Universität Ulm
Aus der Abteilung Innere Medizin II
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Inhibition of postangioplasty restenosis using antisense approach

Dissertation zur Erlangung des Doktorgrades der Medizin
Der Medizinischen Fakultät der Universität Ulm

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2003

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Tag der Promotion: 08.05.2003

To my family with deep gratitude for their persistent support of my academic and scientific endeavors

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Abbreviations and Acronyms

Ab	antibody
APS	ammonium peroxidisulphate
AS	antisense
bp	base pair
BPB	bromphenol blue
BrdU	bromo-deoxy-uridine
BSA	bovine serum albumin
CABG	coronary artery bypass grafts
ddW	double destilled water
dGMP	deoxyguanosine monophosphate
DMEM	Dulbecco's modified Eagle Medium
DNA	deoxyribonucleic acid
dNMP	deoxynucleotide monophosphate
DPBS	Dulbecco's phosphate buffered saline
DTT	dithiothretiol
EDTA	ethylenedinitrictetraacetic acid, disodium salt dihydrate
EVAc	ethylene-vinyl acetate copolymer
FGF	fibroblast growth factor
FITC	fluoresceine-isothiocyonate
FCS	fetal calf serum
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
IEL	internal elastic lamina
kDa	kiloDalton
KIU	kallikrein inhibition unit
mRNA	messenger ribonucleic acid
NADH	reduced nicotinamide adenine dinucleotide phosphate
NP	nanoparticle
NP 40	ethylenphenyl-polyethylene glycol (NONINDET P40)
nt	nucleotide
ODN	oligodeoxynucleotide
PAGE	polyacrylamide gel electorphoresis
PCNA	proliferating cell nuclear antigen

PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PFA	paraformaldehyde
PI	propidium iodide
PLGA	poly(DL-lactide-co-glycolide)
PMS	phenanzine methosulfate
PMSF	phenylmethana-sulfonyl fluoride
PTCA	percutaneous transluminal coronary angioplasty
RAoSMC	rat aortic smooth muscle cell
RNA	ribonucleic acid
RNase H	ribonuclease H
SC	scrambled
SDS	sodium dodecyl sulphate
Temed	N,N,N,N-Tetramethyl-ethylendiamin
TRITC	tetrarodamineisothyocyonate
Tris	Tris(hydroxymethyl)aminomethane(TRIZMA BAZE)
XTT	2,3-bis(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell

1 Introduction

1.1 Postangioplasty restenosis

1.1.1 Definition of restenosis

Being first introduced into clinical practice by Gruentzig in 1978, PTCA is now a well-established and frequently performed procedure that has an initial success rate in reestablishing arterial patency of more than 95% (63, 127). Worldwide, more than 1.5 million percutaneous coronary and peripheral angioplasty procedures are being performed annually (171). Although a long-term symptomatic improvement occurs in the majority of cases, the procedure is complicated by restenosis in 10% to 50% (14, 47, 140). Postangioplasty restenosis can be defined as a renarrowing of more than 50% lumen diameter at the site of balloon dilatation (47); restenosis usually occurs within 6 months after the initial procedure (47, 184). Since 1988, the rate of restenosis has fallen to 20% with the advent and widespread use of coronary stents (47, 153). Now, with introduction of intravascular radiation therapy, in-stent restenosis seems to be reduced by another 50% (192), and with the initial preliminary results of drug-eluting stents, the rate of restenosis may be reduced to less than 5% in *de novo* lesions (37, 67). Although these outcomes are truly remarkable, both intravascular radiation therapy and drug-eluting stents have a number of significant limitations (47, 112) and leave the place for the development of restenotic lesion. Despite the apparent success of many therapeutic modalities in animal models (86, 102, 142), attempts to use pharmacological approaches to prevent restenosis in the clinical setting have not been successful (154, 172, 188, 196). A possible explanation for the disappointing results of pharmacological approaches may be related to differences in the drug dose administered in animals and the one required in humans. In addition, the difficulty in providing sustained administration of the agent to the target site may be another reason. The failure has prompted research into alternative modalities of intervention including the use of antisense oligonucleotides therapeutically targeting genes believed to be critical for the pathogenesis of restenosis (102, 103, 122).

1.1.2 Mechanisms of restenosis

When an artery is dilated by angioplasty, there is an "initial gain" in lumen size. Restenosis can best be defined as a "loss of gain" (128) that is, an early or late (see below) return of the vessel lumen to a stenotic state. It is important to note that this

definition describes restenosis in terms of lumen size, but does describe the mechanisms that lead to the changes in lumen size and restenosis. Indeed, the mechanism involved in increasing the lumen size at angioplasty, the "acute gain," is still not entirely clear. Some of the most illustrative findings on the effects of angioplasty on the vessel wall have come from recent ultrasound studies that suggest that only a small amount of actual plaque mass is lost from the lesion site. Rather, most of the acute gain appears to be due to fracture and compression of the plaque, with fractures of the internal elastic lamina and overstretch of the vessel (117, 143, 173).

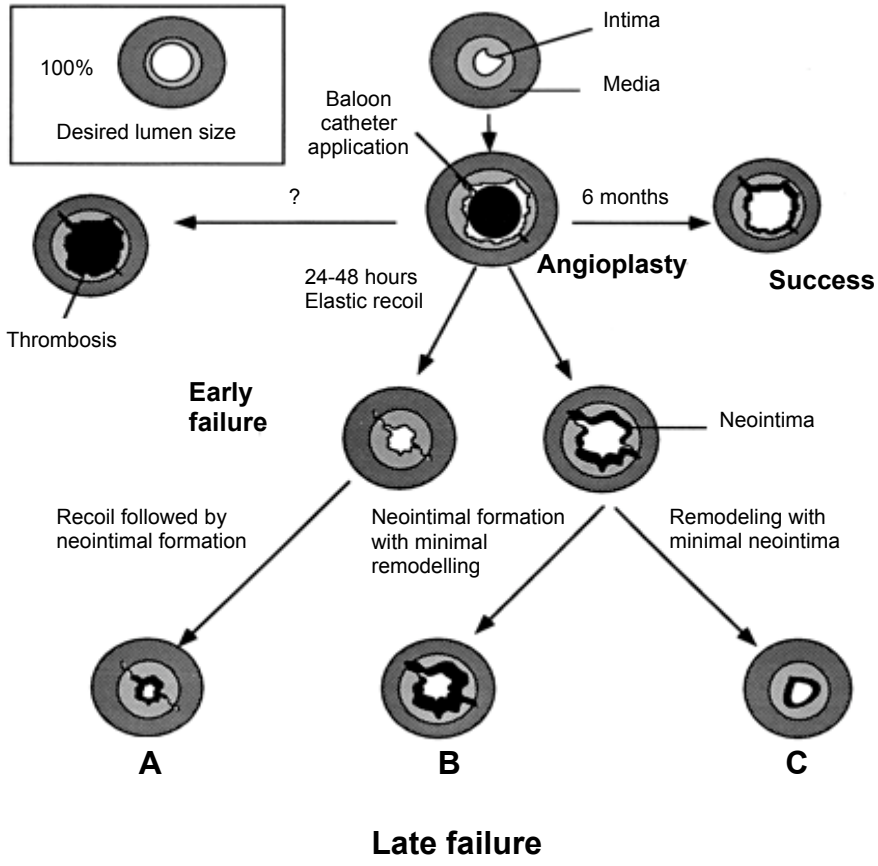

Figure 1-1. Flow chart shows possible outcomes after angioplasty and mechanisms responsible for restenosis. The events leading to both early and late failure after angioplasty are described in the text. It is important to note that an increase in neointimal mass may not necessarily cause restenosis if sufficient remodelling of the vessel occurs, and remodelling also may result in restenosis with minimal increase in neointimal mass. Inset shows desired lumen size (modified after Bennett M (Ref. 8).

After angioplasty, about 70% of patients have a persistently dilated vessel that has the desired lumen size. This persistent gain of lumen can be called "success" (Fig. 1-

1). In contrast, the loss of initial gain in the lumen size can be defined as "failure" and the latter can be divided into early and late forms. Early failure occurs when the lumen is occluded by a thrombus or by rapid recoil of the stretched vessel (18, 109); critically, after the invention of brachytherapy thrombosis at the site of intervention has been observed as late as 6 months following the procedure (192). These early processes occur within hours or at most within a few days after angioplasty and are distinct from restenosis. However, it is very important to remember that subclinical early recoil, combined with the mechanisms discussed below, may also be an important contributor to what appears to be late loss of lumen gain and therefore can be considered as a way of restenotic lesion formation ("Late Failure A" in Fig. 1-1).

The two possible mechanisms for late lumen loss are shown in Fig. 1-1. The first mechanism is remodelling. Remodelling is a normal process that vessels use to maintain an appropriate lumen size or caliber, particularly in response to changes in blood flow (51). The second mechanism of restenosis is neointimal formation and its newly formed mass can narrow the lumen (150). Following angioplasty some degree of neointimal hyperplasia may be tolerated, if remodelling permits some compensatory dilatation ("Success" in Fig. 1-1); in this case an example of positive, outward remodelling takes place. The extent to which neointimal formation will narrow the lumen is dependent on remodelling ("Late Failure B" in Fig. 1-1); in this case outward remodelling is not able to counteract the neointimal formation. Equally, remodelling itself after angioplasty may cause restenosis without a significant increase in the neointimal mass - negative, inward remodelling ("Late Failure C" in Fig. 1-1, 144).

