Modern Medicine. 2014, Vol. 21, No. 3 : 212-219

Chapter Book

ENDOTHELIAL DYSFUNCTION AND INFLAMMATION

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BACKGROUND

Conventional based methods of catheter removal of arterial blockages formed during the process of atherosclerosis often result in production of a series of rapidly occurring events which follow the balloon catheter-induced tearing of the existing atherosclerotic plaques and concomitant arterial damage and luminal destruction and ending with significant lumen narrowing within a period of around 6 months (between 4–10% of cases following endarterectomy and approximately 33% of cases in coronary arteries for example; **Fig. 1**).

The more recent introduction of stents has helped to resolve/reduce some of the problems associated with balloon angioplasty, in that it provides a scaffold which can prevent constriction from the intima, and when coated with anti-proliferative or anti-inflammatory drugs, can significantly slow down the process of in-stent restenosis. However, angiographic restenosis (>50%) and clinical symptomatic restenosis still occurs in 20–30% and 10–15% of patients respectively in the first year after treatment (1), and evidence has shown that there is no significant difference in long-term (3–5 years) follow up regarding subsequent myocardial infarction and death (2).

Within this pathophysiological sequence of events, thrombosis and inflammation can occur at the injury site within 1 week of treatment. These events trigger a cellular response with increased cell proliferation and

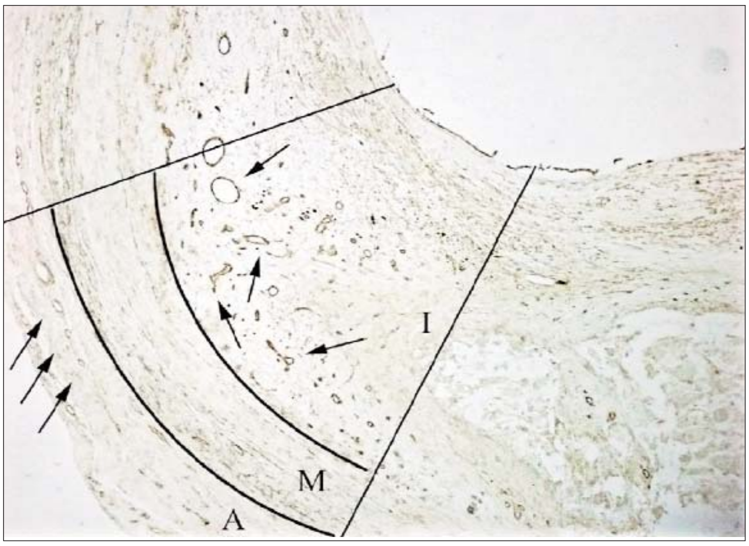

Figure 1. Proliferation of CD105 positive microvessels in the adventitia (A) are associated with intimal expansion and a high concentration of active neovessels in this region Shown: A grade 6 unstable carotid plaque obtained at transplant and stained with antibodies to CD105. Arrows show adventitial active vessels (A) and intimal neovessels (I). M is media.

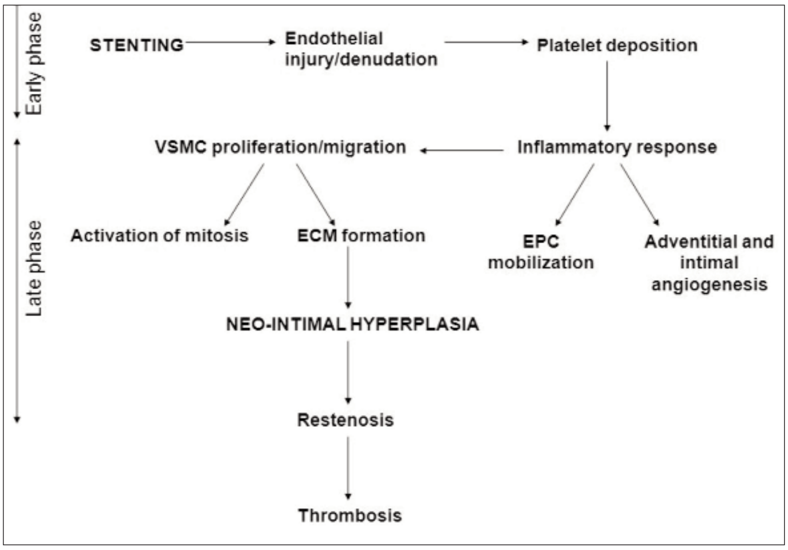

Figure 2. Schematic representation of the major processes which occur following stent implantation beginning with endothelial damage and dysfunction in the early stages and ending with restenosis and thrombosis often within 6 months of application. EPC: Endothelial Progenitor Cells.

migration, extracellular matrix remodeling and finally, remodeling of the arterial intima and neointima involving neovascularization or angiogenesis within 6 months (**Fig. 2**) (3).

