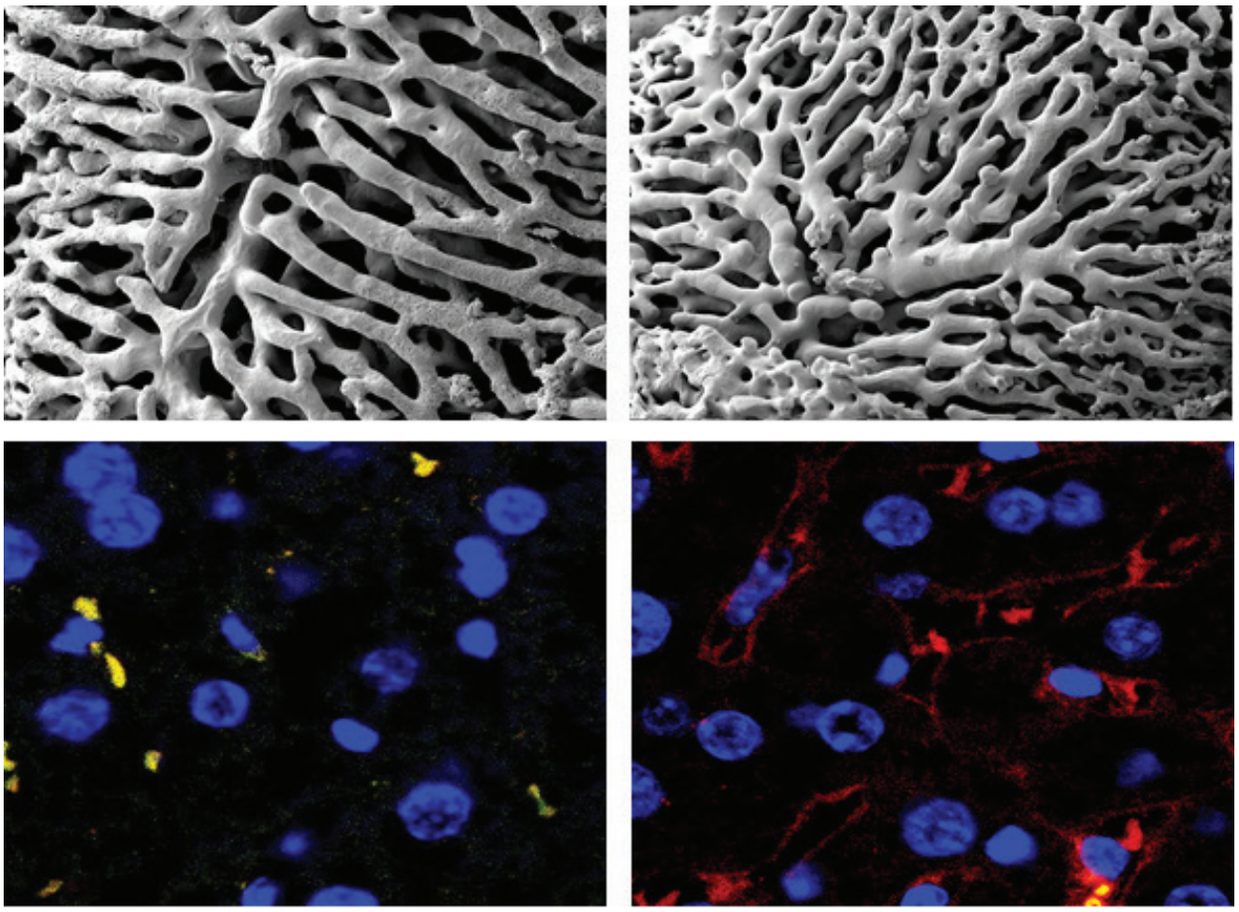

Figure 2. Vascular casts revealed predominant mode of intussusceptive angiogenesis in liver nodular regeneration after Notch1 knockout (B) compared to wild type mouse (A). Immunofluorescence for SDF-1 demonstrated strong sinusoidal positivity for this marker only in Notch1 knockout mouse (D) but not in the wild type (C).

Connecting our observations for SDF-1 positivity and mononuclear cells (MNCs) participation in intussusceptive angiogenesis, we hypothesize that both processes are involved in vessel remodeling. Using our model of chicken area vasculosa, we performed detailed ultrastructural vessel study after application of recombinant SDF-1. A specific behavior of mononuclear cells was detected during this experiment. They were involved in a step-wise process of recruitment and extravasation (**Figure 3**). We determined five distinguished states: 1, MNCs are free in the circulation; 2, MNCs are recruited to the endothelium and rolling under the blood flow; 3, MNCs are bound to the endothelium; 4, MNCs are extravasating; 5, MNCs are localized in the perivascular space.

