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Review

Cardiovascular risk factors impair native collateral development and may impair efficacy of therapeutic interventions

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Animal and early clinical studies of gene therapy for tissue ischaemia suggested that this approach might provide benefit to patients with coronary artery disease not amenable to traditional revascularization. This enthusiasm was then tempered by the subsequent disappointing results of randomized clinical trials and led researchers to develop strategies using progenitor cells as an alternative to improve collateral function. However, the recent publication of several randomized clinical trials reporting either negative or weakly positive results using this approach have led to questions regarding its effectiveness. There are several factors that need to be considered in explaining the discordance between the positive studies of such treatments in animals and the disappointing results seen in randomized patient trials. Aside from the practical issues of arteriogenic therapies, such as effective delivery, vascular remodelling is an extraordinarily complex process, and the administration of a single agent or cell in the hope that it would lead to lasting physiological effects may be far too simplistic an approach. In addition, however, evidence now suggests that many of the traditional cardiovascular risk factors—such as age and hypercholesterolemia—may impair the host response not only to ischaemia but, critically, also to treatment as well. This review discusses the evidence and mechanisms for these observations and highlights future directions that might be taken in an effort to provide more effective therapies.

1. Introduction

Animal and early clinical studies of single angiogenic agents—such as VEGF and bFGF—generated enthusiasm that these agents might enhance collateral development (collaterogenesis) and thereby provide alternative therapies for patients with coronary artery disease not amenable to traditional revascularization. The enthusiasm, apparently justified by the subsequent results of small non-randomized phase-I clinical trials, was then tempered by the subsequent disappointing results of randomized clinical trials, which included the administration of single angiogenic agents either as the protein or as the gene encoding the protein.[1–9] Similar enthusiasm generated by animal and small clinical trials for strategies using progenitor cells to improve collateral function has also been tempered following the publication of larger randomized clinical trials that reported either negative results or, if positive, lacked robust biologic relevance.[10–12]

There are several factors that might explain the discordance between the positive studies in animals and the disappointing results seen in randomized patient trials. First, collaterogenesis involves extraordinarily complex processes leading to the balanced and coordinated expression of many growth factors.[13–15] An example of its complexity is the result of our study in which DNA expression profiling was employed to determine the course of differential expression of 12 000 genes after femoral artery ligation in C57BL/6 mice.[16] Tissue was harvested from the non-ischaemic adductor muscle that lies proximal to the site of ligation but which contains developing collaterals. Ligation caused the differential expression (greater than two-fold) of 783 genes at one or multiple time points: 518 were induced and 265 were repressed.

In addition, collateral remodelling is triggered in part by an increase in shear stress, which occurs in the small, high resistance, low flow arterioles that run parallel to the occluded main arterial channel. The increase in shear stress has multiple effects, among which is the expression of various adhesion molecules and chemoattractant molecules—these contribute to the homing of multiple cellular

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elements with collaterogenic activities. For example, $CD8^+$ T cells are one of the first responders in the processes required for collaterogenesis.[17] When these cells infiltrate the region of developing collaterals, they express IL-16, which contributes to the recruitment of CD4+ T cells and ultimately to the recruitment of macrophages.[18] These cells, including $CD3^+CD31^+CXCR4^+$ T cells (3, below), in turn secrete many of the numerous cytokines that play a critical role in collaterogenesis, such as VEGF, nitric oxide, and monocyte chemoattractant protein 1 (MCP-1).[19] These events lead to endothelial and smooth muscle cell proliferation, migration, vessel remodelling, and synthesis of extracellular matrix (*Figure 1*).

The signalling pathways induced by increased shear stress involve, as noted, the activation of eNOS with the accompanying expression of NO. Mice with impaired NO expression ($eNOS^{-/-}$ mice) exhibit a reduced capacity to develop collaterals in response to acute femoral artery ligation,[20] an observation that early studies showed was not improved by VEGF administration. This suggested that NO acts downstream from VEGF and is critical for this molecule's collateral enhancing activity. Subsequent studies identified AKT phosphorylation of eNOS as a key step in VEGF-induced endothelial cell migration, and in itself leads to NO generation.[21,22] The AKT/eNOS pathway is also believed to be involved in shear stress-induced NO expression, thereby indicating a role of NO not only in angiogenesis, but also in collateral remodelling.[23] In addition, VEGF-induced mobilization of endothelial progenitor cells from bone marrow is reduced in mice deficient in eNOS, an effect that might also contribute to NO-related collaterogenesis.[24] Thus, the capacity of eNOS (a downstream target of several angiogenic molecules and the shear stress signaling pathway) to generate NO importantly contributes to collaterogenesis; conversely, when this pathway is deficient, collaterogenesis is impaired.

