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Non-invasive Monitoring of Angiogenesis in Cardiology

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Abstract

Angiogenesis plays an important role in the pathophysiology of atherosclerosis and after myocardial infarction. Furthermore, angiogenesis has been the focus of many therapeutic strategies. In view of that, a direct and clear understanding of the role of these pathways in the living subject is needed. Molecular Imaging has emerged as a powerful tool to study biological processes non-invasively. In this review, evidence will be presented and discussed on the feasibility of different molecular imaging strategies to study the involvement of angiogenic pathways in the assessment of the atherosclerotic disease and as a tool to assess angiogenic therapy. Focus will be placed on those imaging modalities with the potential to be translated to clinical use.

Keywords

atherosclerosis; myocardial infarction; vascular endothelial growth factor; integrin; angiogenesis; molecular imaging; positron emission tomography; single photon emission computed tomography

Angiogenesis constitutes the formation and/or recruitment of new blood vessels¹, a process that may have different significance depending on the field under study². In Oncology, angiogenesis is associated with providing more nutrients to the tumor and thus tumor growth^{3, 4}, what have led to development of therapies targeted to inhibit angiogenic pathways and reducing tumor size^{5–8}. In Cardiovascular Diseases the formation of new blood vessels can have different significances and can be have either a positive or negative connotation, depending on the pathophysiological background where angiogenesis takes place.

Angiogenesis in Atherosclerosis

Atherosclerosis (ASD) is a complex disease that affects vascular beds throughout the body and patho-physiologically is characterized by the accumulation of cholesterol, different mediators of inflammation, and ultimately fibrosis of the vessel wall and affected myocardium⁹. Furthermore, there is recent evidence on the presence of angiogenesis on both the atherosclerotic plaque as well as of the vasa-vasorum network^{10, 11}, and the important role it plays in the initiation, development and progression of atherosclerotic vascular lesions¹⁰. The neo-vascularization described in early atherosclerosis, similar to that seen in cancer¹², is mostly composed of immature vessels that area characterized by increased vascular permeability^{13, 14}. Clinically accepted therapies for reduction of high cholesterol and ASD (e.g. statin and angiotensin converting enzyme inhibitor-ACEI-therapies) that have been shown to decrease cardiovascular mortality and disease progression, have also been shown to reduce the level of neovascularization in early atherosclerosis^{15, 16}, what suggest an association between neo-vascularization and ASD.

Myocardial infarction (MI) is caused by sudden occlusion of a coronary artery, interrupting blood flow to the downstream myocardium, leading to tissue anoxia and cell death⁹. As a biological response to this insult, a number of biological pathways (e.g. pro-inflammatory and pro-angiogenic mechanisms) are activated^{9, 17}. The activation of these mechanisms has been associated to myocardial remodeling, ultimately determining how the myocardial tissue adjusts to the new tissue “microenvironment”^{18–20}. Angiogenesis has also been shown to be part of the remodeling process²¹, and has been observed mainly on the “border zone” (zone between normal and infarcted myocardium), suggesting that it can be part of the tissue response to the ischemic stimuli. Integrins and vascular endothelial growth factor (VEGF) have been associated to the angiogenic response after MI^{22, 23}.

Integrins

Integrins are a family of proteins expressed by endothelial cells to interact with their extracellular microenvironment²⁴. Integrins are composed by two subunits, α and β . The first and most largely studied integrin is the vitronectin-type $\alpha_v \beta_3$ integrin²⁵. Each individual integrin subunit consists of a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail.

Integrins have many different functions that are carried by different portions of the protein. Integrins are the main receptors for extracellular matrix (ECM) proteins, such as fibronectin, laminins, or collagens, and may play a fundamental role in tissue remodeling²⁶. Besides promoting physical adhesion, integrin ligation also initiates signaling that induces cell spreading, migration, survival, proliferation, and differentiation²⁶.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF)²⁷ is the most prominent member of a family of growth factors, and is composed of seven protein sub-types: VEGF-A, VEGFB, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placenta growth factor²⁸. VEGF-A is a dimer with at least seven homodimeric isoforms (121, 145, 148, 165, 183, 189, or 206 amino acids). VEGF is a family of proteins that have a similar background, with same modifications, which may be responsible for the specific proteins and ligand specificity²⁹.