Little is known about the molecular mechanisms involved in remodelling. As a consequence clear pharmacological strategies are lacking. On the other hand, neointimal formation is the result of VSMC migration from the media or adventitia, followed by cell proliferation and connective tissue formation. High levels of proliferation markers have been shown in atherectomy tissue from both primary and restenotic lesions (167). It is also important to notice that the injured wall may produce new extracellular mass via mechanisms that are independent of proliferation (150). Collagen, elastin, and proteoglycans may all contribute to the lumen loss by forming a mass that occludes the lumen. Finally, a decrease in lumen caliber could occur by retraction or contraction of healing tissue in the wound. This latter

mechanism would produce restenosis even if there was no actual increase in neointimal tissue mass.

1.1.3 Animal models of restenosis and the role of vascular smooth muscle cells in restenosis

Several animal models of restenosis have been developed and these have led to an improvement in our understanding of the cellular mechanisms involved. The anatomical and physiological similarities between human and porcine/non-human primate cardiovascular systems have led to the use of these animals to study restenosis (56, 164). It is possible to use the same balloon catheters and similar inflation pressures as used clinically, and the morphology of the lesion that follows balloon injury is similar to that seen in human postangioplasty restenosis (97). These large animals also allow easy application of site-directed drug delivery systems (porous balloon, coated stent) (48, 70). Advantageously, rabbits are very responsive to atherogenic diets, developing hyperlipidaemia and early lesions soon after initiation (170). There are also inbred rabbit strains with genetic abnormalities of lipid metabolism, equivalent to human conditions such as familial hypercholesterolaemia (the Watanabe heritable hyperlipidaemic rabbit; 169) and familial combined hyperlipidaemia (151). The rat model is not ideal for studying human disease (91, 150), because of the lack of the IEL rupture induced by the 2-Fr-Fogarty-compliant balloon (25, 91) that is most often used to induce restenosis in this animal. On the other hand the ballooned rat carotid injury model, developed by Clowes (25), remains the best characterized model to date (48) and it has proven useful to study the kinetics of VSMC response to injury (25, 167). Recently, a rat carotid artery dilatation model with IEL rupture using a standard PTCA balloon catheter has been tested (90, 91). There are also mouse models of restenosis induced by perivascular electric current application (16) or green light illumination (130). The attractiveness of a mouse model arises from the possibility to use transgenic or knockout animals (42).

The studies empoying balloon rat carotid injury model have led to the definition of four waves describing the VSMC response to injury and to the identification of molecules playing a role in, or interacting with, each of these waves in rats (27, 76, 144). In the Clowes' rat model complete destruction of the endothelium, as well as extensive death of medial VSMC accompanied by monocyte attachment and platelet aggregation (25, 42) comprise the events immediately

following angioplasty. The initial response to balloon injury, called "first wave," consists of medial VSMC proliferation and begins 24 hours after the injury as a response to the local increase of PDGF, bFGF and other cytokines (27, 48), released from dying medial VSMC and thrombi. The migration of VSMC across the internal elastic lamina to form the intima constitutes the "second wave". VSMC are readily observed on the luminal side of the internal elastic lamina 4 days after injury to rat arteries (25, 27). Once smooth muscle cells arrive in the intima of the rat artery, neointimal cells closest to the lumen may replicate for weeks to months (26). This replication is called the "third wave." The neointima can be stimulated to show a further increase of replication by infusion of different mitogens. This increased responsiveness can be called a "fourth wave" (34, 113, 120).

In summary, restenosis is defined as a late lumen loss occurring weeks or months after angioplasty. The potential contributing processes include remodelling, healing of the injured vessel, VSMC proliferation, VSMC migration, and formation of new extracellular matrix (the latter three taken together cover the process of neointima formation). Any component of these interrelated processes is a theoretical target for intervention, and pharmacological approaches to each have been proposed (84, 85).

1.2 Platelet-derived growth factor (PDGF)/platelet-derived growth factor receptor (PDGFR) system and its role in restenosis

1.2.1 Biochemistry of the PDGF/PDGFR system

PDGF is a major motogen and somewhat weaker mitogen for fibroblasts, smooth muscle cells, and other cells (82, 145). Originally, PDGF was identified as a constituent of whole blood serum that was absent in cell-free plasma-derived serum (105, 148, 189); PDGF was subsequently purified from human platelets (4, 38, 83). Although the □-granules of platelets are a major storage site for PDGF, recent studies have shown that PDGF can be synthesized by a number of different cell types, actually all vascular wall cells, with VSMC among others (145).

Human platelets and cultures of cell lines naturally expressing both A- and B-chains contain all three PDGF dimeric forms (homodimers AA, BB, and heterodimer AB), suggesting that the assembly of PDGF dimers is a random process (73, 75, 77). Most cell types expressing PDGF make both A- and B-chains, but the expression of the two chains are independently regulated at the transcriptional as well as

posttranscriptional levels (41). Recently, two more PDGF chains, namely C and D, have been discovered (98, 115), but the knowledge on them, as to their localization, synthesis and function, still needs to be elucidated. As far as their relation to the problem addressed in this study has not yet been reported the main focus will be on the well-studied isoforms.

PDGF isoforms exert their effects on target cells by activating two structurally related protein tyrosine kinase receptors. The □- and □-receptors have molecular sizes of about 170 and 180 kDa, respectively, after maturation of their carbohydrates. Because PDGF isoforms are dimeric molecules, they bind two receptors simultaneously and thus dimerize receptors upon binding (11, 80, 152). The □-receptor binds both the A- and B-chains of PDGF with high affinity, whereas the □-receptor binds only the B-chain with high affinity. Therefore, PDGF-AA induces □□-receptor homodimers, PDGF-AB induces □□-receptor homodimers or □□-receptor heterodimers, and PDGF-BB induces all three dimeric combinations of □- and □-receptors (74, 94).

The three dimeric PDGF receptor combinations transduce overlapping, but not identical, cellular signals. Whereas the heterodimeric receptor complex may have unique properties, most information is available about the functional roles of PDGF receptor homodimers. Both □- and □-receptor homodimers transduce potent mitogenic signals. There is, however, a difference between the receptors regarding their effects on the actin filament system. Whereas both receptors stimulate edge ruffling and loss of stress fibers, only the □-receptor mediates the formation of circular actin structures on the cell surface (45). Both the □-receptor and the □-receptor mediate an increase in intracellular Ca^{2+} concentration, albeit the □-receptor more efficiently than the □-receptor; pretreatment with PDGF-AA reduced the Ca^{2+} mobilization after PDGF-BB stimulation (40). PDGF also inhibits gap junctional communication between cells (88) and exerts an antiapoptotic effect (195). Because there are differences between □- and □-receptors in their binding specificity of PDGF isoforms and in the signals they transduce, the response of a cell to PDGF stimulation will be determined by which of the two receptor types are expressed in the cell. The target cells for PDGF such as fibroblasts and smooth muscle cells express both □- and □-receptors, but generally higher levels of □-receptors (82).

A common theme for activation of tyrosine kinase receptors is ligand-induced receptor dimerization, which juxtaposes the intracellular parts of the receptors and

allows autophosphorylation of tyrosine residues in trans between the two receptors in the dimer (81). The autophosphorylation induced after dimerization of PDGF receptors serves two important functions. On one hand, phosphorylation of a conserved tyrosine residue inside the kinase domains (Tyr-849 in the β-receptor and Tyr-857 in the α-receptor) leads to an increase in the catalytic efficiencies of the kinases, as has been demonstrated for the PDGF β-receptor (46, 99). On the other hand, autophosphorylation of tyrosine residues located outside the kinase domain creates docking sites for signal transduction molecules containing SH2 domains.

1.2.2 Role of PDGF/PDGFR- system in postangioplasty restenosis

Whereas PDGF is expressed at low levels in arteries from healthy adults, its expression is increased in conjunction with the inflammatory-fibroproliferative response that characterizes restenosis (147). Thus studies of balloon catheter-injured arterial tissue (82, 103), coronary arteries after PTCA (177) revealed increased expression of PDGF and PDGFR in these lesions. It was shown that PDGF B-chain markedly increased at the site of arterial injury, peaking at day 7 (sevenfold) and staying augmented until day 21, while PDGF A-chain showed only slight increase (1.5-fold). Likewise, the expression of PDGFR-β increased 3-fold (176). The events were co-localized with vascular smooth muscle cells at the sites of injury. In accordance with PDGF B-chain overexpression cellular proliferation in neointima peaked at day 7, being followed by a dramatic increase of neointimal areas thereafter (176). These observations suggest that PDGF, produced by activated macrophages, smooth muscle cells, or released from platelets in thrombi, is important for the formation of the lesion. The involvement of PDGF in the restenotic process has been confirmed experimentally using balloon catheterisation of rat carotid arteries as a model. In the denuded artery, an increased amount of activated PDGF receptors was seen at the sites of injured vessel wall and the activation of substrate proteins has been shown (1, 138).

The development of specific PDGF/PDGFR antagonists has allowed studies on the effects of such compounds in different animal models of intimal hyperplasia formation. Promising results have been obtained in rat, porcine, and nonhuman primate models of arterial injury through the use of, for example, neutralizing antibodies against PDGF (49). In addition, a low-molecular-weight PDGF receptor kinase inhibitor, AG-1295, was shown to inhibit neointima formation by 35% in a rat

carotid model (50) and by ~50% after balloon injury in porcine femoral arteries (6). The role of PDGF in the restenotic lesions may be to stimulate smooth muscle cells to migrate from the media of the vessel to the intima layer and to proliferate and produce matrix molecules at this site. Antibodies to PDGF inhibited intima thickening by about 50% and anti-FGF antibodies also by about 50%; the combination of anti-PDGF and anti-FGF antibodies resulted in 84% inhibition of intima thickening (149). In this study a significant decrease in intimal hyperplasia (more than 80%) was observed after 8 days of the Ab administration, suggesting the important role of PDGF in the early stages of this process. However, PDGF-AA appears to be less important, since no appreciable inhibition of intimal hyperplasia was observed after administration of PDGF-AA antiserum (78).

