Chapter 17

Therapeutic Angiogenesis: Foundations and Practical Application

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Abstract

Angiogenesis as therapeutic target has emerged since early works by Judah Folkman, yet his "holy grail" was inhibiting vascular growth to block tumor nutrition. However, in modern biomedicine, "therapeutic angiogenesis" became a large field focusing on stimulation of blood vessel growth for ischemia relief to reduce its detrimental effects in the tissues. In this review, we introduce basic principles of tissue vascularization in response to ischemia exploited in this field. An overview of recent status in therapeutic angiogenesis is given with introduction to emerging technologies, including gene therapy, genetic modification of cells ex vivo and tissue engineering.

Keywords: therapeutic angiogenesis, growth factors, cytokines, gene therapy, cell therapy, plasmid, viral vector

1. Introduction

Blood vessel growth is a natural process driven by multiple stimuli of which hypoxia is one of the strongest inducing potent response until O_2 pressure is normalized by the blood coming through de novo formed vasculature. However, a large group of diseases is caused by hypoxic or ischemic state of tissue. These include peripheral artery disease (PAD) and intermittent claudication (IC), coronary heart disease (CHD), myocardial infarction (MI) and ischemic stroke. Accompanied by endothelial dysfunction and age-related reduction of angiogenic response, they result in disabilities and mortality rate of 25–25% annually. Existing strategies for surgical bypass or endovascular interventions have limited efficacy as far as a cohort of non-option patients expands reaching 25–50% after certain extent of disease progression. Moreover, long-term prognosis after most interventions is negative as grafts undergo restenosis and vascular

biocompatible prosthetics are yet to come for wide application. This drew attention of physicians and researchers to the concept of angiogenic therapy to stimulate body's own resource and form new blood vessels to relieve ischemia. During recent decade the field of biomedicine known as *therapeutic angiogenesis* evolved rapidly using protein delivery, gene therapy, cell therapy and tissue engineering for induction of vessel growth and overview of its basic concepts and recent achievements will be presented to the reader in chapters below.

2. Biological foundations of therapeutic angiogenesis

Postnatal growth of blood vessels is mediated by three mechanisms: vasculogenesis, angiogenesis and arteriogenesis [1]. Vasculogenesis is de novo formation of vasculature from specific progenitor or stem cells; however, it is attributed to prenatal period and after birth its role is unclear [2] and major extent of blood vessel formation involves two other mechanisms focusing our attention on them. Molecular and cellular basics underlying these processes became the cornerstones of therapeutic angiogenesis and become the source of novel objects for applied researchers and translational medicine.

2.1. Angiogenesis: hypoxia-driven growth of blood vessels

Angiogenesis is the formation of a blood vessel de novo, yet in contrast to vasculogenesis, it relies on migration, proliferation and sprouting of existing endothelial cells (EC) comprising capillaries. The latter are small (8–15 μm) vessels lacking tunica media responsible for majority of tissue blood supply and O_2/CO_2 exchange [3]. Reduction of tissue O_2 induces angiogenesis response in health (intense exercise, tissue growth, etc.) and in disease: in the case of interrupted or declining supply due to atherosclerotic lesions or anemia [4]. Under normal condition, capillaries are stabilized by autocrine and paracrine stimuli (Notch1 axis, angiopoietins, thrombospondin, angiostatin, transforming growth factor (TGF)-β, etc.) that balance influence of pro-angiogenic cytokines within blood vessels' vicinity (vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF)). Hypoxia dislodges this balance toward angiogenic events and this is mediated by O_2-sensitive system existing in a variety of cells including EC themselves, smooth muscle cells (SMC), pericytes and fibroblasts. Cells respond to hypoxia via a system of hypoxia-induced factors (HIFs) [5]—a group of heterodimeric transcription regulators controlled by O_2-sensitive prolyl hydroxylases. Briefly, stability of HIFs is increased drastically in hypoxic environment resulting in their binding to hypoxia-responsive elements within promoter regions of genes increasing their expression [6]. HIF-dependent genes include a vast array of cytokines stimulating EC proliferation, blood vessel sprouting and, thus, labeled "angiogenic growth factors" [7, 8]. The latter include soluble growth factors associated with EC proliferation and differentiation (acidic FGF (aFGF), basic FGF (bFGF), HGF, VEGFs) [9, 10] and cytokines bound to extracellular matrix (ECM) and released during its cleavage [11]. These changes induce EC proliferation and migration forming a vascular sprout guided by a "tip cell." This cell follows a gradient of concentration and produces matrix metalloproteinases (MMPs) and urokinase (uPA) to cleave the ECM [12], releasing growth factors and basically tunneling ECM followed by "stalk cells" that form a new capillary [13]. After lumen