Similar to many other proteins, VEGF has specific receptors to which it binds to elucidate specific actions. The angiogenic properties of VEGF are mainly mediated via two endothelium-specific receptor tyrosine kinases, Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2)³⁰. VEGF-A isoforms bind to both VEGFR-1 and VEGFR-2, which are mostly present in vascular endothelial cells. VEGFR-1 plays a major role in physiologic and developmental angiogenesis and its function varies with the stages of development, the states of physiologic and pathologic conditions, while VEGFR-2 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF²⁸.

Angiogenesis as Therapy

For more than a decade, significant interest has been placed on therapeutic alternatives (e.g. trans-myocardial laser revascularization) to restore blood flow to affected areas of the heart³¹. Gene therapy with many different growth factors (e.g. fibroblast growth factor, vascular endothelial growth factor-VEGF-) has also been developed in an attempt to provide the “roadblocks” and create new blood vessels in the myocardium^{32, 33}. In the last few years, stem cell therapies are being developed with the goal of creating new vessels³⁴, and/or provide the necessary structure that can sustain a body’s generated pro-angiogenic response.

Despite significant advances in the understanding of the benefits of these therapies, numerous questions remain. Issues like the bio-availability of these genes in vivo, the kinetics of these growth factors in the living subjects are among some of the questions that remain to be answered.

In summary, angiogenesis plays a major role in the pathophysiology of different cardiovascular diseases, and is also the goal of different therapeutic approaches. Integrins and VEGF have been associated with, and used as therapeutic strategies for ASD. To better understand and assess the involvement of angiogenic pathways it becomes imperative to be able to monitor and quantify the angiogenic response in the living subject.

Molecular imaging of angiogenesis

Over the last few years, significant efforts have been placed in developing novel strategies to image and quantify the angiogenic response, whether is to study disease or assess the response to therapy. Development of angiogenesis-targeted molecular imaging probes could serve as a new tool to better understand the role and expression profile of angiogenic-related molecules in many angiogenesis-related diseases, as well as for the assessment of angiogenic therapies.

Imaging of Integrins

As previously mentioned, integrins are transmembrane protein that play an important role in angiogenesis³⁵. Because of that, they have become interesting targets to develop strategies to image the angiogenic stimuli, and through that approach be able to study the pathophysiological states where angiogenesis is present. Due to the transmembrane characteristic of integrins, specially the $\alpha_v\beta_3$ integrin, imaging approaches to image integrins have focused on the development of ligands that will bind to the extracellular portion of the integrin molecule. In other words, an exogenously administered ligand will attach (in a specific manner) angiogenic receptor expressed in the cell surface, which will retain the ligand in the area of interest, and that can be imaged and monitored non-invasively (Figure 1).

With these premises, a number of investigators have developed a number of imaging probes to image integrin expression in angiogenesis. Harris *et al* reported the high affinity and selectivity of ^{111}In -RP748 for the activated conformation of the vitronectin-type $\alpha_v\beta_3$ integrin receptor³⁶. Using ^{111}In -RP748 as the imaging probe, Meoli *et al* demonstrated the feasibility of imaging the activation of the vitronectin-type $\alpha_v\beta_3$ integrin after MI (Figure 2)³⁷ and showed that the expression of $\alpha_v\beta_3$ integrin after MI occurs in the area of the MI, but not in remote zones (Figure 2)³⁷. This constitutes the first report of non-invasive monitoring of angiogenesis in the myocardium. Investigators went further and showed that in areas where angiogenesis was increased (increased uptake of ^{111}In -RP748), there was also increased uptake of ^{99}Tc -59-21 (hypoxia marker), establishing a relationship between hypoxia and angiogenesis in the myocardium³⁸. As the imaging modality, investigators used single photon emission computed tomography, a modality commonly used in the clinics. Studies like those from Meoli *et al* are critical as they set the basis for future non-invasive approaches to study pathophysiology of angiogenesis expression in the myocardium, and at the same time bringing these studies closer to the clinics.

