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Chapter 7

Fibroblasts and Endothelial Cells: The Basic Angiogenic Unit

François Berthod*

Centre LOEX de l'Université Laval, Centre de recherche du CHU de Québec,
and Département de Chirurgie,
Faculté de Médecine, Université Laval, Québec, QC, Canada

One of the key features during the angiogenic process is undoubtedly the interaction between endothelial cells and the extracellular matrix. Adhesion of these cells to the extracellular matrix through integrins regulates their proliferation, survival and migration and is essential for their assembly into vessels. However, this fibrillar scaffold is not sufficient to promote angiogenesis, and necessitates another key element, the fibroblast.

Fibroblasts are so basic and ubiquitous cells in connective tissues that they tend to be ignored. The purpose of this book chapter is to show that fibroblasts are not only the manager of the extracellular matrix, but they play a critical role to support the angiogenic process at the microenvironmental scale, through extracellular matrix remodeling and local delivery of growth factors.

In addition, they also enhance the stability of the nascent capillaries. Indeed, endothelial cells once organized into tubes can induce nearby fibroblasts to differentiate into pericytes that will enwrap the microvessels providing stability and regulating perfusion.

This sequence of events can be recapitulated in vitro using a tridimensional tissue-engineered connective tissue seeded with human microvascular endothelial cells. This model promote the spontaneous formation in standard culture conditions of a network of capillary-like tubes made of endothelial cells that will be stabilized by pericyte recruitment from fibroblasts. Tubes form after 10 days of in vitro maturation and can be maintained for more than 50 days, providing an ideal model for long-term studies on angiogenesis.

* Corresponding Address: Dr. François Berthod, LOEX, Centre de recherche du CHU de Québec, Aile R, 1401, 18e rue, Québec (QC) Canada G1J 1Z4, Phone: +1-418-990-8255, ext. 1705, Fax: +1-418-990-8248, E-mail address: francois.berthod@fmed.ulaval.ca.

In spite of the huge complexity of the angiogenic process, the close cooperation of fibroblasts with endothelial cells through the creation of an intimate local microenvironment truly corresponds to the basic angiogenic unit.

Angiogenesis is a critical step in the assembly of tissues during embryonic development, as well as during wound healing and tumor growth. It is also the biggest challenge in the new emerging field of regenerative medicine in which organ failure aims to be restored by cell or tissue transplantation. In such approach, the first worry is to assess whether any tissue of even less than a few millimeters in size will be able to survive the first days or weeks after graft. The highest risk for the transplant is necrosis mainly due to insufficient blood supply. Since passive diffusion of oxygen and nutrients is known not to exceed about 100 µm, adequate vascularization of a graft thicker than 200 µm needs to be achieved quickly through the process of neovascularization [1]. Unfortunately, this process is too lengthy to promote adequate blood supply of an organ before necrosis occurs [2].

To try to answer multiple clinical needs for organ replacements, regenerative medicine has developed various approaches of tissue engineering to reconstruct these organs. However, whatever sophisticated they can be, they all face the same challenge, to be vascularized fast enough after transplantation to promote survival. Thus, the solution should be the same for all of them, and is based on a better control of angiogenesis.

Actually, even if the neovascularization process could be highly speeded up through growth factor expression or alternative strategies, this will probably not be sufficient to prevent necrosis. It seems that the only valuable strategy to achieve a complete vascularization of a tissue-engineered organ in a few hours after graft is the reconstruction of a prevascular network in the whole organ prior to transplantation. Indeed, it is well known by plastic surgeons that skin autografts or cadaveric skin grafts are vascularized in a matter of hours. This achievement is due to the process of inosculation, in which the capillary network of the graft is able to connect to the host's one very quickly. As soon as both network are connected, blood flow can be established in the whole transplant immediately [3]. Such success of fast vascularization could be obtained with tissue-engineered organs provided that they contain their own capillary network [4]. Thus, building a human capillary network in tissue-engineered organs should be a priority in regenerative medicine, and requires determining how to efficiently promote and control angiogenesis in tissues in vitro.