Platelet-derived growth factor is also involved in neointima formation in other model systems including human saphenous vein cultured *in vitro* (58). Given the role for PDGF in restenosis, the PDGFR-β has been targeted in attempts to inhibit intimal hyperplasia. Neutralizing mouse or mouse/human chimeric monoclonal antibodies against the receptor have been found to inhibit intimal thickening in injured arteries of the baboon, and a murine monoclonal antibodies to inhibit intimal thickening of rat carotid arteries (78). The role of PDGFR-β and the attractiveness of its targeting to inhibit neointimal mass accumulation has been further supported in baboon vascular graft model where mouse/human chimeric antibodies were tested (35) with the intimal areas significantly reduced after treatment with anti-PDGFR-β but not anti-PDGFR-α antibodies. Targeting endogenous platelet-derived growth factor B-chain by adenovirus-mediated PDGFR-β extracellular domain gene transfer potently inhibited in vivo smooth muscle cell proliferation following arterial injury (69).

These observations suggest that inhibitors of PDGF-BB or PDGFR-β may be clinically useful to prevent the restenosis that occurs at high frequency after PTCA.

1.3 Antisense technology

Conceptual simplicity, the possibility of rational design, relatively inexpensive cost and developments in the sequencing of human genome have led to the use of short fragments of nucleic acids, commonly called oligodeoxynucleotide (ODN), either as therapeutic agents or as tools to study gene function. The concept underlying the AS technology is rather straightforward: the use of a sequence, complementary by virtue of Watson-Crick bp hybridisation, to a specific mRNA can

inhibit its expression and then induce a blockade in the transfer of genetic information from DNA to protein. To understand how AS agents suppress proliferation in animal models of restenosis or eventually how they may work in humans, it is necessary to analyse how AS-ODN themselves block gene expression. The synthesis of cellular proteins (whether structural components, enzymes, receptors, or proteins involved in cellular proliferation) occurs via a coordinated sequence of molecular events (Fig. 1-2).

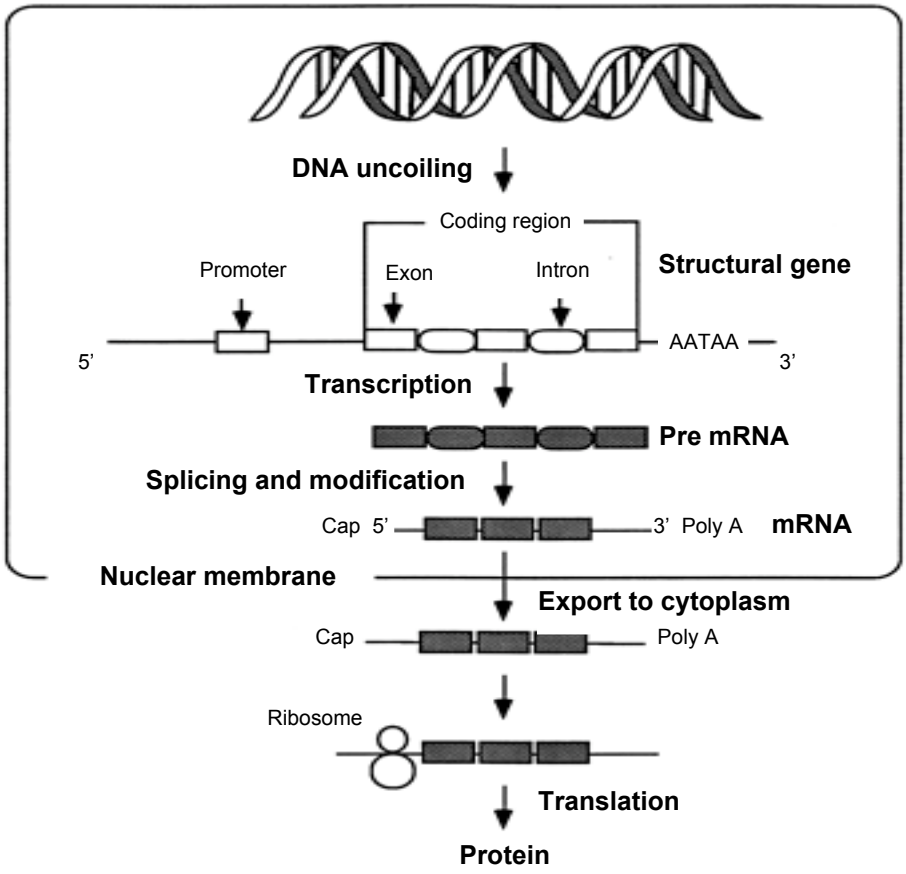

Figure 2. Schematic representation of the events leading to expression of cellular proteins. DNA of the gene to be expressed is uncoiled, and a complementary mRNA copy is transcribed. This mRNA is processed, which involves splicing out of introns (areas of mRNA that are not translated), methylation at the 5' end, and polyadenylation at the 3' end, and exported from the nucleus. The mRNA is then translated into protein via binding and movement of the ribosome complex along the mRNA molecule (modified after Bennett M (Ref. 8).

The AS approach to inhibit gene expression is to block any one of the following processes: uncoiling of DNA, transcription of DNA, export of RNA, RNA splicing, RNA stability, or RNA translation (critically, the first two are usually separated under “antigen” approach).

1.3.1 AS-ODN: mechanism of action

Many studies have used the properties of AS-ODN to inhibit gene expression in cultured cells, and their use has also been extended to whole organisms (30, 63, 135). These studies have shown that AS-ODN targeted to cellular mRNA sequences can reduce target gene mRNA and/or protein product (79) and exert biological effects, manifested usually as a suppression of cell proliferation or differentiation. Therefore, AS-ODN are useful agents for targeting any gene sequence *in vitro* or *in vivo*. AS-ODN are short (usually less than 30 bp) complementary DNA or RNA sequences that will hybridise to a specific mRNA forming a hybrid duplex. Although the precise mechanisms by which AS-ODN reduce target mRNA and protein levels within the cell are imperfectly understood, two main mechanisms have been postulated. First group of ODN includes steric-blocking ODN that physically prevent or inhibit mRNA export to cytoplasm, the progression of splicing, assembly of ribosome machinery or the translation. Evidence for steric interference comes from studies in which AS to the 5' cap of the mRNA has been found to be most effective in inhibiting rabbit □-globin protein synthesis (63). Interference with the splicing, that could be caused by ODN to 5’ or 3’ mRNA regions involved in this process (106), can lead to the lack of expression of a mature protein (60, 89) or, as numerous reports have shown, to the correction of aberrant splicing and the restoration of a functional protein (158, 181).

Second group of ODN includes RNase H-dependent ODN that induce the enzymatic degradation of mRNA. Critically, the majority of AS drugs investigated in the clinic function via an RNase H-dependent mechanism. RNase H is a ubiquitous enzyme that hydrolyses the RNA strand of RNA/DNA duplexes (183). ODN-assisted RNase H-dependent reduction of targeted RNA expression can be quite efficient, reaching 80-95% down-regulation. Furthermore, in contrast to the steric-blocker ODN, RNase H-dependent ODN can inhibit protein expression when targeted to virtually any region of the mRNA (36, 111). The importance of RNase H-induced cleavage of mRNA has been shown in at least four systems, including wheat germ extract (17), rabbit reticulocyte lysate (129), Xenopus oocytes (157), and human leukaemia cells (61).

Mechanism of AS-ODN action is dependent on ODN backbone modification (165, 183). In this light phosphodiester and phosphorothioate ODN function via

RNase H-dependent mechanism while 2'-O-methyl, methylphosphonates, phosphoramidate, morpholino and "second generation" PNA-ODN employ steric-blocking mechanism (164).

1.3.2 Target sequence

A number of theoretical considerations help in the choice of target sequence for AS ODN within a specific mRNA. As most AS-mRNA interactions are proposed to occur within the cytoplasm, areas of the mRNA with no or little (in this case ODN would substitute one of the mRNA strands) secondary structure should offer attractive targets. This frequently means sequences directed to the region around the initiation codon (ATG) of the mRNA; about 70% of all ODN sequences reported in the literature are targeted to this region (5, 61, 174). For interactions involving nuclear mRNA, splice sites involved in mRNA processing and export have also been found to be effective. Other sites that have been found to be particularly effective are related to the 5' cap; the 5' cap is where a number of initiation factors bind for ribosome assembly and ribosome translocation along the mRNA (Fig. 1-2; 107). Despite these considerations, however, a few base-pair shift in target sequence can profoundly affect the ability of an ODN to inhibit gene expression. In addition, sequences directed at different parts of the same mRNA have widely differing activities (2, 21). Although the secondary structure of the mRNA may be partly responsible for differences in hybridisation, the full explanation of this phenomenon is unknown. This makes design of ODN sequences an informed guess at best, and many sequences are usually tested before sequences are chosen that exert maximal suppression of target gene expression (21). The coding region itself appears to be somewhat less targetable. In fact, compared with the 5'-untranslated or initiation codon regions, there exist just a few examples of ODN able to induce an AS effect when targeted to coding regions (68, 131, 141). This may be attributable to the ability of the ribosomal machinery to unwind the ODN from its targeted mRNA.