In light of the complex processes described above, the administration of a single agent in the hope that it would lead to lasting physiological effects may be far too simplistic an approach. Cell therapy attempted to circumvent this limitation, as progenitor cells secrete multiple cytokines and growth factors, many of which have known angiogenic potential. The negative, or at best modest, efficacy of randomized cell therapy trials to date might partly be explained, as with gene therapy—by the complexity of collaterogenesis.[18,25,26] However, other factors including cardiovascular risk factor impairment of the host response must also be considered.

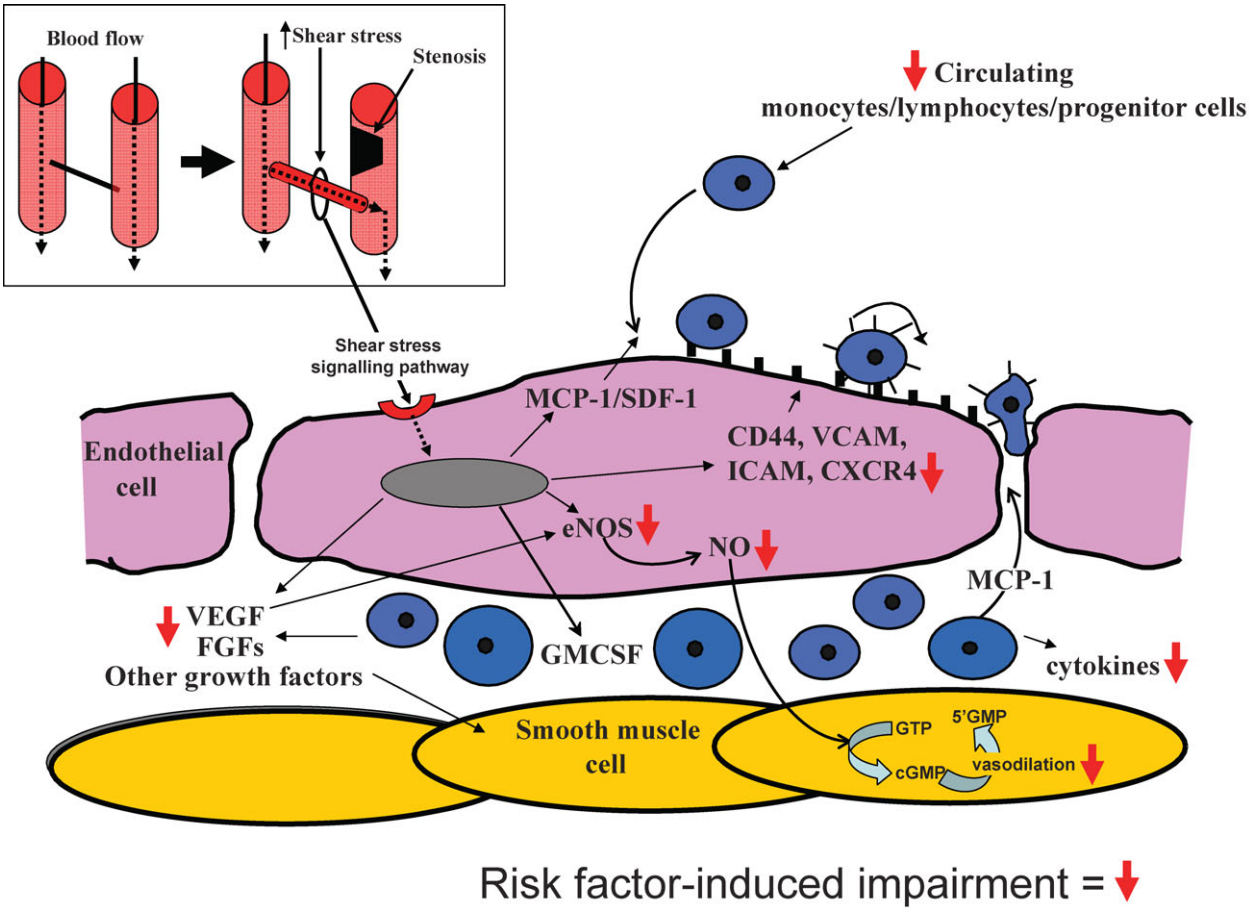

Figure 1 Insert (upper left) illustrates a non-functioning collateral between parallel circuits in the absence of flow obstruction. Following proximal occlusion, there is a pressure drop across the pre-existing collateral, leading to increased shear stress activating complex intracellular signalling cascade involving, among many others, up-regulation of various adhesion molecules and MCP-1. The latter facilitate migration of circulating monocytes into the subintimal space where they express multiple factors, including VEGF, FGF, transforming growth factor, and PDGF. These products act in a coordinated manner leading to smooth muscle cell growth and vessel remodelling. Red arrows indicate potential negative impact of cardiovascular risk factors on the steps involved in native collateral development.

1.1 Risk factor-induced impairment of native collaterogenesis and response to therapy

Many of the risk factors predisposing to atherosclerosis (present in most patients who participate in such trials) impair the innate collaterogenic responses to arterial obstruction and tissue ischaemia (*Figure 1*). Thus, studies have suggested that age, hypercholesterolemia, genetic susceptibility, diabetes, and smoking, frequently present together, all adversely affect the *intrinsic* capacity for collaterals to develop. Although not yet proven, an *intervention* that enhances collaterogenesis in a young healthy mouse or rat may, like intrinsic collaterogenesis, be ineffective in a patient in part because of an inability to overcome the host resistance to therapy.[27]

1.1.1 Potential mechanisms

Risk factor-induced impaired collaterogenic capacity could involve any of the multiple steps involved in collateral development: depressed mobilization of bone marrow-derived progenitor cells, decreased angiogenic efficacy of the multiple angiogenic effector cells, and/or diminished responsiveness of the target tissue (developing collaterals) to the multiple angiogenic stimuli. If any of these deficiencies exist, they could translate into not only a diminished intrinsic collateral response, but also into an impaired responsiveness of the patient to virtually any angiogenic intervention.

