

Recently Patented Applications of Homologous Cellular and Extracellular Agents as Therapeutics or Targets for the Prevention of Restenosis Post-Angioplasty

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Abstract: Currently available drug-eluting stents have been shown to reduce the prevalence of in-stent restenosis. However, their use is limited by their enormous cost and unwanted side effects associated with both drugs, sirolimus and paclitaxel, presently used to coat most of the stents clinically available. Due to their lack of selectivity with respect to targeted cell types these drugs do not only inhibit vascular smooth muscle cell proliferation underlying neointima formation, they also compromise endothelial repair increasing the risk for subacute thrombosis following implantation of drug-eluting stents. Accordingly, there is need for new cost-effective agents capable to inhibit restenosis without clinically relevant, unwanted side effects.

In the present paper a selection of the most important patent applications published within the last 3 years and claiming the use of homologous cellular and extracellular agents as therapeutics or targets to prevent restenosis are reviewed. Such agents include c-Jun, the focal adhesion kinase (FAK) and its inhibitor FAK-related non-kinase (FRNK), estrogen receptors, variants of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) as well as some so far poorly characterized factors supposedly involved in the control of cell proliferation, inflammation and apoptosis. Such agents promise to be cost-effective and, in some cases, potentially devoid of unwanted side effects. Clinical long-term studies have yet to support such notions.

Keywords: Restenosis, neointima, angioplasty, drug-eluting stent, endothelial repair, vascular smooth muscle cells, subacute thrombosis, vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), protein associated with restenosis inhibition and secreted (PARIS), focal adhesion kinase (FAK), focal adhesion kinase-related non-kinase (FRNK), c-Jun, estrogen receptors, cost-effectiveness.

INTRODUCTION

The clinical problem of restenosis after angioplasty including in-stent restenosis remains partially unsolved. Vascular smooth muscle cell (SMC) proliferation and matrix formation are the predominant factors responsible for the narrowing [1]. Remodeling plays an additional role if balloon dilation is performed without implantation of a stent preventing it. Endothelial damage caused by angioplasty is another factor contributing to restenosis [2]. Many genes that control cellular activities potentially involved in restenosis like cell proliferation, differentiation, migration and matrix deposition have been determined by molecular biology. This has led to new approaches to treat arterial damage, and consequently, there is today a real possibility that restenosis may be prevented by the supply of certain protein activities or specific nucleic acids modulating gene expression, e.g. via gene or protein transfer [3]. Such approaches aim to produce a local and specific effect on certain relevant target cells, i.e. to particularly reduce smooth muscle cell proliferation or matrix formation or promote re-endothelialization.

The cellular activities relevant for restenosis may be modulated by extracellular factors which include growth factors, or by cellular factors including those localized within the cytoplasm and those anchored in or at the plasma membrane. In general, therapy can be aimed to achieve a reduction in smooth muscle cell proliferation and migration either by inhibition of normally occurring growth stimulators or augmentation of natural growth inhibitors; alternatively, therapy can intend to kill cells. On the other hand, re-endothelialization should not be compromised. Therefore, one of the most challenging requirements of new approaches will be to facilitate or at least not compromise endothelial cell (EC) proliferation, migration and differentiation allowing for fast endothelial repair. These requirements can be exemplified by the case of C-type natriuretic peptide (CNP). CNP is a physiological endogenous vasodilator secreted by ECs [4, 5]. On the one hand CNP inhibits the proliferation and migration of SMCs via local paracrine and autocrine mechanisms through the cyclic guanosine 3, 5-monophosphate (cGMP) cascade. Consequently, the destruction of ECs during and after percutaneous transluminal angioplasty (PTA) among other effects reduces the concentration of endothelium-derived relaxing factors, such as CNP or nitric oxide, allowing for SMC proliferation and restenosis. On the other hand, CNP induces endothelial growth *in vitro*, which would potentially accelerate re-endothelialization following PTA [6, 7]. According to these pleiotropic effects CNP is a prototype extracellular factor

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which has the potential to become a therapeutic for the prevention of restenosis [8-12].

The aim of this article is to review a selection of the most important patent applications of the last 3 years claiming the use of homologous cellular and extracellular agents to attenuate the restenosis process and discuss their specific properties in comparison to agents already used today in clinical practice and predominantly applied via drug-eluting stents or more rarely using other administration routes such as catheter-based local delivery.