In Vitro Models to Study the Angiogenic Process

The pro or anti-angiogenic potential of a compound can be tested using one of the various in vivo models to study angiogenesis, such as the corneal assay, the chick chorioallantoic membrane assay (CAM) or the Matrigel plug assay [5]. However, in vivo models share the major drawbacks to be influenced by multiple different parameters such as oxygen tension, inflammation or encapsulation that are difficult to control. These results are also difficult to extrapolate to human angiogenesis. In vitro models have been developed to focus on the angiogenic process independently of confounding factors such as inflammation, humoral modulations or indirect effects via another cell type or biological process. In order to study angiogenesis in a physiological environment, endothelial cells need to be cultured in a three-dimensional matrix to achieve tube formation with an internal lumen. This matrix can be

made of various molecules from collagen type I to fibrin [6, 7]. Matrigel, a matrix secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, is a popular substrate to promote capillary-like tube formation using human endothelial cells [8-11] However, the major drawbacks of these models are the need to add high concentrations of growth factors (VEGF and FGF, or endothelial cell growth supplement) to the culture to achieve capillary-like tube formation, and the fact that the capillary network is organized over a few hours and not stable more than a couple of days [8]. A so rapid and evanescent capillary formation does not correspond to a normal angiogenic process occurring in vivo that usually takes days to be achieved. This is probably due to an excessive amount of growth factors that induce a boost on capillary-like formation ultimately closer to pathological than normal angiogenesis. To be recapitulated in vitro in conditions mimicking as close as possible the in vivo environment, human endothelial cells have to be co-cultured in association with their usual partner in connective tissues, the fibroblast.

Fibroblasts Modulate Angiogenesis Through Secretion of Various Molecules

Fibroblasts have been shown to promote angiogenesis through secretion of various angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), Angiopoietin 1 (Ang-1), Angiopoietin-2 (Ang-2), and hepatocyte growth factor (HGF) [12-19]. From these angiogenic factors, VEGFs and FGFs are the best characterized and achieve together a synergistic angiogenic response. They promote endothelial cells survival, migration and proliferation, basement membrane degradation, and endothelial cell sprouting and patterning [12, 13, 20].

VEGF and FGF can bind to extracellular matrix (ECM) molecules such as heparan sulphate proteoglycans through their heparin binding sites. Binding of growth factors by ECM is not only a storage capacity, it is of major importance to establish guidance cues for the control of vascular growth patterning through concentration gradients [20].

Fibroblasts not only control angiogenesis through secretion of VEGF, FGF-2, or Ang-1 and -2, but also produce MMPs and their inhibitors, tissue inhibitor of metalloproteinases-1 (TIMP-1). Moreover, they produce numerous ECM proteins that, once cleaved, release anti-angiogenic fragments. Collagen type IV, a component of the capillary basement membrane, can be cleaved into arresten, canstatin, tumstatin and α6NC1 that are all anti-angiogenic peptides targeting specific integrins [21]. In this respect, TIMP-1, which is uniquely secreted by fibroblasts, has been shown to increase capillary formation through inhibition of MMP-9 that induces the release of tumstatin by cleavage of collagen IV [22]. Collagens type VIII, XV, and XVIII, as well as fibronectin, thrombospondin-1 and 2, and perlecan have also been shown to release anti-angiogenic fragments following cleavage [21].

Thus, it is clear that the nature of the ECM in which endothelial cells are embedded in any in vitro model will have a major impact on angiogenesis and the growth factor requirements to promote it. VEGF and FGF-2 promote capillary formation with lumen from endothelial cells cultured in a collagen gel [23]. In contrast, it was recently showed that the fibroblasts-derived growth factors that promoted endothelial cell sprouting in a fibrin gel to a similar extent than coculture with fibroblasts was a combination of Ang-1, angiogenin, HGF,

transforming growth factor alpha (TGF-α and tumor necrosis factor (TNF), while these factors failed to achieve lumen formation [19]. Capillary-like tube formation in fibrin gels necessitated secretion of Collagen type I, SPARC and insulin-like growth factor–binding protein 7 (IGFBP7) to be realized.

Thus, to efficiently reproduce the angiogenic process in vitro, it is crucial to combine endothelial cells with fully functional fibroblasts in an environment promoting extracellular matrix deposition.

Basic Principles to Promote Extracellular Matrix Deposition by Fibroblasts

Connective tissues constitute the basic structure of most of the body organs. Interwoven collagen fibril bundles form the three-dimensional architecture of the tissue on which various molecules and cells can attach. Collagen fibrils promote tissue strength while the elastic system provides elasticity and glysosaminoglycans, resistance to compression. Over these basic but essential mechanical properties, the extracellular matrix (ECM) is made of a large variety of proteins and glycosaminoglycans that play crucial roles in the vascular network development. It was shown that a loss of function in fibronectin, laminins, collagens type I, III or IV, perlecan, to name a few, can cause vascular abnormalities and embryonic or perinatal death [20]