1.3.3 Advantages of the AS approach over the other inhibitory strategies

There are many ways of how cell proliferation can be inhibited pharmacologically, so what are the advantages of using AS approach compared with conventional inhibitors?

The first major advantage of synthetic AS-ODN, at least in theory, is the potential for design of agents with target specificity. The hybridisation of base sequences between nucleic acids is very specific; only the complementary base (C-G, A-T) should be bound. Because the mRNA of related proteins often have areas lacking significant homology, this specificity of base pairing means that an AS sequence of bases should target only a single mRNA, without affecting the mRNA of other genes. As evidence of this specificity, studies have shown that mRNA can discriminate between ODN that differ by one or two bases (8, 87, 186). In the latter two of these studies, changes in a c-myc AS sequence of only two bases resulted in almost complete loss of activity. This specificity of binding is greater than can be achieved with most conventional pharmacological inhibitors, which frequently act on a variety of proteins with different binding affinities.

The second advantage is that AS-ODN targeted to specific mRNA are much easier to design and synthesize than any previous class of drugs. The structures of ODN are relatively simple, consisting only of oligomers of the four base pairs. Since the target sequence is known, rational drug design against a target is theoretically obvious without screening thousands of products as occurs with pharmacological agents. Finally, if delivered properly, the effects of ODN-based drugs should be highly localized. ODN are taken up into cells and are trapped in the intracellular compartment (54). Any ODN that remain outside the cell or undergo exocytosis is likely to be rapidly degraded by serum nucleases. This mechanism may help to restrict local delivery of AS-ODN to the site of delivery. Indeed, site specificity is a very important consideration if one is to target replication in restenosis. Other potent antiproliferative drugs used in cancer chemotherapy almost always have systemic side effects that would be unacceptable in the treatment of restenosis. However, site-specific inhibitors of proliferation cannot be used in the vessel wall because no smooth muscle–specific pathway has been shown to exist. This problem is successfully overcome using site-directed drug delivery systems such as porous balloons or drug-coated stents.

1.3.4 Limitations of the AS technology

1.3.4.1 Cellular uptake and stability

A potential problem of AS delivery into cells or tissues relates to uptake and stability of sequences. In cell culture, ODN are usually microinjected into cells or added to the culture medium, whereupon they are taken up into cells. Microinjection is feasible only for small numbers of cells, and therefore most studies in cultured cells use direct addition to the culture medium. However, the exact mechanism of ODN entry into cells by use of this method is unclear. ODN are typically 15 to 30 bp long with molecular weights from 4500 to 9000 Da. ODN are also polyanions and cannot passively diffuse across cell membranes. Uptake depends on length of ODN, overall charge and hydrophilicity/lipophilicity (which in turn depend upon chemical modifications of the ODN; see below), and concentration of ODN. Uptake is also an energy-requiring process that is optimal at 37°C (32). Studies using fluorescent acridine–labelled ODN have suggested that uptake of unmodified sequences is by a mechanism consistent with receptor-mediated endocytosis, and two surface proteins (34 and 80 kDa) have been identified that may mediate the process (116, 193). However, this route of uptake has yet to be conclusively proven, and it is also likely that the predominant method of uptake differs among the modified ODN (32). For instance, it has been shown that the 80-kDa protein binds phosphodiester and phosphorothioate ODN, but not methylphosphonates (116, 165). At relatively high ODN concentration (□M range), these receptors are saturated, and the pinocytotic process assumes lager importance. Movement of ODN across cell membranes is also not a one-way process. ODN exocytosis has been demonstrated in a number of cell types (32) being temperature dependent, optimal at 37°C.

Uptake of both phosphodiester and modified ODN is generally an inefficient process (64, 163, 165). More specifically, naked ODN tend to localize in endosomes/lysosomes, where they are unavailable for AS purposes. Numerous experiments have demonstrated that the *sine qua non* of AS activity appears to be nuclear localization. To improve cellular uptake and ODN spatial and temporal activity, a range of techniques and transporters have been developed. Simultaneously, the use of these vectors increases the stability of ODN against nuclease digestion and permits the use of far lesser (approximately 1/10th) concentrations of ODN.

Endo-lysosomal escape has been reported for a number of vectors used in gene therapy including viruses, fusogen peptides, cationic lipids, and cationic

polymers (9, 15, 24). Viruses and peptide toxins use a fusogen peptide to cross the endosomal membrane and reach the cytosol (31). Nonviral vectors such as cationic lipids and polycations protect the DNA by either retarding the transfer of DNA from endosomes to lysosomes or destabilizing the endo-lysosomal membranes (187). However, these carriers suffer from a number of limitations including immunogenicity, toxicity, and instability *in vivo* (119). It has been shown (109, 156) that polymeric NP formulated from the biodegradable polymer PLGA are able to cross the endosomal barrier and deliver the encapsulated therapeutic agents into the cytoplasm. NP are colloidal systems that typically range in diameter from 10 to 1000 nm, with the therapeutic agent either entrapped into or adsorbed or chemically coupled onto the polymer matrix (108). The PLGA-NP formulation with a therapeutic agent entrapped into the polymer matrix provides sustained drug release. The degradation products of PLGA are lactic and glycolic acids that are formed at a very slow rate and are easily metabolised in biological systems via the Krebs cycle and are eliminated (135). PLGA has previously been used for drug, protein and gene delivery applications because of its biocompatibility and sustained-release properties (28, 72, 110). Thus, PLGA-NP offer the advantages of safety, the ability to carry different classes of therapeutic agents, and the possibility of sustained intracytoplasmic delivery. A sustained therapeutic effect of an NP-encapsulated low molecular weight drug with cytoplasmic receptor (dexamethasone) and sustained gene expression with DNA-loaded NP as an example of a macromolecular therapeutic agent was demonstrated (139). The escape from endo-lysosomal compartment has also been shown leaving the latter intact (139).

PLGA-NP can be internalised by VSMC in an energy-dependent manner, which suggests an endocytotic process (134) that has been reported to run in energy-dependent and saturable manner (168). In another study NP uptake was significantly reduced after inhibition of clathrin vesicles but not caveolae. Clathrin-mediated transport has been previously reported for active receptor-mediated endocytosis (59). Recently, a nonspecific mechanism, possibly by fluid-phase pinocytosis, has been suggested (139). It was also noticed that intracellular uptake of the NP is unaffected by serum (139) and hence PLGA-NP are suitable for *in vivo* applications.

In addition to generally poor uptake, the instability of ODN has been a significant problem in their use *in vitro* and their potential use *in vivo*. ODN are very

sensitive to degradation by exogenous and endogenous nucleases (phosphodiesterases) (191). These enzymes are widespread, with significant activities being demonstrable in serum (178, 190) and the presence of nucleases has previously precluded the use of unmodified ODN in studies of whole cells (87). To improve stability against nucleolytic phosphodiesterases, the phosphate backbone of the ODN has been chemically modified in a variety of different forms (178, 180). Compared with the unmodified phosphodiester linkage, chemical modifications such as phosphorothioate (Fig. 1-3) bonding have improved nuclease resistance by up to 10-fold, thereby reducing the concentration at which a biological effect can be observed (18, 126). It is also believed that individual phosphodiester linkages may be employed in an AS-ODN, particularly if it is protected at the 3' and 5' termini with, for example, three PS linkages (164). Despite the use of modified ODN, the inhibition of gene expression using AS-ODN is an inefficient process. Studies using unmodified ODN have required concentrations of 50 to 100 μmol/L to inhibit gene expression by 90% (33, 146) representing very high molar ratios of ODN to mRNA.

1.3.4.2 Other biological effects of ODN

Another problem concerning specificity of action of ODN is that of possible sequence-independent pharmacological effects of large amounts of duplex or interactions of ODN sequences with cellular proteins. Another potential source of a non–AS inhibition of biological processes relates to the fact that ODN can bind to cellular proteins in a sequence-specific manner. The binding of ODN, designated aptamers, to proteins can alter that protein's biological activity (12). Suppression of protein activity has also been documented *in vivo*, indicating that the ODN can be used pharmacologically without any specific AS action being implicated (118). In fact, the binding of ODN to specific proteins might actually provide a much more specific inhibition of a target protein than AS-ODN binding to the equivalent mRNA. Although the binding between protein and ODN is dependent on the sequence of ODN, it is not yet possible to predict which proteins will bind with which ODN sequences. Thus, sequence-specific binding to a protein may result in inhibition of an expression of a non-targeted gene when AS-ODN are used. ODN can also bind proteins in a non-sequence-specific manner, including CD4 protein kinase C-□1 and albumin (194). Although the binding constants of ODN to proteins via non–sequence-specific interactions are usually significantly lower than those of a natural ligand, this is not

always the case. Thus, introduction of an ODN into a cell may affect the function of a wide range of proteins, which may be responsible for the biological effect observed.