CLINICAL BACKGROUND

Cardiovascular disease is still the leading cause of death. Most frequently atherosclerotic disease is underlying with its manifestations coronary artery disease, cerebrovascular disease and peripheral artery disease. Atherosclerotic disease accounts for a large proportion of health care cost which in part is a result of increasing numbers of angiographies usually combined with therapeutic angioplasties with or without stent implantations.

Percutaneous transluminal angioplasty was introduced more than 25 years ago [13] as a non-surgical revascularization technique for patients with coronary or peripheral artery disease. In comparison with surgical bypass grafting, the procedure is far less invasive and therefore better tolerated as it has been demonstrated in clinical trials [14]. Hence, several years ago, the number of angioplasty procedures has exceeded the number of bypass operations performed. There are currently at least 2.0 million cases of angioplasty carried out worldwide.

However, the value of angioplasty currently remains limited by the development of restenosis in approximately one third of patients, necessitating repeat procedures or surgery [15, 16]. Despite the development of alternative interventional devices such as laser or atherectomy catheters, and intravascular bare metal stents, the problem of restenosis has not been convincingly solved in the long-term [17]. The pathophysiological process of restenosis has been investigated in detail in many models; yet, no systemic pharmacological treatment has been shown to significantly reduce the incidence in patients [18]. Only recently, the problem of restenosis appeared partially resolved with the introduction of drug-eluting stents bringing restenosis rates down to approx. 5%. However, recent reports describe the formation of subacute thrombosis after implantation of drug-eluting stents, which again limits their application [19-22]. Subacute thromboses may be the result of the toxicity of drugs used on stents, incomplete lesion coverage by the stents, or delayed re-endothelialization following endothelial damage [21, 23]. Thus, an incomplete healing of vascular tissue following angioplasty and stent implantation may prolong the period of thrombogenicity associated with the enhanced risk of occlusion of arteries resulting in myocardial infarctions or even death or acute peripheral artery occlusions with an increased risk of amputations. As a consequence, the efforts to develop alternative therapies have been intensified. Particularly, agents with differential effects on the proliferation of SMCs and ECs are of interest to circumvent this thrombotic sequela. Another limitation of drug-eluting stents available today is the extremely high cost preventing

the use of such stents in many cases where they may be clinically indicated due to increasing budget restrictions in patient care. Thus, less expensive alternatives are urgently desired.

PATHOPHYSIOLOGY OF RESTENOSIS

The analysis of animal models revealed, that four overlapping stages occur in the development of restenosis, which in some ways resemble the wound healing process [24]. At the time of injury the inflammatory and thrombotic phases begin and continue for hours. These are followed by a proliferative phase, with a peak at about seven days post-injury. Finally, there is a phase of formation of extracellular matrix from one week onwards. In the proliferative and matrix formation phases, many of the vascular SMCs found in the neointima have undergone a phenotypic change from the contractile quiescent state to the synthetic proliferative state. A variable response to the vascular narrowing is adaptation and alteration of arterial shape, termed remodeling, which as yet has not been fully evaluated in animal models [25].

Early research focused mainly on the histological changes in the arterial wall after injury and on the distribution of extracellular proteins, particularly growth factors, without providing much information about their function. With the advent of directional atherectomy as a treatment for restenosis more than fifteen years ago, it has been possible to obtain fresh restenosis tissue [26]. In combination with cell culture experiments and the application of contemporary molecular techniques at the RNA and protein level this allowed to reveal many of the cellular and molecular processes underlying the process of restenosis. Now it is clear that local stimulation of cell proliferation by extracellular growth factors produced either by certain cells in order to act on neighbouring cells (called paracrine), or produced by cells in order to act on themselves (autocrine) is fundamental to the process of restenosis.

Extracellular (growth) factors bind to cell surface receptors and can initiate the cascades of signal transduction which eventually leads to cellular processes as proliferation, migration and differentiation. Components of the cascades include various membrane-associated and cytoplasmic protein kinases, coupling proteins (e.g. *ras*) and effector proteins. Apart from the possibility to primarily manipulate the extracellular events leading to restenosis there are many intracellular control points, where gene manipulation may be possible in order to prevent restenosis. For example, vascular SMCs leave the quiescent state and proliferate by progressing through the cell cycle after balloon angioplasty of human arteries. This cell cycling is driven by proto-oncogenes which are transiently switched on upon stimulation by growth factors. These again are antagonized by cell cycle inhibitory proteins, such as p21 and p27. Accordingly, adenovirus-mediated p21 gene transfer for example has been used to inhibit neointima formation in a rat carotid artery model of angioplasty [27].