These imaging strategies have also been used to assess angiogenesis in other vascular territories. Hua *et al*, using a ^{99}Tc -labeled peptide that binds to the $\alpha_v\beta_3$ integrin, described the kinetics of $\alpha_v\beta_3$ integrin in a murine hind-limb ischemia model³⁹. Similarly, Lee *et al*. used a ^{125}I -labeled RGD compound to track the expression of $\alpha_v\beta_3$ integrins after peripheral ischemia⁴⁰. Furthermore using Ultrasound as the imaging modality and microbubbles labeled with $\alpha_v\beta_1$ integrin, Leong-Poi *et al*, confirmed previous observations and showed that integrin expression is upregulated in a model of hind-limb ischemia⁴¹. In addition to their biological

significance, these studies showed how these different imaging modalities can be used to study biology non-invasively across different vascular beds.

Imaging of VEGF Receptors/VEGF

In order to understand the role of the different biological pathways in the angiogenic stimuli, it is imperative to be able to image the different angiogenic pathways involved. As mentioned before, VEGF/VEGFR is the main angiogenic pathway and has been strongly related to the angiogenesis described in ASD and after MI.

Imaging of VEGF Receptors

Using the imaging approach depicted in Figure 1, Cai *et al* have developed an imaging probe (^{64}Cu -DOTA-VEGF₁₂₁) for positron emission tomography (PET) that binds selectively to the VEGFRs, and have shown the feasibility of VEGFR imaging in a cancer model⁴². More recently, our laboratory has translated this approach to monitor the expression of VEGFRs after MI⁴³. In a rodent model of MI (permanent coronary artery ligation), we showed that MI is associated with increased uptake of this probe-as a surrogate of increased VEGFR expression (Figure 3)-. In these studies we followed the binding of ^{64}Cu -DOTA-VEGF₁₂₁ for 4 weeks, and showed that there is an early expression of VEGFRs after MI (peak binding occurred during the first week), and then the expression decreased reaching levels not different from baseline by the fourth week. This is the first study that describes non-invasively the expression of VEGFR over time after MI and because it uses a clinically applicable imaging modality (PET) constitutes an important step towards the application of these modalities in the clinics.

As with integrins, imaging of VEGFRs has also been performed in the peripheral vasculature. Lu *et al*, using a ^{111}In -VEGF₁₂₁ labeled molecule, showed that ischemic tissue had increased tracer uptake-as a surrogate of VEGFR expression- compared to sham-operated or contralateral muscle, and that this uptake was increased as early as 3 hours⁴⁴. More recently, our laboratory has used the ^{64}Cu -DOTA-VEGF₁₂₁ probe to monitor VEGFR expression in a murine model of hind-limb ischemia⁴⁵. Furthermore, using VEGFR-1 and -2 mutants we demonstrated that VEGFR-2 is the main VEGF receptor activated after hind-limb ischemia, and showed that exercise training lead to increased VEGFR-2 expression and improved exercise tolerance in these animals.

When put together, the above mentioned studies on integrin- and VEGF-imaging, these studies are of critical importance for a number of reasons: on one side, they serve as proof of concept for imaging angiogenic biological pathways across different vascular territories. Furthermore, they provide important information on the biology that underlies these pathophysiological states in a non-invasive manner. Lastly, and because most of these studies have been performed using clinical imaging modalities, they open the door for the transition of these modalities to the clinics.

Imaging of VEGF Therapy

As mentioned above different therapeutic strategies are targeted to the expression of VEGF, as a mediator for the angiogenic stimuli. Until recently, most of the studies used to assess the effect of these therapeutic strategies have been based on histology or assessment of organ function (e.g. using Echocardiography for left ventricular ejection fraction-LVEF-). While the assessment of LVEF is important and is routinely used clinically, by itself it does not provide information on the actual expression of the growth factor. Recent developments in molecular biology now permit the non-invasive imaging of trans-gene expression^{46, 47}. Wu *et al* demonstrated the feasibility of monitoring VEGF gene therapy, in a rodent model of MI, using PET⁴⁸. In these studies, the investigators used a dual cassette strategy, where one gene cassette delivered the therapeutic gene (VEGF), while in the other one a reporter gene (thymidine

kinase, a PET reporter gene) is used to report the expression of the therapeutic gene (Figure 5). The authors demonstrated a good correlation between the reporter gene signal and the VEGF trans-gene expression, enabling them to use the reporter gene to monitor VEGF expression in the living subject (Figure 6). Wagner *et al* use a similar strategy to monitor the trans-gene expression of therapeutic VEGF in a large animal model of MI⁴⁹, bringing these strategies closer to clinical applications.