1.3.5 Data from preclinical and clinical studies using AS approach

Over the last 2 decades a plethora of experiments has been published, employing AS approach to inhibit gene expression at the mRNA level both in tissue culture and *in vivo*. These data led to the introduction of phosphorothioate ODN into clinical therapeutic trials. At the present time the most promising of these are G3139, an 18-mer targeted to the initiation codons of the bcl-2 mRNA and ISIS-3521, a 20-mer targeted to the 3'-untranslated region of the protein kinase C-□ isoform. A phase I study of G3139 (Oblimersen, Genasense™, Genta Inc.) in combination with chemotherapy has been performed in patients with refractory or relapsed acute leukaemia (125). Randomized clinical trials are currently underway to evaluate the efficacy and tolerability of oblimersen in combination with cytotoxic chemotherapy in chronic lymphocytic leukemia, multiple myeloma, malignant melanoma, and non-small cell lung cancer. In addition, nonrandomized trials are under way to evaluate oblimersen in non-Hodgkin's lymphoma, acute myeloid leukemia, and hormone-refractory prostate cancer. Preclinical data also support the clinical evaluation of oblimersen in additional tumor types, including chronic myelogenous leukemia and breast, small cell lung, gastric, colon, bladder, and Merkel cell cancers (104). In advanced lung cancer, early results with ISIS-3521 when added to combination chemotherapy have raised speculation that life expectancy may be prolonged as much as 50% (57). Promising results on ISIS-3521 initiated in October 2000 phase III trials in combination with carboplatin and paclitaxel for the treatment of non-small cell lung cancer (114).

AS constructs directed against cell cycle regulators - such as c-myb, c-myc, cdc-2, cdk-2, proliferating cell nuclear antigen (101, 160) and retinoblastoma protein (19) or blockade of intracellular signal transducers (e.g., Ras and Raf kinase) (23) - have diminished intimal thickening with varying degrees of efficacy in animal models of restenosis. Among the AS-ODN reported to date, E2F decoy AS-ODN has undergone the most extensive evaluation with successful completion of preclinical and toxicology studies, as well as phase I/II human trials (66, 123). E2F is a transcription factor and it is believed that blocking the cell cycle could prevent neointimal hyperplasia *via* suppression of VSMC proliferation. Indeed, the first clinical

trial (PREVENT-I) on human bypass vein grafts demonstrated that E2F decoy ODN inhibited PCNA and c-myc expression and blocked VSMC proliferation of the graft (123). PREVENT-I studied a cohort of patients at high risk for lower extremity graft failure in a prospective, randomised and controlled fashion. The study results met the primary end points, which were safety and feasibility. A second larger phase IIB clinical trial was performed to study the effect of E2F decoy ODN administered following CABG. The result demonstrated a 30% to 40% decrease in CABG failure as documented by angiography and intravascular ultrasound (66). Two phase III trials evaluating efficiency in CABG and peripheral arterial bypass are currently underway.

1.4 Aim of the study

The present study has been designed and conducted to study the possibility of postangioplasty restenosis inhibition by means of AS approach to target the related PDGFR-β mediated events in vascular SMC. For this purpose, AS (and corresponding SC) ODN have been designed complementary to the regions of predicted initiation codons at nt 77 and 269 of rat PDGFR-β mRNA. Partially phosphorothioate ODN have been employed in both naked and PLGA-NP formulations. In *in vitro* model of isolated RAoSMC has been established and possible ODN cytotoxicity and the impact of the ODN on cell proliferation have been studied. To elucidate the underlying mechanism of ODN action we further investigated the influence of the ODN on the expression of PDGFR-β and its PDGF-BB-induced activation.

2 Methods and materials

2.1 Reagents and materials

FCS, XTT, PMS, sodium-orthovanadate, TRIZMA BASE, Zn acetate, BPB	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
ODN sequences	Alpha DNA, Montreal, Canada
PMSF, saccharose, PFA, Triton X-100, Temed, methanol, CCl_3COOH, glycerol, EDTA, DTT, Tween 20, skim milk powder, APS	MERCK KgaA, Darmstadt, Germany
NP40	USB Shell, Cleveland, Ohio
PDGF-BB	TEBU GmbH, Offenbach, Gemany
SDS	ICN Biomedicals Inc., Aurora, USA
DPBS, DMEM, L-glutamine, penicillin, streptomycin, gentamicin, rainbow marker	Invitrogen Corporation, Paisley, Scotland
BSA	SERVA Electrophoresis GmbH, Heidelberg, Germany
Mowiol 4-88	Calbiochem-Novabiochem Corp., La Jolla, USA
NaOH, HCl, NaCl	Mallinckrodt Backer B.V., Deventer, The Netherlands
Acrylamide/N,N-Methylene-bis-acrilamide	Bio-Rad Laboratories, Hercules, USA
microscopy slides, microscopy slide coverslips	Menzel–Glaser GmbH, Saarbrucken; Germany
6-, 12-, 24, 96-well plate; polysterene round-bottom tube; polypropylene 15, 50 ml conical tube; cell scraper; 10 and 25 ml serological pipet	Becton Dickinson and Company, Franklin Lakes, USA
tissue culture flasks (75 cm^2)	Greiner Labortechnik GmbH, Frikenhausen, Germany
trypsin/EDTA solution	Biochrom AG, Berlin, Gemany
pipette tips, 10 ml combi tips, safe-lock tubes	Eppendorf AG,

	Hamburg, Germany
glycin	AppliChem GmbH, Darmstadt, Germany
sterile scalpel	P.J. Dahlhausen&Co. GmbH, Köln, Germany
POLYVIALS V	Zisser Analytic, Bruchsal, Germany
Ultima Gold	Packard Bioscience B.V, Groningen, The Netherlands
trasylol	Bayer Vital GmbH, Leverkusen, Germany
fungizone	Bio Whittakker Co., Walkersville, USA
disposable cuvettes	Brand GmbH, Wertheim, Germany
rabbit anti-human PDGFR-□ antibody, mouse anti-phosphotyrosine (PY99) antibody, HRP-conjugated goat anti-rabbit antibody, HRP-conjugated goat anti-mouse antibody	Santa Cruz Biotechnology Inc., USA
rabbit anti-human von Willebrand factor antibody	DAKO A/S, Glostrup, Denmark
FITC-conjugated goat anti-rabbit antibody, TRITC-conjugated goat anti-mouse antibody	Dianova GmbH, Hamburg, Germany
mouse anti-human □-smooth muscle isoform of actin antibody	Progen Biotechnik GmbH, Heidelberg, Germany
5-Bromo-2'-deoxy-uridine labeling and detection kit III	Roche Diagnostics GmbH, Mannheim, Germany
[^{3}H]thymidine, Hybond-C extra, Hyperfilm MP	Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany

2.2 Cell culture studies

2.2.1 Cell culture media

For all the studies the basic medium used was DMEM with high (4500 g/l) glucose concentration. Medium#1 (isolation medium), medium#2 (cultivation medium) and medium#3 (starvation medium) were prepared with the following additives: penicillin 100 units/ml, streptomycin 100 µg/ml, fungizone 2,5 µg/ml, gentamicin 2,5 µg/ml, 2 mM L-glutamine – and differed each from the others only in FCS concentration as follows: medium#1 – 20% FCS, medium#2 – 10% FCS, medium#3 – 0,5% FCS.

2.2.2 Cell isolation and culture of rat aortic smooth muscle cells

RAoSMC were isolated by "explant" technique from thoracic aortas of female rats (Rattus Norvegicus, strain Sprague Dawley) weighing 300-400 g. All the rats were in their 2nd pregnancy and were dissected at the Department of Anatomy and Cell Biology, University of Ulm. Freshly dissected blood vessels from animals euthanized less than 5 min before were placed in ice cold sterile DPBS. The vessels were aseptically cleaned with removing fat and connective tissue (adventitia) and the lumen was opened longitudinally with a scalpel blade. The lumen surface was gently scraped with a scalpel blade to dislodge endothelial cells. Afterwards the vessel wall was cut with a scalpel blade into 4 mm^3 pieces (explants). The explants were placed into 6-well plates and pressed with their inner surface against the bottom. To promote cell adhesion, the 6-well plates were coated for 2 hours with fibronectin (1 µg/cm^2) in DPBS. After application of medium#1 the explants were left undisturbed for 1 week in a humidified incubator at 37°C and 5% CO_2 atmosphere to let the cells outgrow. Then the explants were aspired and medium was changed every two days afterwards. When the cells reached confluence they were subcultured with 0.05% trypsin/0.02% EDTA solution in DPBS (applied for 2 min) and passaged into plastic flasks with 75 cm^2 size with the medium changed every two days afterwards. The cells beyond the first passage were cultured in medium #2.

Cells to be passaged were detached by exposure to 0.05% trypsin/0.02% EDTA solution in DPBS for 2 min in the incubator with following trypsin inhibition by medium#2. Next, cells have been transferred into appropriate cell culture dishes at a desired dilution.

Cells in passages 3-5 have been used for all the experiments.

Cell identity has been confirmed by light microscopy (characteristic ‘‘hill and valley‘‘ growth pattern) and immunocytochemistry (positive staining for smooth muscle □-actin).

2.2.3 Immunocytochemistry and fluorescence microscopy

RAoSMC have been seeded at 20% confluence onto microscopy slide cover glasses in medium#2, let to attach and spread for the next 4 days with the medium changed every 2 days. Cells were fixed for 20 min at room temperature in 3.5% PFA (prepared in DPBS), washed once with DPBS, and then permeabilized by a 10-min exposure to 0.1% Triton X-100/PBS solution. The cells have been washed once with DPBS, exposed for 30 min to DPBS solution containing 2% glycin (to bind PFA acidic groups) and blocked for another 30 min with a DPBS solution containing 2.5% FCS (further in this assay – blocking solution). The primary Ab has been added, incubated for 1 hour at room temperature, and then removed by washing with blocking solution. Next, specific secondary Ab has been added, incubated for an additional hour at room temperature and washed off with blocking solution. The cover glasses have been mounted onto glass microscopy slides with Mowiol 4-88 as an antifade agent. Immunofluorescence microscopy has been performed on confocal laser scanning microscope LEICA DM IRBE (Leica Lasertechnik GmbH, Germany) and images have been processed with LEICA TCS NT software.