By increasing our understanding of the regulation of events at the level of the proteins and nucleic acids, it has been possible to develop alternative strategies to prevent restenosis. However, it is recognized that the results of

preclinical investigations based on such strategies must be viewed cautiously [25]. Many early therapeutic strategies showing potential *in vitro* or in animal models have not translated into reductions in restenosis rates when subsequent large controlled studies in patients were carried out [28].

PATENTS COVERING CELLULAR AGENTS AFFECTING RESTENOSIS

The application of several cellular factors known or supposed to be involved in the control of proliferation, survival, migration or differentiation of vascular cells have been covered by earlier patents in the context of restenosis prevention. These include for example the cyclin dependent kinase inhibitor p21 [29]. Here we present the principles underlying the applications of three cellular agents for restenosis prevention published as patent applications within the last three years (Table 1).

FRNK

Laser et al [30] (Table 1) disclosed the use of a known homologous inhibitor of the focal adhesion kinase (FAK) to prevent/treat restenosis. The inhibitor is the FAK-related non-kinase (FRNK) that is envisioned to be applied in a gene therapy approach by an adeno-associated virus vector.

The background of this invention is as follows: The migration of vascular SMCs from the tunica media into the tunica intima of the dilated vessel is one of the most important factors contributing to the development of restenosis [31]. Apart from the intracellular contractile machinery, cell migration depends on the continuous formation and resolution of specific contacts between the cells and the extracellular matrix. These contacts, enabled by integrins as cellular receptors for proteins of the extracellular

matrix, are accumulated at specific sites which are referred to as focal adhesion points [32]. However, focal adhesion points are not only structural elements but mediate the transduction of signals important for the control of adhesion-dependent growth and the regulation of cell motility into the cell. Since integrins do not have an enzymatic activity themselves, integrin-mediated signal transduction is mediated by integrin-associated enzymes. The protein tyrosine kinase focal adhesion kinase (FAK) is one of these integrin-associated enzymes [33]. It is expressed in adherent cells such as vascular SMCs [34]. The formation of cell-matrix contacts, mechanical stress or the addition of various growth factors cause the activation of this kinase.

A C-terminal so-called focal adhesion targeting domain (FAT) which mediates the localization of FAK to integrin rich focal adhesions is essential for the function of FAK [35]. In SMCs and some other cell types the C-terminal region of FAK is expressed as a separate transcript and protein. Due to its missing kinase activity this protein is called FAK-related non-kinase (FRNK). FRNK is a dominant-negative version of endogenous FAK since it comprises the FAT domain and is therefore recruited to the focal adhesion points where it competes with FAK for binding sites [36]. Therefore, FRNK and derivatives thereof can be used to inhibit FAK-dependent cellular events such as SMC migration and thus the formation of neointima in restenosis.

The therapeutic use of an intracellular protein requires an highly efficient vector system for the transfer of the gene encoding this protein. In addition, the expression of the transgene construct should be controlled by a sufficiently strong and specific promoter to ensure high level expression in the required cell type. The inventors therefore prefer the

Table 1. Homologous Cellular and Extracellular Agents Affecting Restenosis

agent (protein, gene)	localization	application rationale	demonstrated cellular effect	primary mechanism of restenosis prevention	patent (US or international version)
FRNK	cellular	therapeutic agent	inhibition of SMC migration	inhibition of neointima formation	WO2005014835
c-jun	cellular	target for DNazymes	inhibition of SMC proliferation	inhibition of neointima formation	US20050119213
estrogen receptor	cellular	therapeutic agent and target for 17 E	inhibition of SMC and promotion of EC proliferation	inhibition of neointima formation and promotion of re-endothelialization	US20050065065
VEGF145 variant	extra-cellular	therapeutic agent	stimulation of EC proliferation; no effect on SMCs	promotion of re-endothelialization	WO2004055156
FGF-2 variants	extra-cellular	therapeutic agent	stimulation of EC migration and tubulogenesis	promotion of re-endothelialization	WO2004069298
PARISs	extra-cellular	therapeutic agent	inhibition of SMC proliferation	inhibition of neointima formation	US2004242470

use of the adeno-associated virus, in particular adeno-associated virus type 2 [37], as the most suitable vector system. Concerns by others [38] that adeno-associated virus vectors are only suitable for the transfection of ECs in *vasa vasorum* are not justified according to the inventors. Vascular SMC-specific promoters as the "smooth muscle 22 alpha-actin promoter" [39] are preferred by the inventors to direct the expression of FRNK and derivatives thereof into this cell type. Regarding the application of such adeno-associated viruses the inventors envision coated stents and catheters.