Thus, in patients with multiple risk factors: (i) mobilization strategies using such agents as GCF or GCSF may not optimally mobilize effector cells from the bone marrow; (ii) reduced responsiveness of developing collaterals would interfere with the beneficial effect of any intervention; and importantly; (iii) there is good evidence that risk factors cause dysfunctionality of the very stem and progenitor cells that are not only involved in intrinsic collaterogenesis responses, but also are the focus of an increasing number of trials testing the hypothesis that autologous administration of these cells will enhance collateral development.

2. Individual risk factors and their effects on collateral development

2.1 Mechanisms of impaired vascular responses in the elderly

Clinical studies demonstrate adverse outcomes following myocardial infarction in the elderly.[28,29] Although multiple mechanisms undoubtedly contribute to the higher risk, aged mice have impaired collaterogenesis; it is therefore likely that inadequate collateral development contributes to the adverse outcome of older patients.

The transcription factor hypoxia-inducible factor (HIF) plays a pivotal role in coordinating tissue response to ischaemia.[30,31] Under hypoxic conditions, HIF proteins bind to DNA hypoxia-responsive elements (HRE) augmenting the transcription of several factors including VEGF, VEGFR2, insulin-like growth factors, erythropoietin, nitric oxide, and many of the enzymes involved in glycolysis.[32,33] Under normal oxygen tension HIF protein is almost instantaneously degraded by an oxygen-dependant ubiquitin–proteasome complex. In young animals, the onset of cellular hypoxia rapidly switches off this pathway and the intra-nuclear levels of HIF protein increase.[34,35] However, although HIF mRNA levels are equivalent in smooth muscle cells from senescent rabbits exposed to hypoxia when compared to younger animals, levels of HIF protein itself are significantly lower than in younger animals.[36] It is postulated that the normal suspension of proteasomal degradation that stabilizes HIF proteins under hypoxic conditions fails to occur with increasing age, although the exact molecular defect as yet remains obscure. In a further study of the effect of aging on HIF signalling, although HIF protein levels in liver, kidney, and brain extracts appeared to increase appropriately with the onset of tissue ischaemia, the formation of HIF/HRE complexes was profoundly reduced.[37]