2.3 ODN design

AS ODN have been developed complementary to the regions of predicted initiation codons of rat PDGFR-□ partial mRNA (Rattus Norvegicus, strain Sprague Dawley, GenBank, accession Z14119). Potential ATG initiation sites have been predicted employing a web site programm ATG^{pr} (www.hri.co.jp/atgpr/ed). AS sequence specificity has been confirmed through GenBank using Blast resource. Corresponding SC sequences have been designed. The sequences were 18 bases long and have been synthesized by standard phosphoramidate chemistry and HPLC-purified.

Two AS sequences revealing the highest prediction specificity have been chosen:

1) complementary to the region (nt71 to nt88) of predicted initiation codon at nt77 (ATG77):

- AS 5'-*C*G*TTCCCACATCACT*G*G*-3';

- SC 5'-*C*G*TTTCCCACATCTAC*G*G*-3';

2) complementary to the region (nt266 to nt283) of predicted initiation codon at nt269 (ATG269):

- AS 5'-*C*G*GGAGGAAGCCCATG*G*T*-3';

- SC 5'-*C*G*GAGAGAGGCCGTCG*A*T*-3'.

In the signed (*) nucleotides phosphodiester backbone linkage (non-bridging oxygen) has been substituted with phosphorothioate one.

Incorporation of ODN into PLGA-NP has been performed at Department of Pharmaceutics (School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Israel) employing a double emulsion system and solvent evaporation technique.

All the ODN have been aliqouted and stored in the following buffer: 10 mM Tris, 1 mM EDTA (prepared in ddW, pH 7.4) - at –20°C.

2.4 Cell viability assays

2.4.1 XTT conversion method

To assess possible naked ODN influence on cell viability the method based on the cellular conversion of XTT into a formazan product by the action of NADH-generating dehydrogenases found in metabolically active cells has been used. RAoSMC were seeded in a 96-well plate at a density of 3000 cells/well in 100 µl of medium#2 and let to attach for the next 48 hours then medium was changed for the medium#3 with ODN; control samples have been treated only with the medium containing corresponding buffer. At the end of the tested period 40 µl of 0.01% XTT/125 µM PMS (the latter used to solubilize the formazan product) solution (prepared in DPBS) have been added for the additional 2 hours and the sample extinction has been measured at 450 nm (with the reference wave-length at 690 nm) in a microtiter plate reader SUNRISE 1000 (TECAN Deutschland GmbH, Crailsheim, Germany).

2.4.2 PI staining of cellular DNA and FACS analysis

To study the impact of encapsulated ODN on cell viability the method of DNA staining with PI has been used. RAoSMC have been seeded in a 6-well plate at a density of 60000 cells/well in 100 µl of medium#2 and let to attach for the next 48

hours, then the medium has been changed for medium#3 with encapsulated ODN at 3μM concentration for the next 48 hours; control samples have been treated only with the corresponding buffer. Cells have been washed once with DPBS/EDTA, detached by exposure to 0.05% trypsin/0.02% EDTA solution, samples collected and centrifuged at 1200^{-1} at 20°C. Pellets have been washed once with DPBS by centrifugation at 1200^{-1} at 20°C and resuspended in 1 ml of staining buffer (0.1% sodium citrate, 0.1% Triton X-100, 50 μg/ml PI, prepared in double distilled water) with the following transfer into polysterene round-bottom tubes where they stayed for the next 3 hours (4°C, in dark). Measurement has been performed on FACSCalibur (Becton Dickinson and Company, Franklin Lakes, USA). The data was processed on CellQuest Pro software.

The total of 10000 events have been counted and the ratio between DNA from living cells and total DNA has been calculated.

2.5 Cell proliferation assays

2.5.1 BrdU labelling assay

Cell proliferation under naked ODN administration has been evaluated with BrdU incorporation based DNA-synthesis assay. RAoSMC have been seeded in a 96-well plate at a density of 3000 cells/well in 100 μl of medium#2 and let to attach for the next 48 hours then the medium was changed for medium#1 with ODN. The cells were serum-starved for 24 hours prior to stimulation. Following the pre-treatment with ODN the cells were stimulated with PDGF-BB (10 ng/ml) for the next 24 hours and then BrdU labelling solution (prepared in DPBS, end concentration 10 μM) was added for another 4 hours. Next, the cells were washed twice with DPBS containing 10% FCS (further in this assay – washing buffer) and precooled (stored at -20°C) fixative solution (70% ethanol, 0.5 M HCl prepared in ddW) was applied for 30 minutes at –20°C followed by washing 3 times with the washing buffer and exposure for 30 min at 37°C to 100 μl nuclease solution (prepared in buffer containing 66 mM Tris, 0.66 mM MgCl2, 1 mM 2-mercaptoethanol). After washing off the nuclease solution with 200 μl of the washing buffer 3 times 100 μl of anti-BrdU peroxidase-conjugated antibody solution (DPBS containing 10 mg/ml BSA) were added for 30 min at 37°C. Antibody solution was removed by washing (3 times 200 μl of the washing buffer) with the following incubation with peroxidase substrate for 30 min in dark place.

The sample extinction was measured in a microtiter plate reader SUNRISE 1000 at 405 nm with a reference wave length at 490 nm.

2.5.2 [^{3}H]thymidine incorporation assay

Cell proliferation in the presence of encapsulated ODN was assessed with [^{3}H]-thymidine incorporation based DNA synthesis assay. RAoSMC were seeded in a 24-well plate at a density of 20000 cells/well in 500 µl of medium#2 and let to attach for the next 48 hours then the medium was changed for medium#1 with ODN. The cells were serum starved for 24 hours prior to stimulation. Following the pre-treatment with ODN cells were stimulated with PDGF-BB (end concentration 10 ng/ml) for the next 24 hours and then pulsed with [^{3}H]thymidine (250 nCi/well) for another 2 hours. DNA was precipitated with 5% CCl_3COOH solution for 30 min, washed twice with ice-cold double distilled water and solubilized in 1 M NaOH for 10 min with gentle agitation with the following neutralization with equal volume of 2 M HCl. The samples were collected and transferred into plastic vials POLYVIALS V for liquid scintillation (Ultima Gold) based measurement. Samples were thoroughly shaken and amount of incorporated radioactivity was assessed on 1219 RACKBETA (LKB Instrument GmbH, Grafelfing, Germany).

2.6 Protein isolation and SDS-PAGE

To study the impact of ODN on PDGFR-□ expression and its phosphorylation, RAoSMC have been cultured in 12-well plates. In all the experiments the cells at 70-90% confluence have been used. When the receptor phosphorylation was studied cells have been serum-starved in medium#3 for 24 hours prior to stimulation with parallel administration of ODN. Next they have been stimulated with 10 ng/ml of PDGF-BB for another 24 hours. All the following steps were run at 4°C. The cells were washed once with DPBS (in case of phosphorylation studies with 100 µM sodium-orthovanadate in DPBS) and then solubilized in the lysis buffer (1% NP40, 10% glycerol, 150 nM NaCl, 20 mM Tris/HCl pH 7.4, 10 nM EDTA, 100 µM orthovanadate, 1000 KIU trasylol, 1 mM PMSF, 1 mM Zn acetate prepared in ddW) for 30 min with gentle agitation. That was followed with scraping of the cell remnants from the plates and sample centrifugation (13000^{-1}; 4°C). The latter was done to remove the cell debris. After the sample transfer into new tubes the protein concentration was measured with Bio-Rad Protein Assay (Bio-Rad Laboratories,

Hercules, USA) on ULTROSPEC 3000 (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany).

30 µg of total protein from each sample were mixed with equal volume of SDS-PAGE sample buffer (2 nM Tris/HCl pH 8.8, 60% Saccharose, 4% BPB, 4% SDS, 10 mM DTT) and then boiled for 5 min with gentle agitation. Protein separation was performed under reducing conditions by SDS-PAGE (1 hour; 150 V; buffer composition: 2.88 % glycin, 50 mM Tris/HCL pH 8.7, 0.1 %SDS prepared in deionised water) on Mini-Protean 3 (Bio-Rad Laboratories GmbH). The gel composition was as follows: stacking gel: 0.125 M Tris/HCl pH 6.5, 0.1% SDS, 4% acrilamide/N,N-methylene-bis-acrylamide (29:1), 0.05% APS, 0.01% Temed; resolving gel: 0.375M Tris/HCl pH 8.8, 0.1% SDS, 7.5% acrylamide/N,N-methylene-bis-acrilamide (29:1), 0.05 %APS, 0.01 % Temed.

2.7 Western blot analysis

Following SDS-PAGE the proteins were electroblotted (2 hours; 396 mA; 4°C; buffer composition: 1.44% glycin, 25 mM Tris, 5% methanol) onto nitrocellulose membrane Hybond-C extra, washed twice for 15 min with 0.01% Tween 20 in DPBS (further in this assay - washing buffer); all the following steps were performed at room temperature. Non-specific binding sites were blocked with 5% skim milk solution in the washing buffer for 1 hour; the rest of the milk solution was removed with the washing buffer (twice, 15 min). Primary antibodies were applied in the washing buffer containing 1% BSA, washed off (4 times, 15 min each). For the detection of PDGFR-□ and its phosphorylation antibodies of rabbit and mouse origin correspondingly were used. Specific secondary HRP-conjugated antibodies (goat anti-rabbit and anti-mouse at the dilutions 1:30000 and 1:10000, correspondingly) in the washing buffer containing 1% BSA were added for 1 hour with the following washing 6 times, each 15 min long. Autoradiography was performed with the exposure for 5 min to Super Signal West Dura Extended (Perbio Science Deutschland GmbH, Bonn, Deutschland) and read on LAS-1000 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

2.8 Statistical analysis

The two-tailed unpaired Student's test was used to analyse the significance of differences in mean responses between the various treatment groups. Differences were accepted as significant at p values less then 0.05.