c-Jun

In an invention by Khachigian *et al.* [40] (Table 1) methods and compositions for reducing c-Jun-mediated cellular processes predominantly aimed at the prevention of restenosis are provided.

The basic region-leucine zipper protein c-Jun was shown to be transiently induced following arterial injury in animal models [41]. It builds homodimers and together with other bZIP proteins also heterodimers which contribute to the formation of the AP-1 transcription factor. Previous investigations have linked AP-1 with proliferation, tumorigenesis and apoptosis. However, it was also demonstrated that AP-1 has a role in opposing processes such as tumor suppression and cell differentiation [42]. Consequently, the downregulation of c-Jun expression by genetic approaches does not necessarily reduce cell proliferation. In agreement, Kanatani *et al.* [43] demonstrated that the downregulation of c-Jun by antisense oligonucleotides dose-dependently reduce the growth-inhibitory effect of dexamethasone and TGF β . However, in vascular SMCs c-Jun activity appears to stimulate cell proliferation as indicated by recent reports which demonstrated that c-Jun NH2-terminal kinase/stress activated protein kinase (JNK) is expressed by SMCs in human and rabbit atherosclerotic plaques [44]. In addition, vascular injury leads to a transient increase of c-Jun expression and dominant negative JNK inhibits neointima formation after balloon injury [45].

Based on these findings and notions, the inventors provide a strategy to inhibit neointima formation by the application of so-called DNazymes capable to cleave the c-Jun mRNA. To justify the preference for this technology they refer to known disadvantages of the use of antisense oligonucleotides and ribozymes. Whereas the antisense technology suffers from non-specific activities and toxic side effects, ribozymes are highly prone to intracellular degradation which significantly limits their effectivity. DNazymes on the other hand share the principle advantage of ribozymes, i.e. the catalytic nature which makes them to inherently efficient and specific tools. However, due to the fact that these molecules consist of DNA they are much more resistant to degradation within the cell as ribozymes [46].

Using a DNzyme targeting c-Jun, the inventors were able to abrogate neointima formation in rat carotid arteries although the DNzyme they constructed not only abrogated SMC proliferation but also blocked endothelial cell replication and angiogenesis.

Estrogen Receptor-Alpha and -Beta

A patent application by Tanguay and Sirois [47] provides methods and specific nucleic acids which can be used to modulate the expression of estrogen receptors to aid vascular healing and endothelial recovery after injury (Table 1).

The invention is based on the following facts and notions. It has been demonstrated in several animal studies that estrogen absence increases and estrogen treatment reduces neointima formation after vascular injury [48]. One of the inventors showed in a previous study that local delivery of 17 β -estradiol (17 E) reduced neointima formation caused by balloon dilation in a porcine model [49]. The protective effects of 17 E were found to be associated with effects such as endothelial nitric oxide synthase-mediated (eNOS)-dependent relaxation of coronary vessels [50], tumor necrosis factor- release, inhibition of platelet aggregation [51], beneficial changes in the plasma lipid profile [52], inhibition of SMC proliferation [53] and migration [54] and reduction of extracellular matrix synthesis.

The two known estrogen receptors (ERs), ER α and ER β , are expressed in all vascular cell types [48]. It was demonstrated in the past that the effects mediated by these receptors are based on their direct involvement in the regulation of gene transcription in the nucleus (genomic signaling). However, recently it became obvious that 17 E can also directly modulate cytoplasmic signal transduction (non-genomic signalling) [55, 56]. There is evidence that some of the vascular protective effects of 17 E mediated by ERs are due to a non-genomic mechanism involving activation of eNOS through the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/Akt pathways [57, 58]. In agreement, the inventors showed that 17 E stimulates the phosphorylation of p38 and p42/44 MAPK and the proliferation and migration of ECs under *in vitro* conditions whereas p38 and p42/44 MAPK activation, cellular proliferation and migration were inhibited in vascular SMCs [59]. Since they also could demonstrate by the application of an antisense approach that the observed effects on ECs were ER α -dependent whereas those on SMCs were mediated by ER β [60] they claim a strategy to prevent restenosis based on the upregulation of ER α in ECs and ER β in SMCs to enhance the responsiveness of the two cell types to 17 E. This is envisioned to be achieved by the transfer of recombinant DNAs encoding ER α and ER β via adenoviral vectors, for example. The simultaneous or subsequent application of 17 E or related estrogen derivatives should prevent the development of restenosis.