Extensive neovascularization is often seen in recurrent endarterectomy/coronary artery specimens, together with fibrin-rich surface thrombi in association with intraplaque thrombi, and of particular interest, it has been shown that plaques with an abundance of smooth muscle cells (SMC) are more likely to develop greater neointimal growth after surgery, compared with macrophage and lymphocyte-rich lesions (4). Understanding the processes responsible for mediating endothelial dysfunction and activation and stimulation of remodeling through neovascularization may help us to design specific inhibitors to slow down the process of neointimal formation. Firstly, it is important to understand the relationship between inflammation and endothelial cell activation since these processes in combination are largely responsible for development of unstable atherosclerotic lesions which may translate to the process of restenosis.

INFLAMMATION AND ANGIOGENESIS IN TYPICAL ATHEROSCLEROTIC PLAQUE FORMATION

The atherosclerotic process is initiated early in life, when there is already evidence of cholesterol-containing low-density lipoproteins accumulating in the intima and activation of the endothelium. Leukocyte adhesion molecules and chemokines promote recruitment of monocytes and T cells. Monocytes can differentiate into macrophages and up-regulate pattern recognition receptors, including scavenger receptors and toll-like receptors.

Scavenger receptors mediate lipoprotein internalization producing foam-cells. Toll-like receptors are important transducers of activating signals that lead to the release of cytokines, proteases, and vasoactive molecules, and are considered to be an important link between inflammation and cardiovascular disease (5). For example, deficiency of the toll-like receptor 4 (TLR-4) protein reduces macrophage recruitment in association with reduced cytokine and chemokine levels (6). T cells in lesions recognize local antigens and mount T helper-1 responses with secretion of pro-inflammatory cytokines that contribute to local inflammation and growth of the plaque (7).

Vasa vasorum (VV) density, their proliferation, medial-intimal infiltration, and concurrent adventitial inflammation are strongly associated with advanced lesions suggesting a strong link between the two processes (8). This is backed up by various studies conducted using animal models of atherosclerosis which, due to their rapid development times, are more akin to the processes associated with restenosis following direct arterial damage in man. Plaque neovascularization correlated with the extent of inflammation in hypercholesterolemic apoE mice and inhibition of vessel formation reduced macrophage accumulation and plaque progression (9). Similarly, transfection with murine soluble VEGF-R1 inhibited early inflammation and late neointimal formation, suggesting that generation of neovessels and the inflammatory response are

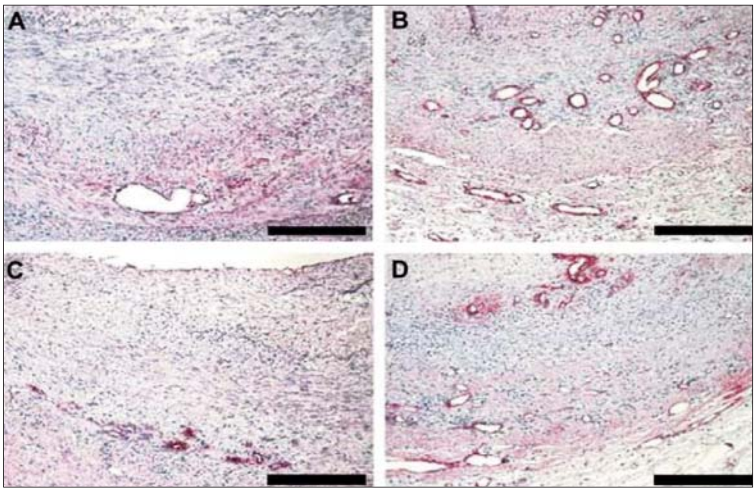

Figure 3. Intima+media and adventitia microvessel angiogenesis. Photomicrographs of microvessel angiogenesis 28 days after coronary angioplasty and VEGF or LacZ gene transfer with the needle injection catheter. Adjacent cross sections of the coronary arteries were immunolabeled with an antibody to vWf showing ECs (endothelial cells) of intima+media (A) and adventitia (B) microvessels from VEGF-transfected artery and ECs of intima+media (C) and adventitia (D) microvessels from LacZ-transfected artery. Note the significant microvessel angiogenesis in the adventitia of VEGF-transfected compared to LacZ-transfected arteries and the similar angiogenic microvessel response in the intima+media area after (peri)adventitial VEGF and LacZ plasmid/liposome transfer, respectively (bar = 200 μm). Reproduced with permission from Oxford Journals and taken from the article by Pels K et al. Cardiovasc Res 2003;60:664-672.

inter-linked and perpetuate in a continuous cycle (10) (**Fig. 3**).