3 Results

3.1 The effect of ODN on RAoSMC viability

3.1.1 Use of naked ODN

To evaluate, if there could be any impact of naked ODN on cellular viability XTT conversion method has been employed.

Naked ATG77 and ATG269 ODN (AS and SC) did not affect the viability of RAoSMC at the highest concentrations of 30 μM that have been tested in this assay as it is shown in Figure 3-1. The ODN appeared to be non-toxic within the time period up to 48 hours for which they have been applied to the cells. Only slight decrease has been observed in case of ATG269-AS with a drop from to 92% in the presence of 30 μM (not statistically significant).

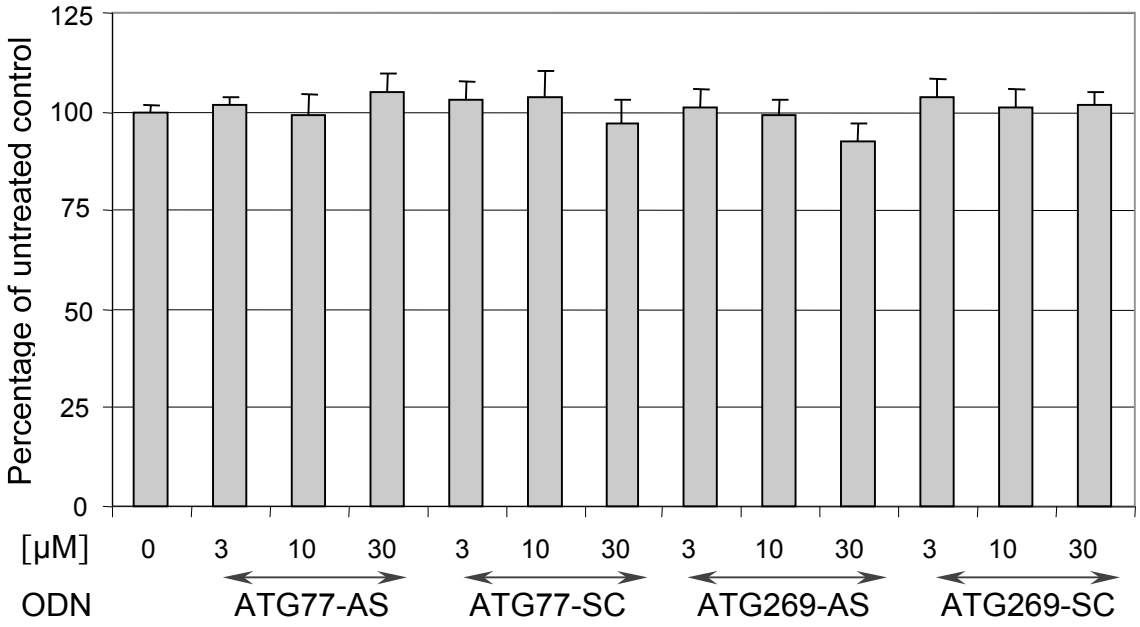

Figure 3-1. XTT conversion based RAoSMC viability assay in the presence of naked ODN.
RAoSMC have been incubated with the designated concentrations of naked ATG77/269 ODN (AS and SC) for 48 hours. Then, the cells have been let to metabolize XTT for 2 more hours. “0 μM” stands for the group of untreated control (cells cultured only in the corresponding medium with the buffer used for ODN aliquoting). Extinction has been measured and the one for the untreated control has been assessed as 100% viability.

3.1.2 Use of PLGA encapsulated ODN

For the assessment of PLGA-NP encapsulated ODN on RAoSMC viability the method of DNA staining with PI followed by FACS analysis of DNA size distribution has been used. For that purpose the cells have been incubated with 3 μM concentrations of the encapsulated ODN.

DNA pattern in the control cells is presented in Figure 3-2A with a characteristic peak for DNA from the mitotic cycle (M) that stands for DNA from living cells. DNA pattern in the ODN-NP treated cells did not significantly differ from the one in the control cells. Blank NP themselves did not affect cellular viability either. Taken together, this data implies that ODN-NP do not affect viability of RAoSMC at the concentration of 3 μM when applied for 48 hours.

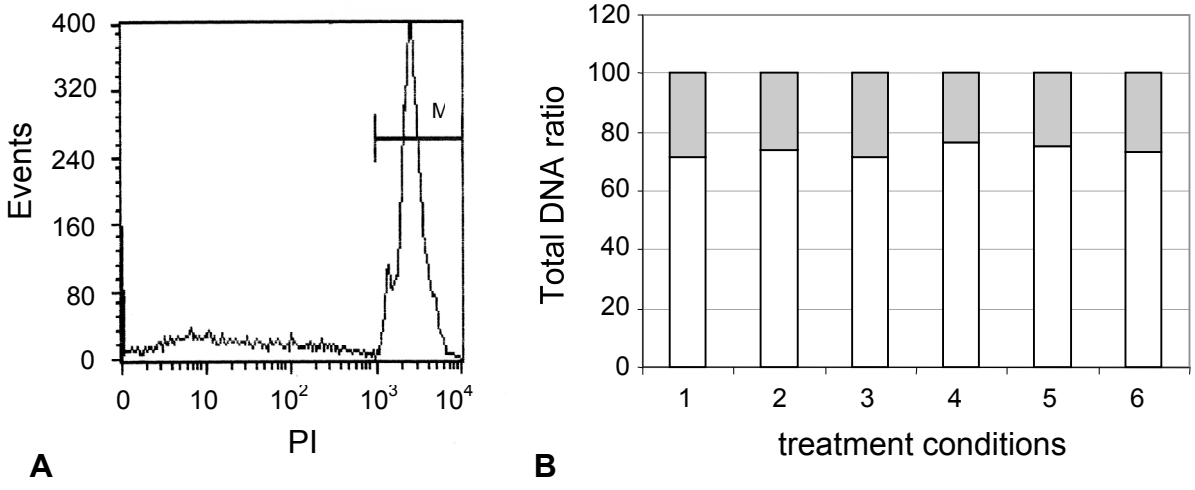

Figure 3.2. Results of PI DNA staining based RAoSMC viability assay in the presence of ODN-NP.
RAoSMC have been treated with PLGA-encapsulated ODN at 3 μM concentration for 48 hours. Cell viability has been assessed with PI staining (3 hours) of cellular DNA and followed by FACS analysis. Logarithmic plot (A) shows DNA from living cells (mitotic cycle – M peak) that is being selected. Data is presented (B) as the total DNA ratio (empty parts of the bars represent DNA from living cells – M peak on the logarithmic plot, filled parts represent fragmented DNA). DNA (the total of 10 000 events counted) from every sample is assessed as 100%. Treatment conditions: 1 - untreated control (cells cultured in corresponding medium with the buffer used for ODN aliquoting); 2 - ATG77-AS; 3 – ATG77-SC; 4 – ATG269-AS; 5-ATG269-SC; 6-Blank NP (without ODN).

3.2 The effect of ODN on DNA synthesis in RAoSMC

3.2.1 Use of naked ODN

To test how RAoSMC proliferation is affected by the administration of the naked ODN the method of thymidine analogue BrdU incorporation into newly synthesized cellular DNA has been employed.

The inhibition of DNA synthesis following administration of naked ODN is summarized in Figure 3-3. Naked ATG77 ODN, both AS and SC, revealed about the same inhibitory potential that was rather unspecific. Namely, they both have decreased unstimulated and PDGF-BB-stimulated DNA synthesis already at 3 μM with an even stronger inhibition at 10 μM However, ATG77-SC turned to be at least as potent as the corresponding AS sequence. 20% inhibition of DNA synthesis was observed in unstimulated samples and 80% inhibition in the stimulated ones following the incubation of the cells with the naked ODN for 24 hours. DNA synthesis in the PDGF-BB-stimulated samples dropped to the level of corresponding unstimulated ones for the both concentrations.