PATENTS COVERING EXTRACELLULAR AGENTS

Three important recent patent applications claiming the use of extracellular factors known or supposed to be involved in the control of proliferation, survival, migration or differentiation of vascular cells in the context of restenosis prevention are listed in Table 1 and discussed below.

VEGF145 Variants

In a patent application by Neufeld [61] (Table 1) vascular endothelial growth factor (VEGF) variants are disclosed that can be used for the prevention of in stent restenosis. The

invention has the following background: In contrast to many other polypeptide growth factors which are active on a wide range of cell types, VEGFs predominantly act on the various types of vascular cells and bone marrow-derived cells due to the binding to VEGF receptors (VEGFRs) that are primarily expressed on the surface of these cells [62]. VEGFRs have distinct biological roles and expression patterns. VEGFR-2 is considered to mediate most of the effects of VEGF on ECs, i.e. mitogenic, angiogenic, survival, and vascular permeability effects. In agreement, re-endothelialization after vascular injury is primarily the result of VEGF interaction with VEGFR-2 on ECs [61]. This interaction leads to the generation of nitrogen mono oxide (NO) which in turn impairs vascular SMC migration and proliferation [63]. In contrast, VEGFR-1, is the receptor variant predominantly affecting SMCs and leukocytes [64] and therefore, its activation by VEGF can promote neointima formation [65]. Consequently, a VEGF variant that would activate VEGFR-2 but not VEGFR-1 receptor signalling should be ideally suited to prevent neointima formation and restenosis.

The VEGF gene family comprises at least five genes encoding VEGFA, VEGFB, VEGFC, VEGFD and placenta growth factor (PIGF) [62]. Apart from the existence of multiple genes alternative exon splicing further enhances the complexity of VEGF isoforms. In the case of VEGFA, the prototype member of the family and relevant variant for this patent application, the known isoforms are distinguished by the presence or absence of the peptides encoded by exons 6 and 7 of the 8 exons of the VEGFA gene [66]. Whereas the protein domain encoded by exons 1-5 is basically required for the recognition of VEGF receptors, the absence or presence of the peptides encoded by exons 6 and 7 appear to modulate the receptor recognition pattern and affect the heparin and extracellular matrix (ECM)-binding ability of the respective VEGFA isoform. Accordingly, the known VEGFA isoforms can be functionally discriminated with respect to their VEGF receptor-, heparin- and ECM-binding properties as summarized in Table 2.

Of these VEGF isoforms VEGF145 is the one that appears to be the most suitable agent to be used to prevent restenosis. On the one hand it is secreted (in contrast to

VEGF189) and on the other it is expected to accumulate and concentrate on the denuded surfaces of vessels injured by balloon dilation or stent implantation since it binds tightly to the extracellular matrix. However, due to the fact that VEGF145 binds and activates VEGFR-1 it may also promote the proliferation and migration of SMCs and thus could contribute to neointima formation. Work by Li et al [67] demonstrated that the VEGFR-1 binding activity of VEGF165 can be abolished by site directed mutagenesis without compromising the capability to bind and activate VEGFR-2. Correspondingly, the inventor has generated by site-directed mutagenesis a mutated VEGF145 variant that lacks the VEGFR-1 binding capacity while it is still capable to bind and activate VEGFR-2. The activities of such variants can be tested in cell proliferation assays using endothelial cell lines expressing VEGFR-1 or -2.

The inventor envisions to apply such VEGF145 variants as *in vitro* purified recombinant proteins or *via* a gene therapy approach by the transfer of gene constructs encoding these VEGF proteins. In addition to plasmid vectors various viral vectors are provided. All usual application routes, e.g. numerous systemic routes and local delivery *via* coated stents, as well as compositions with additives/adjuvants used for classic pharmaceuticals are also envisioned for the application of such proteins or gene constructs.