Advantages and Disadvantages of Current Imaging Strategies

Most of the current imaging strategies to image angiogenesis are based on monitoring the binding of labeled probes on the cell surface receptors (Figure 1), and use that as a surrogate of receptor expression, and most importantly, of a certain pathway activation. Use of cell surface receptors as surrogates of pathway activation is important as in many cases, as integrins and VEGF receptors are an integral part of the activation of that pathway, and in many cases mediate function. From the imaging perspective, exogenously administered labeled probes do not have to cross the cell membrane, what may facilitate the interaction between the ligand and the recipient (i.e. receptor)^{46, 47}.

On the other side, cell surface receptors may not be representative of the complete intra-cellular angiogenic story. For example, VEGF activates a number of downstream pathways, whose action may remain intracellular and determine cell surface actions, beyond those related to VEGFRs⁵⁰. In that case, imaging cell surface receptors may not be sufficient to accurately study a given angiogenic pathway. From the signal-acquisition perspective, receptors can only bind to a certain number of molecules (ligands), what may limit the signal that remains in the area of interest^{46, 47}.

The studies by Wu *et al* and others elegantly illustrate how genetic therapies can be monitored non-invasively. This strategy ensures that the delivered therapeutic gene (VEGF in this case) actually gets transcribed and translated in the protein of interest. When assessing gene therapy effects, caution should be exercised, as increased trans-gene expression of a therapeutic gene is the first step, but may only be a part of the biologic response in the living subject. Other pathways, may act by deactivating or further activating the angiogenic signal. From the imaging perspective, these strategies use an enzyme-based approach, which results in increased signal (one enzymatic molecule can interact with a large number of administered probes, amplifying the signal)^{46, 47}. On the other side, for the enzyme to interact with the exogenously administered probe, the latter has to cross the cell membrane, what may limit the protein-probe interaction. Future, advances in reporter gene design and development of novel and more sensitive probes may result in improved signal-to-noise ratios.

Role of Angiogenesis Imaging in the Clinic

Clinical imaging of angiogenic pathway activation will be important in many aspects. Angiogenesis has been shown to be involved in the pathogenesis of atherosclerosis. Imaging of VEGF and related pathways can provide a non-invasive assessment of the activity of atherosclerosis in the heart. However, current limitations in spatial resolution and sensitivity of clinical systems may preclude an accurate study of angiogenic pathways in the myocardial microvasculature. With the current imaging technology, clinical imaging of angiogenesis appears more plausible for imaging of angiogenesis in the peripheral vascular territories (e.g. carotid artery, femoral artery).

Imaging of VEGF and related pathways will likely also play a clinical role in post MI-myocardial remodeling, what determines patient outcomes. Patients are routinely placed on a number of therapies to modulate LV remodeling (e.g. statins, ACEIs). Currently, the best way to assess the effect of remodeling and which therapies are appropriate is to measure left

ventricular size, left ventricular ejection fraction, and monitor patient outcomes. However, we are not able to monitor the biology of LV remodeling, such as the development of neo-vascularization, and other factors that can be involved in such response. Using molecular imaging, we will be able to monitor the post-MI state and assess the therapeutic effect of different therapies on LV remodeling.

Another area where imaging of angiogenesis will also be very important will be on the assessment of genetic therapies. Whether it is to evaluate gene or cell therapy, molecular imaging of angiogenesis (using strategies as in Figure 1) will provide invaluable information on the effect of these therapies (using imaging strategies as in Figure 5) and will likely lead to improvement of different strategies.

Summary

Angiogenesis plays an important role in the pathophysiology of many pathophysiological states. Furthermore, it is the target of many therapeutic strategies. Thus, it is imperative that investigators have ways to assess the presence of absence of angiogenesis, as well as methods for gene expression and product (e.g. formation of new blood vessels) quantification.

Molecular Imaging is a powerful emerging tool for the quantification of biological processes, with perturbation of the tissue under study. We have presented evidence that different “biochemical signatures” of angiogenesis can already be detected non-invasively.