In addition, it has been shown that JAK-2 phosphorylation is less responsive to SDF-1 in EPCs derived from patients with CAD compared with healthy volunteers.[38] The CXCR4 receptor is an important mediator of EPC homing to sites of vascular injury, and its downstream signalling cascade (which includes JAK-2 phosphorylation) influences the migratory and angiogenic capacities of cultured human EPCs. It has not been determined which of the risk factors, or combination of risk factors, is responsible for this defect, but age may be one such factor.

Changes in the cellular inflammatory response to ischaemia occur in older animals. Migration of human inflammatory cells across the endothelial cell barrier is an essential component of the vascular response to ischaemia, and although migratory lymphocytes ($CD4^+$ and $CD8^+$ subgroups) may be enriched in elderly subjects, increases in transendothelial migration are not observed.[39] Similarly, T-lymphocyte infiltration into ischaemic skeletal muscle was four-fold lower in elderly rabbits compared to controls.[40] The critical role of T-lymphocytes in modulating tissue responses to ischaemia is now well established.[18] As well as a direct source of pro-angiogenic growth factors such as VEGF and FGF, T-lymphocytes also stimulate endothelial cells to produce VEGF through a CD40-derived pathway.[41,42] Thus, dysfunctional lymphocyte trafficking is likely to be a significant contributor to the impaired responses observed in older animals.[36]

The augmentation of growth factor release in response to ischaemia is also blunted in older animals. In addition to impaired VEGF release mediated through HIF pathway abnormalities and to impaired inflammatory cell trafficking, there are also age-related deficiencies in the release of other angiogenic growth factors such as platelet-derived growth factor (PDGF).[43] A paracrine relationship exists between cardiomyocytes and cardiac endothelial cells, with myocytes inducing endothelial cells to release PDGF, an important collaterogenic cytokine. However, this pathway is dysfunctional in older animals, with a significant reduction in cardiac PDGF production under normoxic conditions.[44] Defects in the expression of other pro-angiogenic growth factors, including angiopoietin-1 and -2,[45] and reduced levels of Flt-1 and Flk-1 (VEGF), have been described in association with aging.[40,45]

Thus, the impairment in the ischaemic vascular response observed in the elderly is due to abnormalities in many signalling pathways, and as such presents a significant challenge to address therapeutically.

2.2 Impaired collaterogenic responses associated with hyperlipidaemia

The potential implications of a relationship between lipid status and vascular remodelling are profound. LDL particles are directly toxic to endothelial and smooth muscle cells and inhibit their proliferation and migration with the effects reversed by HDL.[46,47] This *in vitro* effect has been shown to have biological relevance, in that collateral formation in response to hindlimb ischaemia in hypercholesterolemic rabbits is reduced compared to controls.[48,49] Studies of the relevant molecular pathways have shown that oxidized LDL inhibits VEGF-induced endothelial cell migration in part by inhibiting Akt/endothelial NOS pathway.[50] In addition, ox-LDL reduced endothelial cells' bFGF mRNA and protein levels, DNA and total RNA syntheses, and cell replication rates in a time and concentration-dependent manner.[51] These observations were completely restored by bFGF and, conversely, a bFGF-neutralizing antibody inhibited total RNA synthesis to a similar extent to that induced by ox-LDL.[52] Thus, it appears that the inhibitory effects of ox-LDL on endothelial cell proliferation are attributable in part to reduced bFGF expression. This defect in FGF signalling would appear to provide mechanistic justification for trials employing FGF as an angiogenic agent. However, several randomized clinical trials of intracoronary FGF in patients with stable angina failed to show significant improvement in objective measures of myocardial ischaemia,[2,53] results compatible with the concept that collaterogenesis cannot be achieved by the administration of a single collaterogenic molecule.