FGF-2 variants

An invention by Prats *et al.* [68] (Table 1) aims at the promotion of re-endothelialization by the use of certain fibroblast growth factor (FGF)-2-derived mutant proteins to eventually prevent restenosis. Apart from VEGF, FGF-2 is the most powerful angiogenic factor [69]. However, to stress the higher potential - from their point of view - of the FGF-2-based approach to prevent restenosis, the inventors state that VEGF increases endothelial permeability and the deposit of lipids thereby promoting progression of atherosclerotic plaque and refer to a paper by Celletti *et al.* [70].

The FGF family consists of at least 22 homologous proteins [71, 72]. FGFs act on a variety of cell types by stimulating mitogenesis and/or by inducing morphological changes and differentiation. FGF-1 (or acidic FGF) but

Table 2. Characteristics of VEGF Isoforms

VEGF isoform	structure*		secretion	stimulation of ECs	heparin binding	ECM** binding	receptor binding
	exon 6	exon 7					
VEGF121	-	-	+	+	-	-	VEGFR-1 VEGFR-2
VEGF145	+	-	+	+	+	+	VEGFR-1 VEGFR-2
VEGF165	-	+	+	+	+	-	VEGFR-1 VEGFR-2
VEGF189	+	+	-	+	+	+	VEGFR-1 VEGFR-2

*with respect to presence or absence of exon 6 and 7 encoded sequences

**basement membrane-like ECM (extracellular matrix) produced by corneal ECs

predominantly FGF-2 (or basic FGF) are known for their activities on vascular cells. FGF-2 promotes tubulogenesis, i.e. the formation of capillaries and stimulates the proliferation and the migration of ECs and vascular SMCs [69]. Five FGF-2 isoforms of 18, 22, 22.5, 24, and 34 kDa have been identified. These are generated by an alternative translational initiation processes [73, 74] which lead to different N-terminal sequences. The N-termini of the four high molecular mass (HMM) isoforms confer nuclear translocation. Accordingly, these isoforms of FGF-2 act in an intracrine manner that can influence tumor progression and stimulate cell proliferation under low-serum conditions *in vitro* [75-77]. In contrast, the smallest FGF-2 (18kDa) isoform is predominantly cytoplasmic or excreted and imparts the autocrine and paracrine effects of FGF-2 which consist in the stimulation of cellular proliferation and migration [76]. However, after binding to its transmembrane receptor the 18kDa FGF-2 isoform is internalized together with its receptor [e.g. 78] and translocated into the nucleus. Although several findings suggest that the nuclear translocation of extracellularly acting FGF-2 is required for its effects in various cell types [e.g. 79] the molecular mechanisms by which the extracellular FGF-2 exerts its intracellular effects are largely unknown.

The four HMM and the internalized 18 kDa FGF-2 isoforms were identified as components of large protein complexes [80]. Using the yeast two-hybrid system the inventors identified a previously unknown protein, named translokine which is a specific intracellular binding partner of the 18 kDa FGF-2 variant [81]. As part of their invention they demonstrated by the use of the siRNA technology that translokine is required for the nuclear translocation of the 18kDa FGF-2 isoform. The invention predominantly relies on the finding that certain FGF-2 derived proteins (FGF-2 mutants) which are unable to interact with translokine and therefore cannot be translocated to the nucleus, do not stimulate the proliferation of ECs, whereas their tubulogenesis activity is preserved. Since wildtype FGF-2 also stimulates the proliferation of vascular SMCs such mutants may be used to promote the reendothelialization of the vessels denuded by angioplasty or stent implantation by preserving the tubulogenic and migrating capacity of ECs, without promoting neointima formation.

The inventors state that FGF-2 mutants, generated by site-directed mutagenesis and defective in nuclear translocation activity can be identified by the subcellular fractionation of fibroblasts (such as NIH 3T3) or ECs (such as ABAE) treated with haemagglutinin(HA)-tagged FGF-2 mutant candidates. To check the defect of such mutants in stimulating the proliferation of SMCs and ECs standard cell proliferation assays can be applied. The tubulogenesis activity of the FGF-2 mutants can be evaluated by methods measuring the capacity of ECs to form capillary structures.