1. MC free in circulation–nonadhesive to endothelial cells
2. MC tethered to endothelium and rolling under force of blood flow
3. MC bound to endothelium and migrating
4. Extravasation of MC from blood vessel
5. MC in perivascular space–stabilizing function

http://dx.doi.org/10.5772/66761

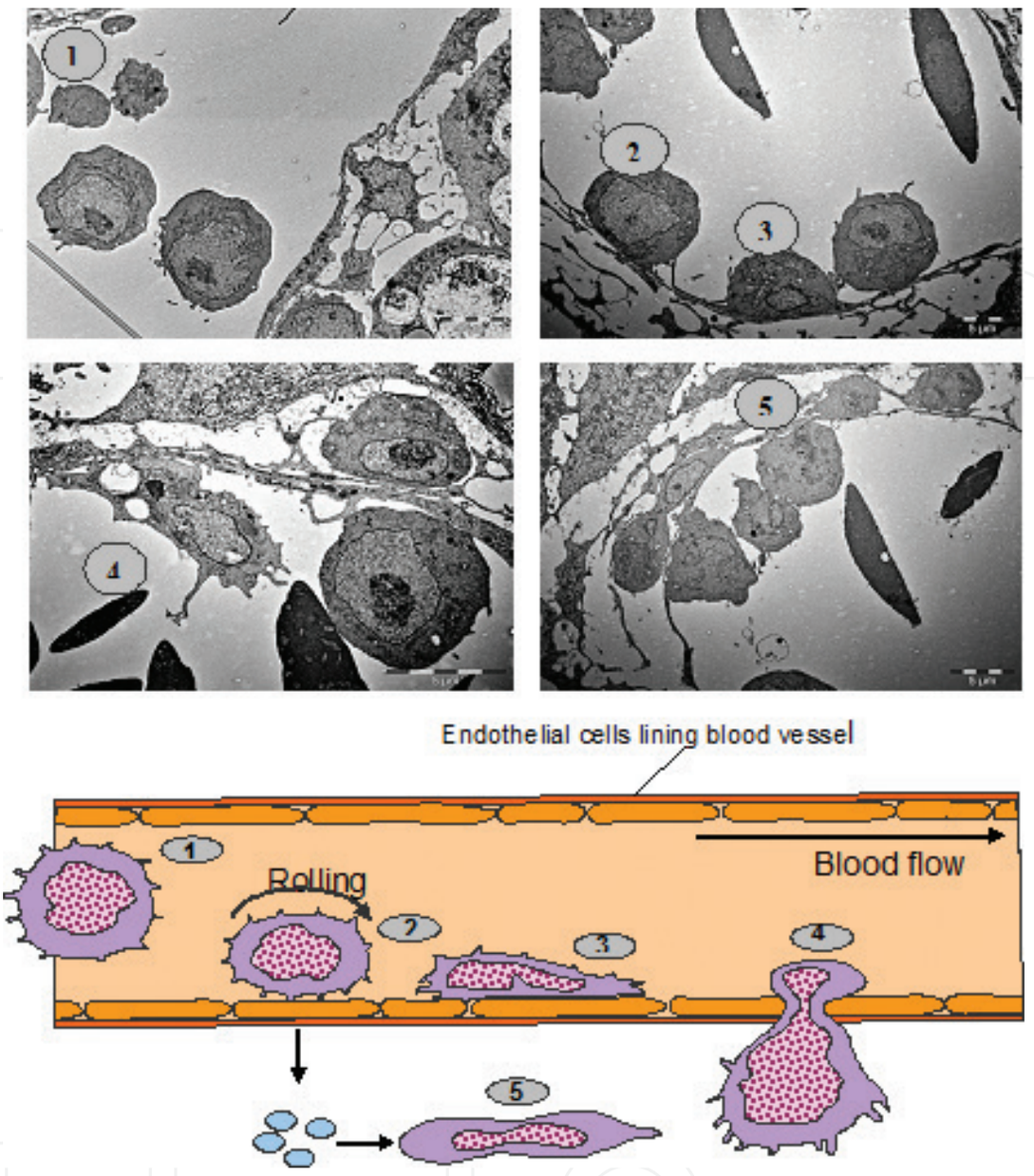

Figure 3. Transmission electron microscopy of chicken area vasculosa after the application of recombinant SDF-1 and the proposed model for mononuclear cells (MC) extravasation.

5. Summary

- Angiogenesis is a complex process involving essential signaling pathways (for instance VEGF, bFGF, Notch, etc.) in vasculature, as well as additional players such as bone marrow-derived mononuclear cells.
- Intussusceptive angiogenesis (IA) is a well-documented and widely spread mode of angiogenesis, occurring both during normal development and in pathological conditions.

- Our preliminary results suggest that IA is most probably synchronized by chemokine factors since intussusceptive growth was associated with the recruitment of mononuclear cells.
- The intensive studies in the past few years allowed distinguishing subpopulations of mononuclear cells existing in the adult bone marrow and circulating in peripheral blood which support angiogenesis.
- During endothelial repair after vascular injury and during tumor angiogenesis mononuclear cells do not seem to be involved in reendothelialization stressing rather their supportive role over trans-differentiation.
- We have demonstrated the endothelial expression of SDF-1 in liver of Notch1 knockout mouse, whereby it was associated with intussusceptive angiogenesis.
- We suggest that this chemokine factor is most probably essential for the recruitment of mononuclear cells, participating in step-wise process of extravasation and stabilizing the formation of intussusceptive pillars.

Acknowledgement

This work was supported by Contract No IZ73Z0_152454 of SNSF, Switzerland.

Author details

Ivanka Dimova[1]* and Valentin Djonov[2]

*Address all correspondence to: ivanka.i.dimova@gmail.com

1 Department of Medical genetics, Medical University Sofia, Sofia, Bulgaria

2 Institute of Anatomy, University of Bern, Bern, Switzerland

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