It is known that inflammatory infiltrates enhance recruitment of monocytes, secrete matrix metalloproteinases and increase the expression of γ-interferon (from t-lymphocytes) which may weaken the fibrous cap; similarly, they can induce synthesis of angiogenic tissue angiotensin-converting enzyme, growth factors, interleukin-8 and tissue factor (11). The importance of the inflammatory response was demonstrated in vivo where oral treatment of apoE-/LDL-double knockout mice with the anti-inflammatory compound 3-deazaadenosine prevented lesion formation (12). A strong correlation has been shown between macrophage infiltration, intraplaque haemorrhage and rupture-prone thin-cap lesions with high microvessel density, whilst these features are not common in calcified or hyalinized human arterial plaques, suggesting a strong link between neovascularization, inflammation and thrombosis (11,13). The phenotype of plaque neovessels could also be important in determining stability of a developing plaque, for example, new vascular networks, and immature vessels with poor integrity and no smooth muscle cell/pericyte coverage would likely act as sites for inflammatory cell infiltration, inflammatory cell leakage and intraplaque haemorrhage respectively (9). The correlation of focal collections of inflammatory cells with areas of intraplaque neovascularization and haemorrhage, suggests that release of growth factors and cytokines by macrophages and leukocytes may have a key role in modulating the vascularization process (14). Evidence for the existence of hotspots or “neovascular milieu” was found in lesions from ApoE-/- mice where the density of VV correlated with the presence of inflammatory cells rather than plaque size. Deposition of RBC membranes within the necrotic core of plaques has also been shown to result in an increase in macrophage infiltration and therefore may further potentiate the inflammatory response (9,15).

Perhaps surprisingly, hypercholesterolaemia may also be a key factor associated with proliferation of VV in coronary and carotid vessels at early stages of plaque development. Williams JK (16) first demonstrated that the presence of atherosclerosis in hypercholesterolemic monkeys induced an increase in blood flow through the VV and plaque regression caused by removal of the high lipid diet reduced the VV concentration and blood flow to the coronary media and intima. High cholesterol levels are associated with increased serum VEGF expression and may cause up-regulation of growth factor receptors on both endothelial and smooth muscle cells (17). Furthermore, oxidized LDL (ox-LDL) generated in response to pro-oxidative cellular changes can exacerbate the inflammatory response, since engulfment of intact apoptotic cells was reduced in the presence of ox-LDL and in its absence, rapid phagocytosis suppressed macrophage inflammatory cytokine release, suggesting a link between high lipid levels, inflammation and possibly angiogenesis (17). *In vitro* studies have demonstrated that stimulation of HUVEC with ox-LDL up-regulates adhesion molecules (including intracellular adhesion molecule -1 (ICAM-1), E-selectin and P-selectin), inflammatory proteins including interleukin-6 (Il-6), thrombotic factors including tissue factor and remodeling proteins such as matrix metalloproteinase (MMP)-2 and MMP-9, many of which are also stimulators of angiogenesis (18).

Medial and intimal thickening induced by hypercholesterolemia may result in a limited supply of oxygen and nutrients reaching these areas from either the lumen and/or VV, resulting in a hypoxic environment. Since the major outcome of hypoxia is increased vascularization, intraplaque vessels may proliferate in association with this potent stimulus. Hypoxia-inducible factor (HIF-1) is expressed in hypoxic regions of expanding and developing plaques, and directs migration of endothelial cells (EC) towards the hypoxic environment via direct HIF-1 binding the regulatory gene of Vascular Endothelial Cell Growth Factor (VEGF) and subsequent induction of VEGF transcription (19). VEGF is a potent angiogenic growth factor, stimulating EC mitogenesis and blood vessel formation via activation of intracellular signaling intermediates including mitogen-activated protein kinase 1/2 (MAPK1/2) and src. Increased expression of VEGF and its receptors in hypoxic areas, in association with interaction with cell membrane integrins including $\alpha5\beta3$, is one of the main causes of vessel leakiness (20). Leaky plaque VV have been identified by ultrastructural visualization of defects between endothelial tight, gap and adherens junctions, and VEGF is known to affect junctional adhesion molecule expression, block gap junctional communication between adjacent endothelial cells and disrupt tight junctional communication through a src-dependent pathway (21).