This strategy to identify and characterize FGF-2 mutants displaying the wanted properties is exemplified in the published patent application by a specific mutant named Nb1a2. Nb1a2 displays uncoupling of differentiation, i.e. tube formation, and proliferation activities which was shown to be due to a lack in its translocation towards the nucleus. This phenotype of Nb1a2 is in agreement with data

published by Kudla *et al.* [82] who demonstrated that artificial stimulation of the FGF receptor 1 (FGFR1) without FGF-2 causes differentiation but not proliferation. To provide proof for the hypothetical potential of Nb1a2 to prevent neointima formation, the inventors used an electric injury mouse model [83] of the distal part of the carotid artery. The effect of Nb1a2 on reendothelialization and neointima formation was analysed in comparison with FGF-2 and a control (saline). As it was expected FGF-2 as well as Nb1a2 stimulated re-endothelialization to a similar degree whereas only Nb1a2 prevented neointima formation in this model.

Protein as well as DNA sequences of other FGF-2 mutants supposedly exhibiting such properties are provided by the inventors.

PARISs

In a patent application published by Fujise and Mnjoyan [84] (Table 1) the discovery of certain soluble proteins ("PARISs") secreted by vascular SMCs and able to inhibit their growth is described. Methods for the use of these PARISs to prevent restenosis and atherosclerosis progression are disclosed in this patent application. The name "PARIS" stands for the phrase "protein associated with restenosis inhibition and secreted". Since some of these PARISs also appear to be normally secreted by other cell types, including non-vascular SMCs, this group of proteins holds promise as inhibitors of cell growth in a variety of tissues, and consequently may find use in treating or preventing cell proliferation in several other proliferative disorders such as cancers.

The individual proteins PARIS-1, PARIS-2, PARIS-3 and PARIS-4, have little or no common homology or mutual family associations. Each of these proteins has been previously assigned another name and their implicated biological functions are different from the bioactivity disclosed in the patent application by Fujise and Mnjoyan [84] (Table 3) for the first time, i.e. their inhibitory effects on the proliferation of SMCs.

The inventors state that no other molecules have been previously described that are secreted from vascular SMCs and inhibit SMC proliferation. The following discoveries and investigations led to the identification of these PARISs: It had been observed in a number of animal surgeries that the extent of neointima formation in a standard carotid artery balloon injury restenosis model, as described by Clowes *et al* [85] is different in the Harlan and Sasco substrains of Sprague-Dawley rats. Based on histological data (number of nuclei in the neointima in hematoxylin eosin-stained sections) it was hypothesized that this difference reflects a genetic, strain-specific difference that is expressed in the SMCs. Cell culture experiments confirmed this assumption. They revealed, that although the SMCs from both substrains were morphologically indistinguishable the growth rate was significantly different between both. The incubation of the cells from the Sasco substrain displaying the higher growth rate and a vascular SMC line (A7r5) with conditioned media from the slowly growing cells of Harlan rats demonstrated that the latter secreted soluble substance(s) which retard the growth of SMCs.

Table 3. Proteins Associated with Restenosis Inhibition and Secreted (PARISs)

name	synonyms	NCBI accession number (nucleotide)	previously known function	homology family
PARIS-1	neuronal pentraxin 1	U18772	pro-apoptosis	amyloid P protein
PARIS-2	MIC-1 (GDF-15, PDF)	NM-019216	anti-inflammatory	TGF- superfamily
PARIS-3	BTG2	M60921	anti-proliferative	unknown
PARIS-4	Fractalkine, soluble	AF030358	anti-adhesion	CX ₃ C chemokine

To identify the hypothetical growth-controlling gene(s) responsible for the differential behavior of the two cell populations, the inventors performed microarray analyses with RNAs obtained from both populations grown under identical conditions. The Affymetrix rat microarray (RGU344) that was used contained 7000 full-length sequences and 1000 EST clusters. From the genes displaying differential expression between the slowly and fast growing cells the inventors selected those that were at least 3-fold upregulated in the slowly growing cells and encode proteins known or assumed to be negative regulators of cell growth, secreted, soluble and not ECM-bound. This led to the identification of PARISs 1-4 (Table 3). None of these molecules has ever been implicated before in the growth regulation of SMCs.