formation occurs normalized blood supply switches off hypoxic stimuli, "tip cells" lose their phenotype and proteolytic potential [14] commencing microenvironment stabilization. Expression of tissue metalloproteinase inhibitors and Dll4-Notch1 axis [15] induction in EC is followed by reestablishment of a balanced state between pro- and antiangiogenic molecules in the tissue leaving a new capillary-sized blood vessel [16]. However, it should be mentioned that this sequence of events never occurs as a perfectly tuned mechanism. "Stub" branches are formed and must be removed, certain "tip cells" fail to form a sprout and maturation of vascular network includes dissociation of certain anastomoses [16], which overall describes angiogenesis as a dynamic process modulated by multiple stimuli [17]. Finally, under influence of stabilizing signals from surrounding EC, pericytes and stromal cells, the vascular bed returns to normal steady state.

2.2. Arteriogenesis: shear stress-induced vascular remodeling

Arteriogenesis is triggered by rise of shear stress after an occlusion and induces collateral vessel remodeling forming a bypass 20–100 μm in diameter with developed tunica media. Arteriogenesis may occur gradually (e.g., in increasing stenosis of a large-caliber artery) or can be triggered by a rapidly developed occlusion with both situations are to result in effective blood flow delivery "around an obstacle" to distal portions of the limb or organ [18]. Certain studies show that collateral remodeling can be reversible till certain point of this process in case shear stress drops to normal after thrombolysis or surgical thrombectomy [19]. Effective arteriogenesis may bypass up to 30–40% of basal blood flow in critical stenosis and thrombosis, which is sufficient for tissue survival. However, its efficacy is drastically reduced in disease and with aging [20]. Smoking-related hypercoagulation, hypertension and diabetes also limit arteriogenic response resulting in critical level of ischemia and tissue loss [21].

After pressure rise in collaterals above the site of thrombosis, shear stress induces EC membrane deformation and flow-sensitive ion channels activate downstream MAP-kinase (ERK1/2, Rho, etc.) phosphorylation and expression of, growth factors, adhesion molecules and chemokines (interleukin-8, macrophage chemoattractant proteins, etc.) [20]. Eventually leukocytes begin to "roll" on EC surface resembling inflammatory changes of vascular function and infiltrate the collateral's wall [22]. Pivotal role in wall thickening is played by monocytes and their differentiated forms—macrophages and dendritic cells. Their function is not limited to ECM and basal lamina cleavage by MMP and uPA production to destabilize the collateral and make it "flexible" [23], but they seem to profoundly change the properties of the blood vessel by induction of SMC proliferation and hypertrophy [24]. Under these influences, media thickness may increase 3- to 4-fold and collateral vessel's volume can enlarge up to 20-fold [25]. Moreover, monocytes produce a wide spectrum of angiogenic and mitogenic cytokines, some of which have antiapoptotic properties required for tissue protection [26]. The role of monocytes and macrophages has been especially emphasized in cardiac arteriogenesis where immunosuppressive steroid hormones [27], anti-inflammatory therapies and even aspirin [28] have been shown to negatively impact the outcomes and collateral remodeling. Toxic depletion of monocytes by clodronate reduced arteriogenesis in cryo-injured myocardium and led to decreased ventricular function and higher mortality [29]. As collaterals increase shear stress stimulus is relieved and EC reduce production of chemokines and lose their "adhesive" phenotype. Macrophages limit production of proteolytic enzymes and start

ECM reconstruction producing collagens, laminin and elastin and forming adventitial and medial portions of a new arterial vessel.