Oxidized phospholipids such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (Ox-PAPC), also prevalent in atherosclerotic lesions, can also up-regulate VEGF expression. In addition, they can regulate leukocyte-endothelial cell interactions and induce expression of inflammatory cytokines from local endothelial cells, monocytes and macrophages (22).

Hence, a large number of signaling intermediates could be operating within the micro-environment of rapidly remodeling restenotic lesions, but due to the complexity of this process, can we really expect to produce a solution to prevent re-formation of hyperplasia and stent thrombosis by blockade of individual signaling pathways, or do we need an approach using combinational therapy?

THE INFLAMMATORY RESPONSE AND VASCULAR REMODELLING DURING RESTENOSIS

As mentioned briefly above, one of the earliest signs of damage following removal of existing plaque by balloon catheters or implantation of a stent is initiation of the inflammatory reaction. Both methods of treatment result in the induction of a systemic inflammatory response, the extent of which can be measured using the marker C-reactive protein, higher levels generally suggesting a worse clinical outcome (23). Insertion of stents/drug eluting stents may exacerbate endothelial dysfunction and delay vascular healing, a critically important process for protection of the intima against subsequent immune cell infiltration and hyperplasia (24,25). In fact, endothelial dysfunction and incomplete neointimal coverage of the stent struts are the main cause of subsequent late stent thrombosis (26). Present conventional research is therefore directed to delivery of therapeutics to attenuate the inflammatory response, alleviate endothelial dysfunction, and produce and maintain a protective and coherent endothelial cell barrier.

Several “generalized” studies have attempted to provide an overview of transcriptional changes occurring following arterial balloon injury. The most useful example, carried out by Zhang (27), employed a rat carotid artery balloon-injury model and measured changes in gene transcription from 1-28 days after injury. They found rapid induction of a large number of pro-inflammatory genes including tumor necrosis factor-alpha (TNF-α) followed by an increase in expression of important angiogenic molecules including CD44 and Cxcl12. They concluded that many of the genes for de-regulated via altered production in mesenchymal stromal cells which could therefore be a potential target for clinical intervention.

INFLAMMATION, ENDOTHELIAL CELL ACTIVATION AND PROLIFERATION. THE KEY TO PREVENT RESTENOSIS?

Attempts to inhibit inflammation have met with some success using both animal models of restenosis and in humans. Although some of these studies have been performed using a carotid model of restenosis, they should be translatable to coronary disease and hence are relevant to this review. Since leukocyte recruitment mediated by up-regulation of adhesion molecules on the endothelial cell surface is a key instigator of the early stages of this process, Qu (28) applied a lentiviral construct containing siRNA directed against vascular endothelial cell adhesion molecule-1 (VCAM-1) and applied it to rats prior to carotid surgical mechanical de-endothelialization (CSMDE). The importance of inhibiting VCAM-1 was shown by a significant reduction in stenosis/increase in blood velocity, and a decrease in the intima/media ratio compared with control, untreated animals. A similar study employed adenoviral delivery of A20 protein (a zinc finger protein which is a negative regulator of tumor necrosis factor-induced signaling pathways and therefore inflammation) to rat carotid arteries prior to balloon angioplasty (29).

A20 was able to down-regulate adhesion markers and chemokine production (e.g. ICAM-1; monocyte chemotactic protein-1) as well as adventitial neovascularisation. These are all key requisites for macrophage trafficking and their results demonstrated that administration of A20 significantly reduced intimal hyperplasia and macrophage infiltration making it a potential drug candidate for inhibition of restenosis. Other methods that have been used to reduce inflammation include the use of simvastatin coated liposomes delivered intravenously (single injection). Fourteen days after balloon carotid artery injury, a significant reduction in proliferation of monocytes and macrophages was noticed concomitant with suppression of neointimal formation (30). These results suggest that there may be alternative approaches to delivery of drugs for control of restenosis and these will be described in greater detail later in the chapter.