Studies of lipoprotein(a) transgenic mice found a significant inverse relationship between lipoprotein(a) levels and recovery of blood flow after femoral artery ligation.[54] Although the mechanisms for these observations are unknown, lipoprotein(a) binds to endothelial cells and inhibits the activation of transforming growth factor (TGF-β). TGF-β has complex effects on angiogenesis, with both pro and anti-angiogenic activities.[55] Its pro-angiogenic effects appear to be partly modulated by the induction of VEGF expression,[56] and partly by activation of MCP-1, which through its chemotactic effects on monocytes and vascular smooth muscle cells, is an important factor in collateral development.[57,58]

Hyperlipidaemia also influences T-lymphocyte migration following the induction of experimental limb ischaemia. In apoE$^{-/-}$ mice, tissue response to hindlimb ischaemia was markedly impaired with a five-fold reduction in T lymphocyte infiltration.[59] Commensurate with this, VEGF protein expression was significantly reduced. Thus, it seems that as with aging, the effects of hyperlipidemia impair multiple parts of the vascular remodeling cascade.

2.3 Diabetes and collateral vessel remodelling

Although some studies indicate that diabetic patients form coronary collaterals in response to coronary stenoses less effectively than non-diabetic patients with the same degree of arterial disease,[60] other studies investigating the acute recruitment of collaterals during angioplasty have not confirmed these data.[61,62] Nevertheless, diabetics experience a four-fold increase in mortality following an MI, and more frequently develop post-infarction angina, infarction extension, and congestive heart failure.[63,64] These clinical observations can be explained by several effects of diabetes, including the greater prevalence of small vessel disease and its potential influence on collateral development.

Animal models of diabetes demonstrate that diabetic animals have an impaired collateral response to arterial obstruction, and also exhibit altered biology of cellular function that could contribute to this defect. Thus, in non-obese diabetic mice, flow recovery in an ischaemic hindlimb was significantly reduced when compared to control.[65] Although significant reductions in local levels of VEGF were also observed, the cellular or metabolic pathways leading to this observation were not identified. As alluded to previously, a central component of collaterogenesis is intact endothelial function, with endothelial cell activation, proliferation, and migration being crucial to the collaterogenic processes.[66,67] Multiple studies demonstrate the impact of diabetes on normal endothelial function and as such is one logical pathway through which diabetes could impair collateral remodelling.[68,69] One molecular mechanism contributing to impaired endothelial function involves defects in the eNOS/NO pathway due to a risk factor associated increase in reactive oxygen species. Thus, the mitochondrial superoxide overproduction induced by diabetes and hyperglycaemia decreases phosphorylation of the Akt site on eNOS, causing posttranscriptional eNOS inhibition.[70]

Additionally, high glucose concentration alters endothelial cell cytoarchitecture (including the generation of giant cells, and changing the structure and distribution of cellular actin filaments),[71] alters cell proliferation, and induces delays in various phases of endothelial cell cycling.[72] Free radicals associated with hyperglycaemia may also modulate these effects, as co-culture of endothelial cells in hyperglycaemia with several different anti-oxidants restored cell proliferation rates to control levels.[73]

As noted, adequate collaterogenesis requires contributions from several circulating cell populations. Monocytes are of particular importance, adhering to adhesion molecules such as VCAM and ICAM[25,74] that are expressed by endothelial cells, migrating to the subintima, and then releasing many pro-angiogenic and pro-arteriogenic growth factors.[75] However, the migratory response of monocytes to VEGF-A (an important monocyte chemoattractant) is attenuated in diabetic patients.[76] VEGF-A-inducible kinase activity of Flt-1 remains intact in diabetic monocytes, implying that the defect lies further downstream in the signalling pathway. EPCs—another circulating cell believed to be involved in collaterogenesis—not only circulate in lower numbers in diabetic patients,[77] but also support *in vitro* angiogenesis assays less potently than cells collected from non-diabetic patients.[78] Subsequent studies have confirmed the compromised function of EPC precursors derived from diabetics, although the effects appeared to be secondary to hyperinsulinemia rather than hyperglycaemia.[79]

Thus, these animal studies provide some insights into the mechanisms underpinning the impaired development of collaterals in diabetic patients following acute arterial obstruction. Given the complexity of diabetes as a disease process, the studies also indicate, as might be expected, that diabetes probably produces this defect through multiple mechanisms involved in collaterogenesis.