Instead of trying to build an ECM through the culture of fibroblasts into a gel of bovine collagen type I/III, or fibrin, we postulated that the most physiological ECM to enhance capillary-like tube formation by endothelial cells should be produced by the fibroblasts themselves. Indeed, we previously showed that fibroblast embedded in a collagen gel produced 3 times less collagen compared to a monolayer culture, while the collagen secreted in monolayer cultures was mostly released in the medium and eliminated at each medium change precluding ECM formation [24]. To promote collagen fibrillogenesis and collagen fibril bundle assembly, addition of ascorbic acid in the culture medium was necessary. Indeed, ascorbic acid (ascorbate, vitamin C) is an essential cofactor in the hydroxylation of proline into hydroxyproline, which is vital for the collagen triple helix stabilization [25, 26]. This is why in absence of ascorbate, an unstable collagen monomer cannot be polymerized into a fibril. Since the basic structure of the ECM is built upon the collagen fibril network, in absence of such framework, the other molecules will not be able to attach to a solid structure and will also all be lost during culture medium change. The addition of 50mg/ml of ascorbate in the culture medium promote ECM deposition by fibroblasts and the formation of a fibroblast sheet over a 28 days of in vitro maturation [27, 28]. This sheet will ultimately drastically contract when tension forces induced by fibroblasts will exceed those keeping it attached to the plastic. However, this culture process can be used to prepare complex organs when engineered prior to contraction into a tissue through superimposition of several sheets and coculture with additional cell types such as epithelial or endothelial cells [29-31]. These fibroblast sheets can also be endothelialized [32, 33]. Some limitations in the preparation of fibroblast sheets include the tissue contraction, the low thickness of the sheet (about 100 µm) and the length of time for tissue maturation. In order to faster develop tissues that will be thicker, easier to prepare and less prone to contraction, fibroblasts can be seeded in preformed

scaffold designed to adequately correspond to the optimal shape of the organ to reconstruct. These scaffolds can be made of polymers or biologic molecules such as collagens and have to be porous and biodegradable [34]. Once fibroblasts are seeded on the top of the scaffold, they migrate inside to colonize it and spread in the pores. Then they start to produce collagens and various proteins and proteglycans to fill the empty spaces with newly synthesized ECM. These molecules are from human origin if the cells are human, and closely mimic the composition of native tissues since they were produced by the fibroblasts themselves. Indeed, it was shown that specialized molecules such as collagens type XII and XIV were reexpressed by fibroblasts cultured in a collagen sponge, in contrast when these cells were cultured on plastic [35].

Thus, culturing human dermal fibroblasts in a collagen sponge allows the deposition of large amount of ECM with characteristics close from native connective tissue that should be well suited to support angiogenesis.

The Tissue-Engineered Connective Tissue: The Ideal Tool to Study Angiogenesis

To design an ideal model of angiogenesis, this process should mimic as close as possible what happens in vivo and thus might not necessitate extra amount of growth factors to occur, and lead to formation of capillary-like tubes stable over a long-term period. The take-home message here should be to trust the potential of the cells to know themselves how to do the things they are programmed to achieve, providing they are cultured in good conditions. Thus, we should not try to modify them through transduction of various vectors to increase VEGF secretion or prevent apoptosis through BCL2 expression, but just let them do their job. If they don't succeed, it just means the surrounding environment is not enough supportive, and should be improved.

Another basic rule is that simpler is better in cell culture and tissue engineering. This is why we first tried to just coculture human fibroblasts and endothelial cells in a collagen sponge for one month. During the first 10 days, the culture medium was made of 50mg/ml ascorbate with half a standard fibroblast medium with 10% fetal calf serum, and half a standard endothelial cell medium (M199 with endothelial cell growth supplement, or EGM bullet kit), and without any endothelial cell supplement for the rest of the culture.

After 10 days, endothelial cells form capillary-like tubes (CLT) in this model, featuring a closed lumen surrounded by a wall made of endothelial cells. These structures are clearly visible in transmission electron microscopic sections were endothelial cells are characterized by their content of Weibel-Pallade bodies, and the lumen correspond to an empty space without any ECM deposit, but some vesicle membrane debris [36]. These CLTs have been shown by confocal microscopy (after staining with anti-PECAM-1 antibodies) to be organized into a three-dimensional network in the whole thickness of the sponge, whereas there was no blood flow in the lumen [37]. They can be maintained up to 50 days in vitro and represent a valuable model to study various pro or anti-angiogenic molecules. Indeed, since the model does not require extra amount of growth factors to promote angiogenesis, it is still sensitive to pro-angiogenic molecules such as VEGF. In addition, it is enough sensitive to discriminate dual pro- and anti-angiogenic effect of the same molecule depending on its