Typically, we mention "therapeutic angiogenesis" referring gene or cell therapy to relieve ischemia. Nevertheless, one may see that angiogenesis and arteriogenesis share common mediators—namely growth factors and enzymes, ECM components, EC activation, etc. Eventually, for adequate function therapeutic angiogenesis has to rebuild both—medium/large-caliber arteries providing influx of blood and capillary-sized vessels that deliver it to the cells.

3. Therapeutic angiogenesis: methods and approaches

3.1. Protein-based therapeutics

After the discovery of proteins with angiogenic effects, the concept of their therapeutic application was introduced by the 1990s and a vast array of animal studies was published to demonstrate angiogenic efficacy of recombinant protein delivery. Going beyond the VEGF family, experimental works showed induction of angiogenesis by FGFs, HGF, PDGF and placental growth factor (PlGF) in small rodents and rabbits [30, 31]. Injection of these cytokines to ischemic tissue or blood vessels increased perfusion and vascular density. However, promise of this method was questioned as far as achievement of local pharmacological concentration by injection was extremely expensive (especially for human body mass) and half-life of most cytokines was too low to render potent effects [32]. Furthermore, little was known on pharmacokinetics of recombinant proteins delivered intravascularly and their potential involvement in tumor growth and chance of "washout" to systemic blood flow raised safety concerns.

In 2000, the first clinical trials of recombinant human bFGF were initiated in PAD/IC patients after a pilot study showing safety and tolerance of intra-arterial delivery of bFGF solution. Unfortunately, it was halted prior to completion of protocol due to urinalysis data revealing proteinuria in bFGF-treated subjects and no positive changes of endpoints at the moment when the trial was put to a premature end [33]. The final attempt to achieve success in the field was the Therapeutic angiogenesis with recombinant fibroblast growth Factor-2 for intermittent claudication (TRAFFIC) randomized placebo-controlled trial in patients with PAD showing significant improvement in walking time and ankle-brachial index (ABI) in bFGF group. However, safety profile was compromised and yet no cardiac adverse effects or evidence for tumor formation was found in recurrent cases of proteinuria and signs of nephrotoxicity were an issue [34].

These results were as disappointing as valuable for the field and suggested that gene therapy with its local sustained expression of desired protein is the best alternative possible [32]. Recently, no further attempts to implicate protein delivery for therapeutic angiogenesis were made in clinics and advantages of other methods are exploited to patients' benefit.

3.2. Gene therapy for angiogenesis

Gene therapy relies on delivery of genetic information by introduction of nucleic acids to target cells/tissues using vector systems. This results in local expression and production of desired protein over a certain period depending on vector used and properties of tissue. First

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experiments indicating possibility of in vivo gene delivery using simple injection of a recombinant plasmid DNA (pDNA) opened the gate for hundreds of studies published within the last two decades [35].

As far as the "cornerstone" of gene therapy is the vector system, a brief overview of existing options is required. General concept in the field is that all vectors can be divided into "viral" and "nonviral" subgroups covering nearly any possible way of genetic material delivery. Among nonviral vectors, pDNA is the most widely used due to its long-studied safety profile, ease of production and low immunogenicity allowing repetitive administration [31, 36]. Moreover, plasmids are feasible for combined delivery of several growth factors by mixing them in a formulation or generating a multicistronic vector. However, low transfection efficacy (0.5–2.0% in various tissues) in large mammals including human is an efficacy-limiting issue for pDNA [37]. Viral delivery systems comprise a broad spectrum of recombinant or chimeric viruses of different capacity having a great potential. The latter is due to high transduction efficacy and long expression period accompanied by tissue tropism in certain viruses. However, disadvantages are safety issues: immune reactions and risk of carcinogenesis due to integration to host genome. The most widely spread vectors include adeno- [38], adeno-associated [39] and retroviruses, yet in therapeutic angiogenesis, the latter have limited application due to high risk of insertional mutagenesis [40]. Recent progress of molecular engineering allowed development of optimized viral systems exploiting their advantages as well as novel more effective pDNA systems [41, 42].

Period of growth factor-based gene delivery dates back to the seminal study by Dr. J. Isner [43] who used injection of pDNA encoding VEGF-A 165 (VEGF165) isoform to succeed in treatment of a non-option patient with critical limb ischemia. "First in-human" data were supported by Baumgartner et al. who found increased collateral formation after intramuscular delivery of VEGF165 and EC proliferation in amputation material providing proof of mechanism [44]. Later a number of vectors using VEGF-A and its isoforms were evaluated in experimental and clinical trials making it the most intensively studied object in therapeutic angiogenesis.