In the future, we anticipate that novel probes targeting novel pathways will be developed and will help broaden the spectrum of pathways that can be studied non-invasively. That, in conjunction with advances in imaging hardware will be critical for the advancement of the field of non-invasive biological imaging.

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Figure 1.

Schematic representation of the imaging approach used for integrin and VEGFR imaging. After MI, there is an angiogenic stimuli that results in the transcription and translation of angiogenic receptors, what localize in the outer cell surface. A radiolabeled substrate is administered, binds to the cell surface receptor and remains in the region. After the unbound substrate is washed out, the ligand-receptor complex can be imaged non-invasively.

Figure 2.

Imaging of $\alpha_v\beta_3$ integrin after MI using single photon emission computed tomography (SPECT). A, Serial in vivo ^{111}In -RP748 SPECT short axis, vertical long axis (VLA), and horizontal long axis (HLA) images in a dog 3 wks after LAD infarction at 20 min and 75 min after injection in standard format. B, Focal myocardial uptake (anterior and lateral walls) of ^{111}In -RP748 is seen in infarct region on ex vivo images (similar to what was seen in-vivo images). Gamma-well counting demonstrates that uptake of ^{111}In -RP748 was increased in anterior and lateral walls compared to remote areas of the myocardium.

Figure 3.

Top, Representative images at baseline (left), animals after MI (middle left), and sham-operated animals (middle right) showing the difference in myocardial uptake in MI animals compared to baseline and sham animals. The red arrow shows the ^{64}Cu -DOTA-VEGF₁₂₁ signal from the myocardium (seen only in MI animals) and arrow heads show the ^{64}Cu -DOTA-VEGF₁₂₁ signal from the surgical wound (muscle layer), which is present in both sham operated and MI animals. The right panel shows a typical image acquired using the VEGF_{mutant}, with minimal uptake, supporting the specificity of the ^{64}Cu -DOTA-VEGF₁₂₁ probe for the VEGF receptors. Middle, Representative images corresponding to one animal of the MI group illustrating the uptake of ^{64}Cu -DOTA-VEGF₁₂₁ over time (in days after induction of myocardial infarction), clearly showing the time-dependent effect on the uptake of ^{64}Cu -DOTA-VEGF₁₂₁. the red arrow points to the myocardial uptake while the white arrowhead points to the chest wall muscular layer uptake. Bottom, Quantification of ^{64}Cu -DOTA-VEGF₁₂₁ after MI over time, expressed in %ID/gram of tissue. The ^{64}Cu -DOTA-VEGF₁₂₁ uptake was highest at day 3 post-operatively (compared to baseline, day-3), and continues to be elevated until day 17 post-operatively. The ^{64}Cu -DOTA-VEGF₁₂₁ uptake was also significantly different compared to sham operated animals and VEGF_{mutant}. * $p < 0.05$ compared to baseline, ¥ $p < 0.05$ compared to Sham and ^{64}Cu -DOTA-VEGF₁₂₁, ¶ $p < 0.05$ compared to VEGF_{mutant} and ^{64}Cu -DOTA-VEGF₁₂₁.

Figure 4.

A, Schematic of Ad-CMV-VEGF₁₂₁-CMV-HSV1-sr39tk-mediated gene expression. Two separate gene cassettes with CMV promoters (PCMV) driving expression of VEGF₁₂₁ therapeutic gene and HSV1-sr39tk reporter gene separated by polyA tails. Translated product of VEGF₁₂₁ is soluble and excreted extracellularly, whereas translated product of HSV1-sr39tk (HSV1-sr39TK) traps [¹⁸F]-FHBG intracellularly by phosphorylation.

Figure 5.

Noninvasive imaging of kinetics of cardiac transgene expression. A, Gene expression peaked at day 1 and rapidly decreased thereafter. B, Representative rat scanned longitudinally with transaxial $[^{18}\text{F}]\text{-FHBG}$ microPET images shown at similar slice levels of chest cavity. Gray scale is normalized to individual peak activity of each image. Myocardial $[^{18}\text{F}]\text{-FHBG}$ accumulation was visualized at anterolateral wall (arrow) from day 1 to day 14.