concentration, as shown with Tamoxifen (angiogenic at 10 µM and cytotoxic at 40 µM) [38]. The cytotoxicity of a molecule, that will necessarily decrease the number of CLT, can be differentiated from a specific anti-angiogenic effect through observation of the histology of the tissue and/or the total number of cells. Moreover, the most powerful aspect of the model is the possibility to assess the reversibility of the effect [38]. Indeed, the molecule can be tested for one week and followed for one or two weeks after the end of the treatment to analyze whether the effect is stable or reversible. The molecule can also be added at the beginning of CLT formation to see its specific effect on angiogenesis, or after most of the network has been established, to look for an alteration of the pre-existing vascular network. The number of CLT can be quantified by confocal microscopy and the number of endothelial cells versus fibroblasts can be assessed through flow cytometry after staining of endothelial cells with anti-von Willebrand factor antibodies [14].

The Distinct Roles Of Fibroblasts and the Extracellular Matrix in Capillary-Like Tube Formation

Through the analysis of the model and knowing the essential role played by the ECM in the formation of capillaries by endothelial cells, it would be tempting to speculate that ECM is necessary and sufficient to promote angiogenesis in this model, and that the presence of living fibroblasts becomes useless as soon as ECM has been deposited. One easy way to investigate this hypothesis is to prepare a connective tissues in which fibroblasts produce ECM, and then have been killed through repeated freeze-thawing that preserve ECM integrity and three-dimensional organization. However, when endothelial cells are seeded on this matrix, they fail to organize into CLTs, even if the cells are fed with a culture medium conditioned by fibroblasts [14]. Thus, living fibroblasts are necessary to achieve CLT formation, and need to be closely associated with endothelial cells to promote angiogenesis.

Then, is ECM absolutely necessary? Coculturing endothelial cells with fibroblasts in a collagen sponge while preventing fibroblasts to produce ECM is tricky. It could be achieved by avoiding adding ascorbate in the culture medium. This will block collagen fiber formation and ECM deposition. Some ECM can still be observed around a few cells because the fetal calf serum can bring a small quantity of ascorbate, but most of the ECM is lost. In this model, a plateau in the number of CLT can be observed after 24 days of maturation in contrast with a high increase in the control with ascorbate that lead to 10 fold less CLT without ascorbate after 31 days [14]. This low number of CLTs is correlated with a lower proportion of endothelial cells and ECM deposition whereas the total number of cells remains similar. These results suggest that with the same number of fibroblasts in the sponge, the much lower amount of ECM deposited in the ascorbate-deprived tissues limits the number of CLT formed, and induces a decrease in endothelial cell proliferation and/or survival. It was clear on histology and immunohistochemistry observations of the ascorbate-deprived sponges that CLTs were only detected in the areas of ECM accumulation [14].

Thus, if it is clear that the ECM is necessary for CLT formation, ECM is not sufficient to promote endothelial cell assembly into capillaries, even if ECM was made by fibroblasts and is cultured in medium conditioned by fibroblasts. A close association between fibroblasts and endothelial cells seems to be required to trigger CLT formation.

As previously discussed, fibroblasts secrete VEGF and FGF2. We showed that if FGF2 was expressed in a proportion varying from 50 to 200 pg/sponge, the levels of VEGF were much higher, with a peak at 30 ng/sponge after 17 days of maturation, followed by a drastic decrease. However, the quantification of the release of a growth factor from a tissue does not give a good idea of the true concentration of this factor in the microenvironment around cells in the tissue. This local concentration of VEGF and FGF2 might be much higher when fibroblasts and endothelial cells are close from each other, and should be high enough to play a key role in the angiogenic process. Indeed, the blockade of VEGF with specific antibodies markedly decreased the number of CLT (up to 70%).

In conclusion, fibroblasts are essential partners in the formation of capillaries in connective tissues through the deposition of the ECM that support capillary assembly and by secretion of angiogenic factors such as VEGF in the close vicinity of endothelial cells.

Recruitment Of Fibroblasts by Endothelial Cells to Differentiate Into Pericytes

Pericytes are vascular mural cells closely associated with endothelial cells (they share the same basement membrane) that play major roles in the development, stabilization and remodeling of microcapillaries [39-41]. Pericytes develop close interactions with endothelial cells so that the alteration of one cell type affects necessarily the other. Thus, to develop an efficient in vitro model of angiogenesis, the addition of pericytes is essential. However, pericytes are difficult to extract from tissues because their phenotype is highly variable depending on their location in organs. In addition, they might dedifferentiate rapidly after extraction and during amplification in vitro. Finally, if mixed with endothelial cells and fibroblasts in a three-dimensional environment, they still have to migrate in the tissue and spread on newly formed capillaries, a challenge that they have been shown to successfully achieve [42].