Among numerous clinical examples, one may highlight the first "head-to-head" comparison of adenovirus with VEGF165 (Ad-VEGF165) and liposome-packed pDNA-VEGF165 in PAD patients undergoing angioplasty. The trial showed low clinical efficacy of both approaches—Rutherford severity class stayed comparable to control group yet vascular density was increased after treatment [45]. This and other studies using catheter delivery hinted that this method lacks site specificity and intramuscular injection technique was generally adopted. However, initial Groningen double-blind placebo-controlled trial intramuscular injection of pDNA-VEGF165 in PAD patients with diabetes mellitus failed the primary endpoint (amputation rate), yet improvements in ulcer healing, TcO_2 and ABI were observed [46].

Later, Regional angiogenesis with VEGF (RAVE) trial was the first double-blind placebo-controlled trial of VEGF-A 121 isoform (VEGF121). This cytokine is considered to have better solubility than VEGF165 isoform as it lacks a heparin-binding domain [47]. Key feature of this study was an attempt to perform dose optimization of Ad-VEGF121 dividing 105 patients with PAD/IC into three subgroups that received a single session of 20 intramuscular injections of AdVEGF165 delivering low dose (4×10^9 particles), high dose (4×10^{10} particles), or placebo. Final assessment after 12 weeks revealed no significant differences in endpoints between

control and treatment subgroups, yet dose-dependent increase of edema adverse effect was observed. Indeed, since first studies delivery of VEGF-A isoforms was haunted by evidence of edema formation due to its influence on endothelial permeability [48] with certain authors claiming this was a putative reason for low efficacy of therapy [49].

Trials in MI patients were initiated as early as in 1998 using a pDNA-VEGF165 showing good safety profile and no positive changes [50]. It was followed by Kuopio Angiogenesis Trial [51] using a comparative design with Ad-VEGF165 or pDNA-VEGF165 delivery by intramyocardial injection during transcutaneous angioplasty. In this trial, no differences between control and treatment groups were found, yet at 6 months after injection of Ad-VEGF165, myocardium perfusion was higher than pDNA-VEGF165, which was attributed to its high transduction efficacy. EuroInject One trial gave similar results showing no significant improvement of myocardial perfusion after injection of pDNA-VEGF165, yet local contractility was higher than control [52].

Trials using delivery of HGF were initiated and conducted by Dr. Morishita's group aiming to treat PAD by intramuscular injection of pDNA-HGF. Encouraging results in animal models [31, 53] promoted clinical translation and after safety assessment a Phase II trial was initiated comparing single and repeated dose of pDNA-HGF in favor of multiple injections: only this dosing regimen showed improvement of TcO_2 compared to control [54]. Further results in a placebo-controlled I/IIa phase trial showed good safety with no traces of secreted HGF in peripheral blood and repeated injection of 8 mg pDNA-HGF showed significant improvements of secondary endpoints (ulcer size, ABI and pain reduction) [55]. Similar results were obtained in a placebo-controlled trial in PAD patients where by the end of week 12, 70% decrease of ulcer size was observed [56]. Further attempts to increase efficacy included the use of a bicistronic plasmid encoding two forms of HGF named dHGF and cHGF. They were evaluated in animal models showing better perfusion after expression of dHGF + cHGF than each one alone [57]. Clinical trial of this approach in PAD patients showed that multifocal intramuscular injections of 4–16 mg of pDNA-dHGF/cHGF resulted in improvement within 3 months independently of dose: rest and walking pains reduced and a trend toward ulcer healing and increase of TcO_2 was observed [58].

Overall, we may expect HGF-based drugs to become the first widely marketed for PAD—in Japan it has been registered under "Collategene" name and now undergoes stage III clinical trial in PAD cohort. Furthermore, despite HGF has never been tested for MI treatment in clinical settings, preclinical assessments indicate that it may be effective as it has antifibrotic and angiogenic mode of action that can be a good option for this disease or subsequent ventricular failure due to tissue scarring [53, 59].