Since the introduction of metal stents became the most popular method of treatment, experiments have been performed to examine the effects of concomitant anti-inflammatory treatments with effects determined by direct measurements of intimal growth and hyperplasia by coronary angiography as well as histological approaches and measurements of systemic circulating immune cells to examine the anti-inflammatory efficacy. Pesarini et al. (31) treated a group of patients who had received coronary bare metal stents with high dose oral prednisone (a synthetic corticosteroid which acts as an immunosuppressant and is highly anti-inflammatory) over a period of 40 days following the implant. Some of the major pro-inflammatory cytokines including TNF-α, interleukin-6 and NF-κB were significantly reduced as measured in patient's serum as was late luminal loss and this was associated with reduction in their synthesis by circulating monocytes. In another study, inhibition of p-38 mitogen activated protein kinase signaling (which is associated with the stress response) by treatment with the pharmacological inhibitor SB-681323 (28 days), significantly reduced inflammation as measured by decreased circulating levels of C-reactive protein suggesting that direct inhibition of intermediate signaling proteins could also limit post-stent restenosis after percutaneous coronary intervention (32). Although many other studies have examined individual components of the endothelial-inflammatory complex in an attempt to minimize immune cell infiltration and cellular activation following stent the most effective treatment based on a mono- or duel-therapeutic strategy might be to inhibit key adhesion molecules associated with transmission and/or activation of membrane bound receptor-linked primary signaling effectors associated with down-stream pro-inflammatory cascades. An example of this might be inhibition or knockdown of platelet derived endothelial cell adhesion molecule-1 (PECAM-1) together with NFκB and or TNF-α or its receptors. This strategy has shown promise with several published studies, one demonstrating notable reductions in inflammation and restenosis in PECAM-1 knockout mice via a reduction in activation of the NFκB/AKt pathway (33).

Endothelial cell activation and angiogenesis in medium-long-term restenosis: In the early phase response, the composition of neointima covering the metal struts is associated with the healing response and consists mainly of ECM material and vascular smooth muscle cells. However, since in-stent restenosis is associated with longer-term incidents of thrombosis and myocardial infarction, usually 5-10 years after implant, it is important to understand if and how the composition of "plaque like" material changes to become destabilized over this period of time? Takano et al. (34) used intracoronary Optical Coherence Tomography (OCT) to characterize the tissue components in patients approximately 6 months and 5 years after stent implant. They found after 6 months that approximately 60% of the patients demonstrated with neovascularized areas in the persistent zone. In the late phase group, intra-intimal neovascularisation was found in approximately 86% of patients studied. Interestingly they found no direct significant correlation between intraintimal neovascularisation and thrombus formation. However, neovessel development, which was highest in lipid rich areas (associated with intimal disruption and thrombosis), may have a role in promotion of intimal expansion, recruitment of inflammatory cells to the region, subsequent haemorrhage and contribute to plaque instability as occurs during normal atherosclerotic plaque formation. Knowledge of the pathobiological mechanisms which are responsible for promotion of angiogenesis in restenotic plaques could help in preventing their de-stabilization.

It is well known that many of the chemokines and growth factors produced by infiltrating leukocytes/monocytes/macrophages are pro-angiogenic, and that these may also have activating effects on other cells of vascular origin (35). Vogt (36) showed that down-regulation of angio-associated migratory cell protein (AAMP) inhibited smooth muscle cell migration and neointima formation in a porcine model of coronary restenosis, whilst increase in the concentration of more conventional factors such as VEGF and fibroblast growth factor-2 (FGF-2) also leads to peri-adventitial angiogenesis and intimal thickening in animal models of restenosis (37).

Endothelial progenitor cells and re-endothelialization. Although a discussion of the importance of endothelial progenitor cells in the pathobiology of restenosis has been mentioned in other chapters of this book, their importance in re-initiation of and maintenance of proper EC function should be discussed as they could potentially help to re-form the critical luminal barrier to prevent accumulation of inflammatory cells within susceptible hyperplastic regions liable to undergo thrombosis. There is substantial evidence to show that bone marrow-derived endothelial progenitor cells (EPCs) contribute to endothelial repair and neovascularisation in the atherosclerotic environment, perpetuated by signals emanating from local inflammatory cells (38). Also, in patients who have impaired production and lower circulating levels, such as those with risk factors for coronary artery disease such as diabetes, hypercholesterolaemia and smoking, statistics demonstrate an increased risk of cardiovascular events.