All 4 PARISs are poorly characterized with respect to their roles in cellular physiology. PARIS-1 is identical to neuronal pentraxin-1 which is homologous to the acute-phase proteins serum amyloid P protein and C-reactive protein of the pentraxin family. Since it can be detected in conditioned cell culture media by Western blot analysis as its homolog neuronal pentraxin-2 [86] both proteins are apparently secreted. Recently, neuronal pentraxin-1 was demonstrated to be upregulated in cerebellar granule cells undergoing potassium deprivation-induced cell death [87]. Treatment of the cells with antisense oligonucleotides directed against neuronal pentraxin reduced apoptosis further suggesting a role in negative growth regulation and cell death control. Even less is known about PARIS-2. It is identical to macrophage inhibitory cytokine-1 (MIC-1), also known as growth differentiation factor 15 (GDF-15) and prostate-derived factor (PDF) and therewith it is remotely homologous to TGF [88]. Due to the anti-inflammatory role of TGF the inventors speculate that PARIS-2/MIC-1 is an anti-inflammatory protein. PARIS-3 is identical to B-cell translocation gene 2 (BTG-2). Its function in cell growth control is suggested by the fact, that BTG-overexpressing cells are growth-suppressed [89]. PARIS-4 is identical with the extracellular domain of the fractalkine protein. Fractalkine is expressed on the surface of endothelial, smooth muscle and dendritic cells [90, 91]. However, it can be cleaved which leads to the release of its soluble "chemokine head" (PARIS-4). It is assumed by the inventors that soluble fractalkine has an anti-inflammatory effect. The inhibitory role of fractalkine in VSMC proliferation has not been described previously.

PARIS-4 was selected by the inventors for an exemplary, detailed characterization to demonstrate the suitability of the

identified proteins for their intended use as agents preventing restenosis. PARIS-4 was shown to be produced by vascular SMCs from the restenosis-resistant Harlan rat substrain independently from the initial experiments leading to its identification. Recombinant PARIS-4 protein was produced and shown to slow the growth rate of VSMCs *in vitro*. Using an antibody against PARIS-4 the inventors identified a twofold higher PARIS-4 signal in the slight neointima of the restenosis-resistant Harlan rats in comparison with the signal in the pronounced neointima of the restenosis-prone Sasco strain.

Apart from potential applications in therapies of other diseases due to or characterized by unwanted cell proliferation the inventors predominantly envision the use of PARISs for the prevention and treatment of post-angioplasty restenosis and the delaying or arresting of the progression of atherosclerosis. They suggest the use of compositions which contain one or more purified or recombinant PARISs, and may include a suitable carrier (e.g., sterile isotonic saline). Delivery may be achieved by intravenous, subcutaneous or intraperitoneal injection, transcutaneous delivery, local delivery or using PARIS-coated stents.

CURRENT AND FUTURE DEVELOPMENTS

To prevent restenosis by the use of homologous agents on the one hand intracellular events can be manipulated by targeting cellular proteins known to be involved in the regulation of cell proliferation, migration or differentiation directly or indirectly via gene transfer to the most suitable cell type(s) or by the application of specific small nucleic acids (e.g. antisense oligonucleotides, DNazymes) capable to modulate the expression of a target protein. On the other hand extracellular factors can be supplied or their expression can be manipulated by the approaches mentioned above. However, the application of extracellular factors may have the better perspective. It provides the higher flexibility with respect to application routes, e.g. systemic application, since extracellular factors basically can be applied without the need of a vector system. In addition, if gene transfer is envisioned, the efficiency and specificity can be lower when extracellular agents shall be applied since paracrine effects in combination with a strong non-specific promoter of the transgene construct may compensate for low transfer rates. In contrast, the application of cellular agents *via* gene transfer requires a high transfer rate and, if side effects are expected in the case of expression in an unwanted cell type, the use of cell-type specific promoters which may impart only low expression levels under pathological conditions.

Research efforts to improve the potential of therapies based on homologous cellular and extracellular agents are currently directed towards prolonging gene expression locally. One ideal way may become the combination with new developments of biodegradable stents allowing for much higher amounts of agents and less mechanic irritation of the artery due to the resolution of the stent after a certain time.

In comparison with the drugs currently already used to coat stents, i.e. sirolimus and paclitaxel, the advantages of homologous cellular and extracellular agents patented and outlined here potentially consist in the lower costs and in the lower risk for unwanted side effects. Current drug eluting stents are 10 times more expensive than normal stents and are associated with an increased risk for subacute thrombosis.

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