Fibroblast growth factor has been the first used in protein delivery and gene therapy studies were to follow as soon as it gained attention. Therapeutic angiogenesis leg ischemia study for the management of arteriopathy and non-healing ulcer (TALISMAN-201) have evaluated pDNA-FGF-1 in no-option PAD patients [60] and showed improvements as decreased amputation rate within 1 year after treatment [61] and its prospective part showed reduced general mortality in treated subjects [62]. However, phase III placebo-controlled "TAMARIS" (n = 525) trial drew disappointing results and all primary endpoints including amputation events failed to improve after treatment by pDNA-FGF-1 [62, 63]. Similar results obtained in OPTIMIST and

EuroOPTIMIST trials indicated safety and lack of efficacy after treatment and lead to wrapping up of this prospective drug testing. Nevertheless, in a follow-up stage, important safety data showing no increased cancer, stroke, or MI in FGF-treated patients was obtained and positively impacted new proceedings in the field [64].

In MI patients, FGF-4 was delivered using an intracoronary injection of an adenovirus with this gene (Ad-FGF-4) in an Angiogenic gene therapy (AGENT) trial. Result evaluation showed that the only subgroup with reduced size of ischemic myocardium after treatment was female patients when compared to male subgroup. The authors speculated that it may be attributed to higher extent of microcirculatory disorders in females [65, 66] accompanied by fewer critical stenosis typical in men [67]. As far as FGF-4 is known to positively influence endothelial function, this might have been the mechanism for observed changes in the trial. Among other therapeutic factors used for stimulation of angiogenesis, HIF-1α and development endothelial locus-1 (DEL) are both worth a mention as far as they made it to the bedside in recent years using adenovirus or pDNA vectors. However, trials showed minimal improvement in PAD patients and further evaluations were ceased up to date [68, 69].

Overall despite failure to show expected efficacy in clinic, gene therapy is safe and well tolerated by patients showing little evidence although long-term evaluations are yet to be completed. Key obstacle in pDNA-mediated gene therapy relates to transfection efficacy and thus protein production levels after administration [70]. Viral vectors show some promise in solving the problem, yet optimization of dosage regimen, delivery routes and administration protocols also provide a field for further development.

From the point of translational potential, pDNA-based gene therapy has the best safety profile and the best results are definitely yet to come in the following years yet points for improvement are obvious. Efficacy improvement in gene therapy can be achieved by combined approaches basing on the point that angiogenesis is a dynamic process controlled by numerous cytokines, each playing its party in initiation/cessation of different stages. This puts the basis for combined gene therapy to treat ischemia with higher efficacy and it has been supported by experimental findings using VEGF165 combined with another pro-angiogenic growth factor: bFGF [71], PDGF [72], angiopoietin-1 [73], or Stromal cell-derived factor-1α (SDF-1α) [74]. Our previous experience in mouse hind limb ischemia model showed that combination of VEGF165 and uPA [75] or HGF [76] induced angiogenic response more effectively than each factor alone or allowed to reduce pDNA dose for combined delivery [75]. A crucial transcription factor in angiogenesis, HIF-1α, was also used for combined gene therapy with VEGF165 showing good results in animal model [77] as well as bFGF + heme oxygenase-1 (HO-1) [78]. Regarding the latter, it is known that HO-1 is an important regulator of endothelial function with protective function. Its expression is known to induce angiogenesis in ischemic tissues and blockade or knockout reduces EC proliferation and motility and thus capillary growth [79].

Triple combined gene therapy has not been evaluated for angiogenesis, yet a study was published where controlled release scaffolds containing a mix of VEGF165, HGF and angiopoietin-1 or their double combinations were evaluated for enhancing efficacy of endothelial progenitor cell (EPC) therapy. Triple combinations resulted in significantly higher

SMC counts indicating more efficient vessel stabilization due to angiopoietin-1 effects on perivascular cell chemotaxis [80].