2.4 Genetic background in governing collaterogenesis

Less than 50% of patients with critical coronary stenoses develop angiographically visible collaterals.[80,81] Although factors alluded to previously—such as hypercholesterolemia, diabetes, and advanced age—may impair collateral development, studies examining the characteristics of patients have generally found that clinical characteristics only partly correlate with impairment of collateral development.[82,83] One possible explanation for this is that there are important genetic differences that determine the potential for collateral development. However, there are few human data linking genetic background and inter-individual variability in collaterogenesis. Monocytes harvested from patients with poor collateral development produced significantly less VEGF when exposed to hypoxic conditions than monocytes collected from patients with a robust collateral response.[84] Other than suggesting genetic or epigenetic variability as an explanation of this finding, no other insights into mechanisms were revealed.

Several studies of the genetic heterogeneity of vascular remodelling have been conducted in mice. Various mouse strains demonstrate a 10-fold range in the growth of capillaries following growth factor infusion into a corneal micropocket.[85] In addition, the angiogenic response to chronic airway inflammation was also seen to vary widely between different mouse strains, both in terms of absolute vessel formation and in the type of vessel developed.[86] Murine genetic background also effects large vessel remodelling in the carotid artery model, and the response to myocardial infarction.[87,88] Most relevant to collaterogenesis, there is wide genetically determined variability in pre-existing mouse hindlimb collateral morphology, a factor that markedly influenced tissue damage and hypoxia induced by femoral artery ligation.[89] Such variability was compelling demonstrated by Chalothorn *et al.*,[90] who showed that the diminished collateral function seen immediately after femoral artery occlusion in BALB/c vs. C57Bl/6 mice is associated with fewer pre-existing collateral vessels. Most interesting in terms of genetic predisposition, other tissues of BALB/c mice, in addition to the skeletal muscle of the hindlimb, were found to have fewer collaterals, including the small intestine and brain.

2.5 Other cardiovascular risk factors and collateral response

The relationship between other cardiovascular risk factors and collateral response is less well characterized. Obesity does not appear to be related in multivariate analyses.[60] Smoking may impact on collaterals although the data are somewhat conflicting. EPC numbers are lower in smokers (rebounding rapidly with smoking cessation).[77,91] EPC function has been demonstrated to be impaired with reduced migration and adherence.[92] In addition, smoking appears to reduce monocyte VEGF secretion,[93] and also the migratory response of endothelial cells to this growth factor.[94]

Animal studies demonstrate the relevance of these *in vitro* findings. In mice undergoing hindlimb ischaemia, the exposure to cigarette smoke significantly reduced flow recovery with lower hindlimb HIF-1 and VEGF expression.[94] However, although the inference of these animal data suggests there should be a link between smoking and vascular response in patients, the clinical data are mixed: some studies appear to exclude such a relationship while others suggest a close link.[60,95]

2.6 Influence of risk factors on the collateral response to therapeutic interventions

Thus, considerable data from animal models demonstrate that traditional cardiovascular risk factors significantly impair native collateral development in response to arterial obstruction and resulting ischaemia. Although definitive experimental data relating risk-factor induced impairment in *native collateral development* to a parallel resistance to the beneficial effects of *angiogenic interventions* are lacking, there are some preliminary findings linking these two concepts. For example, EPCs derived from elderly patients appear less effective in restoring tissue perfusion than EPCs from younger patients when transplanted into athymic nude mice with hindlimb ischaemia.[96] Additionally, bone marrow mononuclear cells derived from patients with an ischaemic cardiomyopathy (who, when compared to controls, were older and had a greater prevalence of diabetes, smoking, and hypercholesterolemia) exhibited reduced *in vitro* angiogenic capacity and less collaterogenic efficacy in a nude mouse model of hindlimb ischaemia vs. controls.[97]