A much easier and better solution would be that they spontaneously differentiate from endothelial cells or fibroblasts in vitro, a highly unlikely event that we still wanted to investigate.

One challenge in the characterization of pericytes in tissues is to find a valuable marker they express. Some markers were identified that were more or less specific depending on the tissues in which they were observed. Pericytes express α smooth muscle actin (α□MA), as the most widely shared specific marker. But α□MA is also expressed by myofibroblasts in fibrotic tissues and by smooth muscle cells, as a protein typical of contractile cells. Other markers were identified, such as NG2, 3G5 and RGS5 [43-48], but only α□MA and NG2 were found into our hands to be expressed in human skin with a convincing pericyte pattern (exclusive staining around capillaries) [49].

Surprisingly, when human endothelial cells were cocultured with fibroblasts for 31 days in a collagen sponge, αMA-positive cells were detected all around CLTs, while nearly no αMA-positive cell was seen in fibroblast only sponges. The expression of αMA and the close association of these cells with endothelial cells around CLT strongly argued for their pericyte nature. This was confirmed by coexpression by these cells of NG2 with the same localization pattern. In addition, NG2-positive cells were localized around endothelial cells, but inside the boundaries of the basement membrane marker laminin. Moreover, quantification through flow cytometry of αMA-positive pericytes versus vonWillebrand factor-positive endothelial cells showed nearly a 1:1 ratio (12% vs. 15%, respectively), as found in different tissues such as retina [50]. PDGFB is one of the major players in pericyte recruitment around newly formed capillaries [51]. Blocking the PDGFB receptor with the AG1296 inhibitor induced a 5-fold decrease in the number of αMA-positive cells, but also decreased the number of endothelial cells and CLTs, since both pericyte and endothelial cells are intimately associated [49].

Ultimately, to answer the question of the origin of pericytes in our model, we used combinations of GFP-expressing fibroblasts with endothelial cells, and GFP-expressing endothelial cells with fibroblasts. In the first combination, αMA-positive cells also expressed GFP while in the second one, they did not, showing that pericytes derived from the fibroblasts. Since some previous studies showed that pericytes seem to originate from endothelial cell precursors [52-54] while others hypothesized they differentiated from fibroblasts [55, 56], this result shows a convincing evidence that they can derive from fibroblasts, even if one can argue that fibroblasts may constitute a heterogeneous cell population including some mesenchymal stem cells.

This result underlines the high versatility of fibroblasts that can differentiate into myofibroblasts in a wound healing situation, or after stimulation with TGFβ, and into pericytes in close association with endothelial cells and through at least in part induction by PDGFB.

An intriguing aspect of pericytes is their differentiation potential suggesting that they could be multipotent progenitor cells [57-61]. This is an attractive hypothesis since the pericyte localization around microcapillaries represents a strategic position enabling these cells to be rapidly released in the blood circulation to participate to organ regeneration. Then, the differentiation of pericytes from fibroblasts would suggest that fibroblasts may have a much greater differentiation potential than previously thought, which is highly interesting because of the great easiness to extract and culture these cells in vitro.

Conclusion

Fibroblasts are definitely key players in the angiogenic process and capillaries formation, stabilization and functionality. They secrete and build the extracellular matrix which is necessary for endothelial cells survival and organization into capillaries. They promote capillary formation through local production of angiogenic factors such as VEGF. In return, endothelial cells induce fibroblast differentiation into pericytes that stabilize the capillaries and modulate their function. Such an intimate cell-cell interaction is essential in the microvascular network formation as the most basic angiogenic unit. Then, many other

regulation processes modulate angiogenesis at higher levels of control, such as inflammation or tumorigenicity. But the fibroblast-endothelial cell angiogenic unit should be kept in mind as the basic starting point of angiogenesis.

Thus, using conventional in vitro angiogenic models in which endothelial cells are induced to form cord like structures in a collagen or Matrigel matrix with high concentration of VEGF and FGF2 may not efficiently mimic the in vivo situation. These structures are formed in a few hours, and only remains for less than a few days, in contrast with what is known to take several days to occur in vivo and can remain stable for a long-term period. We believe that a good model to study angiogenesis in vitro needs to enable fibroblasts and endothelial cells to closely interact together with minimal external intervention, such a as growth factor supplementation or extracellular matrix embedding. And also needs time to be achieved (at least 2 weeks as in vivo).

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