Authors claimed that the use of VEGF165 + another cytokine typically leads to decreased edema and vascular permeability: this has been shown for a well-known stabilizing cytokines—angiopoietin-1 [73] and HGF [81]. Thus, another rationale for combined therapy is decrease of certain "side effects" observed in "monotherapy" by VEGF as a key player in gene therapy. The latter point is not limited to reduction of adverse reactions, but also arises from a large spectrum of pleiotropic effects of cytokines. For example, VEGF may act as a pro-inflammatory cytokine by induction of nuclear factor κ-B, while HGF [82] or angiopoietin-1 shows antagonistic effects leading to reduction of VEGF-driven cell adhesion and inflammation [83]. Indeed, this has been confirmed in a number of in vitro tests and skin inflammation model indicating that these properties may be utilized for development of next-generation gene therapy drugs for angiogenesis exploiting pleiotropy of cytokines besides their main angiogenic effect. Another approach is delivery of growth factors in two sessions apart in time: for example, pre-treatment by angiopoietin-1 pDNA resulted in better angiogenic response after subsequent pDNA-VEGF165 injection in mouse hind limb ischemia model hinting time of administration as an important factor for efficacy [84].

3.3. Cell therapy and *ex vivo* modified cells

Cell therapy is a promising tool for regenerative medicine and therapeutic angiogenesis using progenitor or stem cells' ability to self-renew and mediate tissue repair. For potential use of cell therapy for vascular repair, one of the most intriguing findings was the discovery of endothelial progenitor cells (EPC) in circulating blood which hinted involvement of postnatal vasculogenesis in perfusion restoration [85]. However, further works sparkled controversy about EPC phenotype, origin [86], role in recovery from disease and even existence. Report by Prokopi et al. [87] claimed that EPC can be false-detected as endothelium-like (CD31/vWF+) monocytes in cultures due to phagocytosis of residual platelets rich with these protein markers.

Clinical trials up to date focus on delivery of bone marrow (BM) cells for induction of angiogenesis. These studies evaluated effects of BM mesenchymal stem cells (BM-MSC) or mononuclear cells (BM-MNC) delivered by intramuscular or intravascular injection in PAD patients. Most studies supported efficacy and indicated improvement in evaluated endpoints: ABI, pain-free walking distance, TcO_2, ulcer healing, or amputation-free period. However, some pivotal trials are to be mentioned in detail for better understanding of the field's status.

First set of crucial data was obtained during "head-to-head" comparison of different cell types to identify the optimal cell source. In a double-blind randomized study, administration of BM-MSC to diabetic PAD patients with foot ulcerations showed efficacy superior to BM-MNC [88]. Subjects that received BM-MSC showed complete ulcer healing 4 weeks earlier than BM-MNC; perfusion assessment, pain-free walking time, ABI and angiography data also spoke in favor of BM-MSC as a more effective cellular angiogenic agent [88].

However, limitation of BM-based treatment is invasive procedure to obtain material and alternative approach was proposed using peripheral blood mononuclear cells (PB-MNC) mobilized by granulocyte colony-stimulating factor (G-CSF) pre-administration. Feasibility of this

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approach was obvious and a trial was initiated to confirm its efficacy compared to BM-MNC enrolling a total of 150 patients split in two groups. After 12 weeks of observation, PB-MNC patients showed significantly higher limb temperature, ABI and reduced rest pains than BM-MNC. Yet no difference was found in TcO_2, ulcer healing rate and amputation frequency hinting that two methods showed comparable efficacy profile with a trend to PB-MNC application due to feasibility and endpoint improvements [89]. Interestingly, a trial of conventional therapy + G-CSF monotherapy was compared to BM cells and in these groups, improvements in ABI and TcO_2 were comparable and significantly better than in conventional drug therapy control. This was an intriguing finding which showed that mobilization of endogenous mononuclear cells (MNC) was sufficient to replace BM grafting and injection [90].

Another source of cells for therapeutic angiogenesis is adipose-derived mesenchymal stromal cells (AD-MSC). Despite sources of mesenchymal stem cells (MSC) are not limited to adipose tissue, these adult stromal cells can be isolated from samples obtained during lipoaspiration or surgery. Taken together with ease of expansion, well-established phenotype and abundance in healthy individuals, it makes AD-MSC an excellent object for autologous and allogeneic use for angiogenesis stimulation [91]. Published experimental studies show that AD-MSC use their paracrine potential for induction of angiogenesis and support of collateral remodeling [92]. This is referred as "bystander effect" to emphasize that AD-MSC render their effects by paracrine mechanism in contrast to previously existing opinion about their significant ability to differentiate into specific vascular cells and EC in particular [93].