Clearly, the clinical implications of such a link are profound, particularly as many of these risk factors co-exist in patients who would be candidates for angiogenic therapy. For example, hyperlipidaemia was present in 70% of patients enrolled into TOPCARE-MI.[98] In the three randomized studies published recently, 17% of patients were diabetic, 68% had a history of hypercholesterolemia, and the patients mean age was 58 years.[10–12] Although not particularly elderly, relatively speaking these patients are far older than the young animals usually used in studies evaluating effectiveness of various angiogenic therapies. Thus, these considerations suggest there are significant obstacles to successful application of collaterogenic therapy to patients; they also provide, at least in part, some insights into why the results of randomized clinical trials reported thus far are disappointing.

3. Future directions

There are several approaches being considered to address the potential limitations imposed by risk factor-induced impairment of many of the mechanisms involved in optimal collaterogenesis. Progenitor cells that are used to induce collaterogenesis can be genetically engineered to correct some of the deficiencies caused by the risk factors. The concept is that if a patient's progenitor cells are transfected with a gene whose gene product is known to be impaired as a result of one or more risk factors, the resulting overexpression of the gene would result in enhanced collaterogenesis. For example, transduction of EPCs with $VEGF_{164}$ appeared to increase their *in vitro* proliferative activity and adhesive characteristics compared to control cells.[99] Systemic injection of the transduced EPCs in athymic nude mice with hindlimb ischaemia enhanced neovascularization and blood flow recovery, and reduced the incidence of limb necrosis and auto-amputation compared to mice receiving non-transduced EPCs. Although this

approach has potential, it remains to be determined whether these or related strategies will be successful in patients with *multiple* defects caused by the presence of multiple risk factors.

A different approach to potentiate the effects of cell therapy and thus overcome host resistance is to promote cell survival and reverse the very poor short-term survival of transplanted cells.[100] Over-expression of Akt1 is one such pro-survival factor and has been studied in a rat infarct model.[101] Transplantation of MSCs over-expressing Akt1 into the ischaemic myocardium restored four-fold greater myocyte volume than equal numbers of cells transduced with the reporter gene lacZ. Another approach to enhance cell survival is to transduce cells with human telomerase reverse transcriptase (hTERT). In a study utilizing this approach, mitogenic capacity and VEGF-induced migration were markedly enhanced in hTERT-transduced EPCs compared to GFP-transduced EPCs.[102] Subsequent *in vivo* transplantation of hTERT-transduced EPCs improved limb salvage and perfusion in comparison with GFP-transduced EPCs.

Another cell-based strategy we are currently testing is based on the concept that the dominant mechanism by which cells exert their collaterogenic effects derive from paracrine activity rather than from their capacity to transdifferentiate into blood vessel cells and anatomically form or expand collaterals.[103] Thus, MSCs express a large number of cytokines and growth factors that are known to exert angiogenic effect and their secreted products increase the proliferation and migration of endothelial cells and smooth muscle cells.[104,105] Other groups have confirmed these data and the importance of paracrine signalling in mediating the benefits of cell therapy for tissue ischaemia.[106,107]

One strategy that derives from these observations is that *cell products* obtained from MSCs acquired from young healthy individuals could be administered to older patients with multiple risk factors, rather than the cells themselves. Because these cell products probably would not be immunogenic (just as plasma from one individual can be administered to another individual), 'allogeneic' transfer would probably be possible. The success of this strategy, as well as any angiogenic strategy, will depend on whether a major portion of the defects in collaterogenesis manifested by a given patient are due to the impaired function of the host's progenitor cells (which would be overcome by injecting the secreted products derived from young normal persons), or are due to impaired responsiveness of the host's tissues to collaterogenic agents.

4. Conclusions

Although there exists a great unmet need to enhance collateral function in patients with refractory angina, human studies thus far have failed to provide compelling data demonstrating that growth factor (delivered by protein or gene) or cell therapy are effective and ready for widespread application. Cardiovascular risk factors are likely to significantly inhibit the effectiveness of such therapies to improve tissue perfusion. Therefore, the development of strategies to overcome this formidable problem is urgently needed.

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