However, Pelliccia (39), showed that higher numbers of circulating EPCs were associated with increased restenosis in patients with stable angina, similarly, significant increases in stem cell mobilization were observed following coronary bare metal stent implantation in patients, and this was associated with activation of MAC-1 (a leukocyte specific integrin) and subsequent production of MMP-940. So here we have a complex situation where increased EPCs can help to repair dysfunctional endothelium and reduce late stage thrombosis, whilst on the other hand, influx of large numbers of EPCs during the inflammatory acute phase following angioplasty/stent implant, could contribute to more rapid development of restenosis. To combat the deleterious effects of an "over" influx of EPCs, Fukuda (41) showed that sirolimus (otherwise known as rapamycin-an immunosuppressant) coated stents although reducing inflammation following implant, also resulted in increased late-stage thrombosis due to a lack of re-endothelialization. When they treated patients' with fluvastatin, which is known to increase the number of circulating EPCs, re-endothelialization was stimulated and the effects reversed. These results suggest that a combinational treatment such as this may be beneficial for patients in receipt of modified coronary/carotids stents.

NOVEL AND EMERGING STRATEGIES/CONCLUSIONS

Integration of nanotechnology into stent design could improve efficiency and time-course of drug delivery. As we have mentioned previously, coating of stents with anti-proliferative, anti-inflammatory drugs such as paclitaxel and sirolimus demonstrates lower rates of restenosis over a 6 month period, but also prohibits normal vessel remodeling, improper integration of the stent into the vessel wall, endothelial dysfunction and incomplete coverage, all of which could result in late-stage thrombosis (42). Nanoporous stent surfaces such as those containing aluminium oxide or carbon nanoparticle matrices have been used to deliver anti-inflammatory compounds, whist nano-texturing may be able to enhance the interaction between endothelial cells and stent surfaces by increasing cell adhesion (43). Similarly, Epstein (44) formulated gadolinium nanosuspensions of aldendronate (a liposomal biphosphonate) inhibited macrophage growth in vitro and also IL-1-β and TNF-α following a single IV dose in a rat model of carotid artery vascular injury (balloon catheter endothelial denudation).

The ultimate aim would be to employ nanotechnology to combine the integration of non-porous stent surfaces which can deliver drugs on a "time release" basis (45) whilst promoting endothelialization thus reducing restenosis and late-stage mortality due to thrombosis (**Fig. 4**).

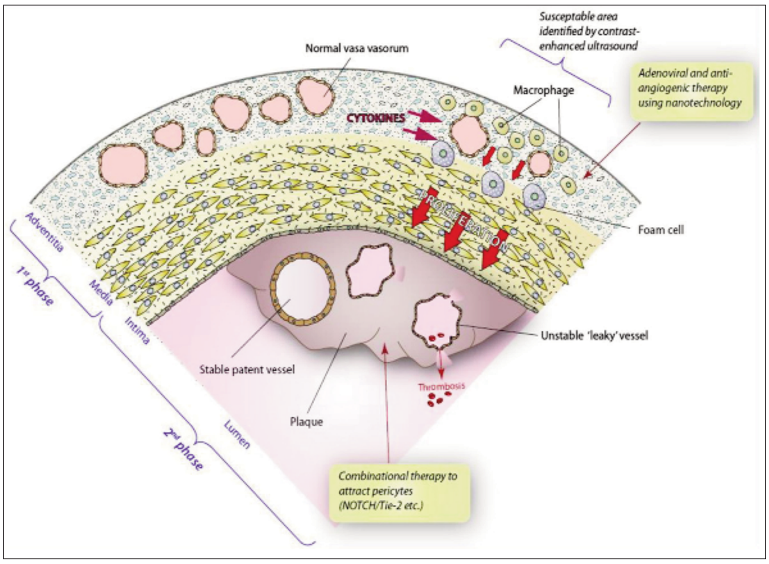

Figure 4. Hypothetical representation of a micro-area of a major artery showing vasa vasorum and potential hypoxic micro-environment responsible for cellular activation, endothelial cell proliferation and ultimately plaque development in restenosis. Phase 1 treatment may involve prevention of vasa vasorum proliferation by administration of targeted siRNA or lentiviral vectors to block relevant gene expression. Phase 2 treatments would be designed to induce stabilization of vessels liable to rupture and bleed by administrating a mixture of factors to induce maturation of fragile vessels. Vascularization in atherosclerosis/restenosis represents a complex interaction between multiple molecules, manipulation of which could benefit patients by slowing down the process of its development and stabilizing developing stenotic regions.

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