These cells have not been evaluated in PAD or MI clinical trials yet and considered to be a very attractive option to complement existing strategies. Certain factors limiting potency of AD-MSC exist including donors' age [94], comorbidities and effects of *ex vivo* culture [95]. However, improvement can be achieved by manipulation of cells' paracrine activity, e.g., by viral transduction to increase expression of cytokines forming an "alliance of gene and cell therapy" for higher efficacy [96]. This approach has become possible after development of effective viral gene delivery systems as far as pDNA transfection in primary human cultures was extremely low or at the level of toxicity exerted by transfection reagents [97]. Modification of cells intended for therapy use in performed *ex vivo* after sufficient amount of material is obtained in appropriate culture condition. Selection of a viral vector depends on safety precautions and vector capacity for genetic material; however, cDNA of most angiogenic cytokines "fit" into commonly used adenoviruses or adeno-associated virus (AAV).

This method has been tested in animal models of ischemia using exogenous delivery of VEGF165 [98], insulin-like growth factor-1 [99], HO-1 [100], or other genes to different types of cells: AD-MSC, EC, BM-MSC, etc. In majority of reports, modification resulted in improvement of response after delivery to ischemic tissue. In our experience, administration of human VEGF165-expressing AD-MSC to ischemic limb of immunodeficient mice resulted in enhanced perfusion and vascular density superior to control cells. Furthermore, muscle necrosis was minimal in this group indicating enhanced blood supply and antiapoptotic effects of VEGF165 as mode of action [98].

Application of modified stem cells for induction of angiogenesis may be limited in coming years unless safety of modification and full extent of its influence on biological properties of cells is understood. *Ex vivo* modified cells are widely used for treatment of oncology and

hereditary disease where benefit for patient overwhelms existing risks [101]; however, for treatment of PAD and MI, additional measures of precaution will be required prior to active clinical trials. Nevertheless, recently a group led by Dr. J. Laird began a phase I trial to evaluate the use of VEGF-expressing MSC in patients with critical limb ischemia. The trial is now ongoing with expected completion in 2017 and preclinical data indicated good safety profile with long-term expression of VEGF in MSC after viral modification [102]. Recent progress in virus biology and gene engineering allowed development of safer vector systems with controlled expression, integration, or directed insertion to genomic "safe harbors" where they induce minimal to none disturbances [103]. Preclinical evaluation of these systems is expected to give more data on long-term impact of modification and facilitate translation.

4. Cell sheets: minimal tissue-engineered constructs

Cell sheets (CS) were first introduced by Dr. Okano's group and occupied a niche between 3D tissue engineering and 2D cell cultures used to obtain therapeutic cellular materials [104]. Briefly, CS is an attached mono- or multilayered xeno-free construct that consists of viable cells with ECM produced by these cells. Application of this method allowed to circumvent a crucial setback observed in a number of experimental works—poor survival of cells used for therapeutic interventions. One of main reasons for this is procedure of detachment by proteolytic enzymes leading to disruption of ECM (along with deposited cytokines) and loss of intercellular contacts resulting in anoikis and high prevalence of cell death aggravated by passage of cells through a catheter or needle causing mechanical damage. Loss of cells implanted to the tissue by injection in suspended form is estimated as 40–75% within the first 3 days [105], while CS limits this damage to minimum keeping the cells viable after delivery and enhancing their engraftment [106]. Furthermore, ECM proteins delivered as a part of the construct are known to have a beneficial impact on regeneration and do not have toxic or immunogenic features of chemical or xenogeneic scaffolds. Generation of CS is possible from MSC, fibroblasts, EC, skeletal myoblasts, induced pluripotent stem cells and cardiomyocytes derived from them, BM cells and cardiac progenitor cells [107]—literally, any adherent cell culture after it produces enough ECM to stand mechanical manipulation [108]. CS can be used to cover a significant surface making it a good technique for superficial lesions, cardiomyoplasty and ophthalmologic and microsurgical manipulations. Numerous clinical trials are being run in Japan these years to reveal their full potential in a wide array of disorders [109].

In relation to angiogenesis, this technique was evaluated in MI models using CS from skeletal myoblasts, AD-MSC, or cardiac progenitor cells showing their ability to generate vascularized additional layer of tissue and facilitate vascular growth in underlying tissue [110, 111]. This resulted in improved ventricular function, limited MI size and fibrosis and favorable outcomes in experimental animals. Comparative study of CS vs. injection of suspended cells showed CS to be superior in terms of most functional and histological endpoints analyzed and using a bioluminescent method, the authors reported higher survival of transplanted rat neonatal cardiomyocytes after CS delivery compared to injection [112]. Recently, clinical

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application of CS from autologous skeletal myoblasts has begun to treat severe heart failure patients with left ventricular assist devices. Delivery of multilayered constructs resulted in ejection fraction increase sufficient to remove the device and postpone heart transplant as well showing good potential of this approach [113].

In limb ischemia and diabetes, CS are generally considered to be a tool for ulcer treatment and indeed numerous clinical trials have been initiated within last years. However, our group has been extensively investigating application of CS as an angiogenic therapy in PAD. We have found that subcutaneous delivery of CS from AD-MSC to mice with limb ischemia resulted in robust angiogenic response and CS were superior to dispersed cells in terms of tissue perfusion and vessel density [114]. This piece of evidence provided basis for CS application in PAD indicating that their potential is not limited to cutaneous healing but that paracrine factors are capable to induce angiogenic response in ischemic muscle. Our data were supported almost at the same time in a study by Bak et al. who used mixed CS from SMC and EC for successful treatment of experimental limb ischemia in mice by subcutaneous delivery [115].

Further improvement of CS potential is possible by application of *ex vivo* modification to express growth factors and discussed above. Our group's experience with viral vectors expressing VEGF165 suggested robust increase of angiogenesis in MI and limb ischemia after delivery of sheets from AD-MSC expressing VEGF165 after viral transduction [114, 116]. Effect of these constructs was superior to control CS and we observed no changes in immune response to genetically modified sheets or cell proliferation/viability within them [114].

Overall, application of CS for therapeutic angiogenesis is a new field and its expansion is expected within next years. These constructs are feasible from a translational point of view as far as they do not contain xenogeneic, artificial, or cadaveric materials circumventing many ethical and safety problems in translation.

5. Concluding remarks

Overall, therapeutic angiogenesis has accumulated a "critical mass" of evidence and approaches that would allow its application in practice within the next 10–15 years expanding the capabilities of treatment. However, possibility to shift from initially used non-option or critical patients may lead to better results in clinical trials, especially in gene therapy, where numerous failures put the whole concept under question several years ago. Development of cell therapy was accompanied by a large framework of regulatory, legal, ethical and industrial work to ensure safety and patients' benefit. Number of clinical trials is growing every year and fortunately no serious evidence for adverse events or other risks for subjects' health and well-being was found up-to-date.

Therapeutic angiogenesis has become one of the pioneer methods in translational medicine and its full potential is yet to be unleashed especially in the field of *ex vivo* modification and tissue-engineered approaches to increase efficacy and ensure safety.

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Abbreviations

AAV	adeno-associated virus
(a/b)FGF	(acidic/basic) fibroblast growth factor
ABI	ankle-brachial (pressure) index
AD-MSC	adipose-derived mesenchymal stem cells
BM-MNC	bone marrow mononuclear cell(s)
BM-MSC	bone marrow mesenchymal stem cell(s)
CHD	coronary heart disease
CS	cell sheet
EC	endothelial cell(s)
ECM	extracellular matrix
G-CSD	granulocyte colony-stimulating factor
HGF	hepatocyte growth factor
HIFs	hypoxia-induced factors
HO-1	heme oxygenase-1
IC	intermittent claudication
MI	myocardial infarction
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell(s)
PAD	peripheral artery disease
PB-MNC	peripheral blood mononuclear cell(s)
pDNA	plasmid DNA
PlGF	placental growth factor
SDF-1α	Stromal cell-derived factor-1α
SMC	smooth muscle cell(s)
TcO_2	transcutaneous O_2 pressure
TGF	transforming growth factor
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
PDGF	platelet-derived growth factor

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