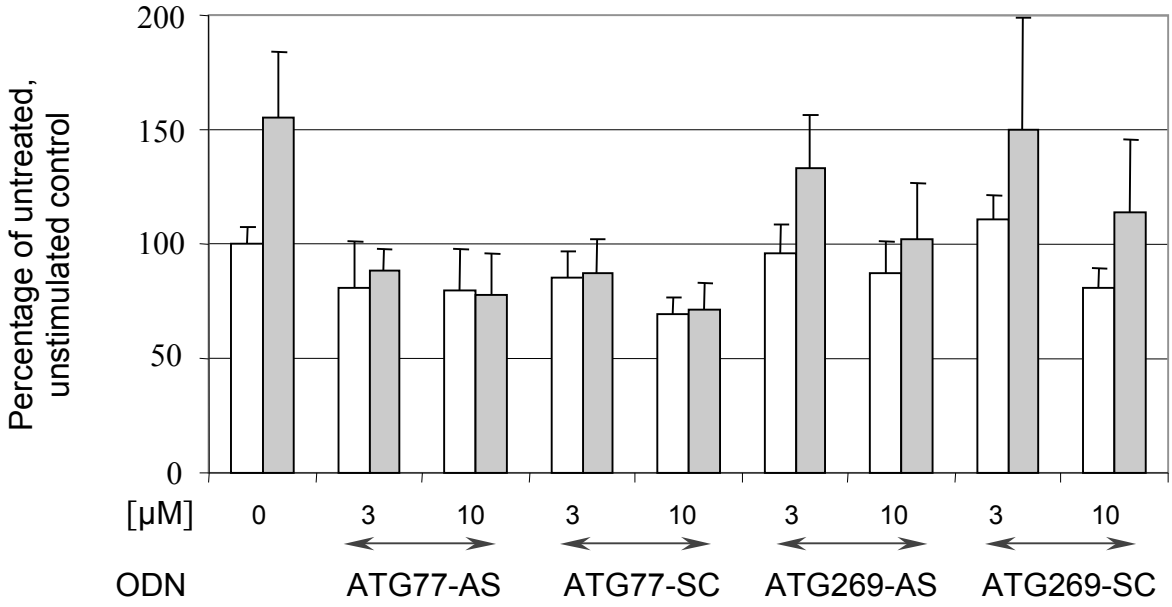

Figure 3-3. BrdU labelling based DNA-synthesis assay in RAoSMC in the presence of naked ODN.
RAoSMC have been pretreated with naked ATG77 and 269 (AS and SC) ODN at the designated concentrations for 24 hours, then stimulated with PDGF-BB (10 ng/ml) for the next 24 hours and BrdU labelled for 4 more hours. “0 μM” stands for unstimulated control taken as 100% DNA synthesis. Empty bars refer to unstimulated cells while grey bars refer to PDGF-BB-stimulated cells.

Administration of ATG269 ODN caused dose-dependent inhibition of DNA synthesis. At the concentration of 3 μM ATG269 ODN slightly inhibited PDGF-BB-induced DNA synthesis with somewhat more potent effect in case of AS-ODN. At the higher concentration of 10 μM both AS and SC ODN further inhibited PDGF-BB -induced DNA synthesis with AS-ODN resulting in about 80% inhibition, while SC-ODN showed only a weak effect. There was no increase in the inhibition at the higher concentration (30 μM) following delivery of ATG269 (AS/SC) ODN (data not shown).

3.2.2 Use of PLGA-encapsulated ODN

To evaluate the influence of PLGA encapsulated ATG77 and ATG269-AS/SC ODN on RAoSMC proliferation, DNA synthesis was assessed by the means of [^{3}H]-thymidine incorporation.

3.2.2.1 ATG77 (AS/SC) PLGA-NP

Encapsulated ATG77-AS and ATG77–SC ODN affected DNA synthesis in RAoSMC in the similar manner (Figure 3-4). At the lower concentration of 0.3 μM they both inhibited PDGF-BB-induced [^{3}H]thymidine incorporation by 60-70% with somewhat more potent effect in the case of SC-ODN. Administration of 1 μM of the ODN-NP resulted in further inhibition reaching 15% of stimulated control for both AS and SC. The concentration of 3 μM did not cause any additional decrease. The effect for all the concentrations of ATG77-AS/SC NP was distinguishable from the one of the blank NP that revealed 30-35% inhibition of PDGF-BB-stimulated DNA synthesis. The unstimulated DNA synthesis did not significantly differ between all the groups.

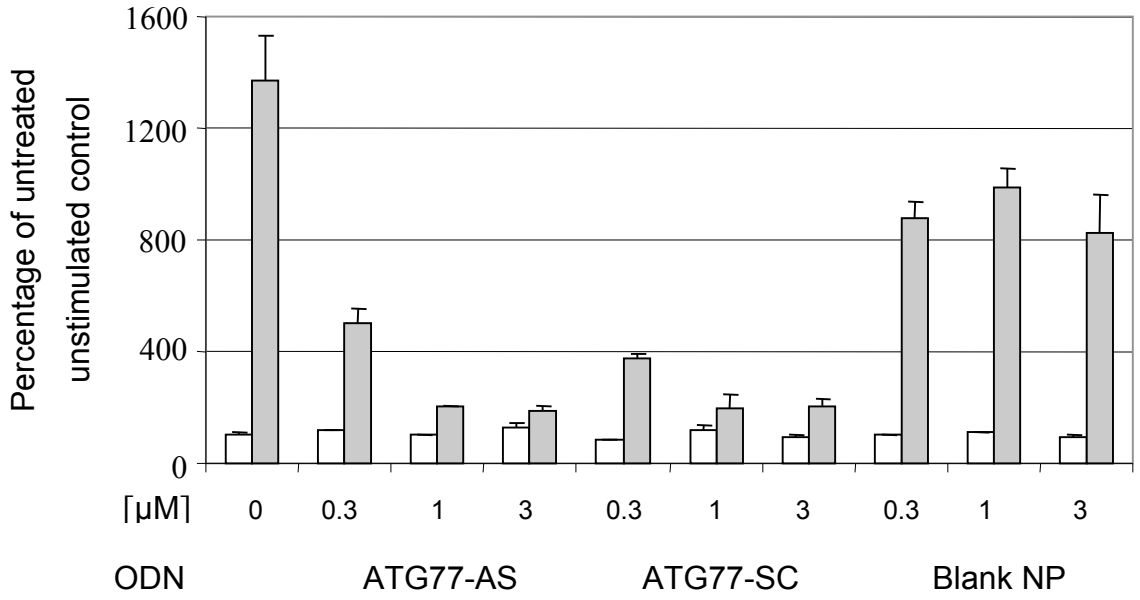

Figure 3-4. [^{3}H]thymidine incorporation-based DNA synthesis assay in RAoSMC in the presence of PLGA-encapsulated ATG77-AS/SC.
RAoSMC have been pretreated with the encapsulated ODN at the designated concentrations for 24 hours, then stimulated with 10 ng/ml PDGF-BB and pulsed with [^{3}H]thymidine for 2 more hours. “0 μM” stands for unstimulated untreated control taken as 100% DNA synthesis. Empty bars refer to unstimulated cells while grey bars refer to PDGF-BB-stimulated cells.

3.2.2.2 ATG269 (AS/SC) PLGA-NP

ATG269 (AS/SC) PLGA-NP inhibited PDGF-BB-induced DNA synthesis in RAoSMC in a dose-dependent manner (Figure 3-5). Namely, at the lower concentration of 0.3 μM they both caused a 30% decrease with a 70% decrease at the higher concentration of 30 μM. Critically, the effect of the AS and SC ODN has been distinguishable from the one of blank NP at the concentrations of 1 μM and 3 μM. Administration of any of ATG269 (AS/SC) NP as well as blank NP did not affect the baseline [^{3}H]thymidine incorporation.

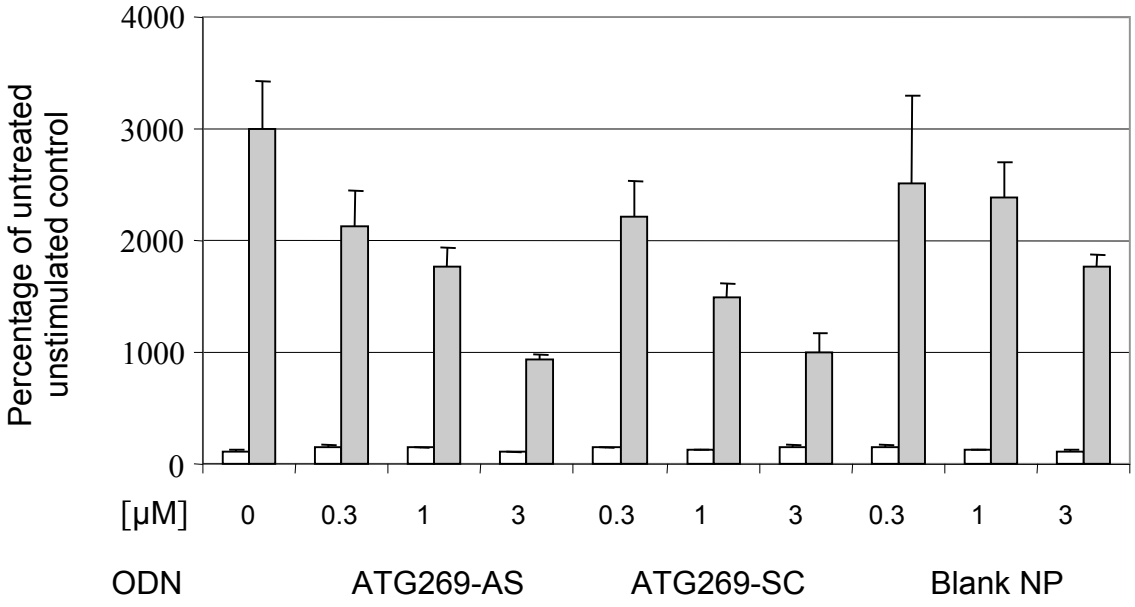

Figure 3-5. [^{3}H]thymidine incorporation based DNA synthesis assay in RAoSMC in the presence of PLGA encapsulated ATG269-AS/SC.
RAoSMC have been pretreated with the encapsulated ODN at the designated concentrations for 24 hours, then stimulated with 10 ng/ml PDGF-BB and pulsed with [^{3}H]thymidine for 2 more hours. “0 μM” stands for unstimulated untreated control taken as 100% DNA synthesis. Empty bars refer to unstimulated cells while grey bars refer to PDGF-BB-stimulated cells.

3.3 Effect of ODN on the level of PDGFR-β expression

To figure out how the tested ODN may influence the level of PDGFR-β expression, RAoSMC have been incubated with naked and encapsulated ODN (AS, SC) to ATG77 and ATG269 of rat PDGFR-β mRNA for the following time periods: 3, 6, 12, 24, 48 hours. To study the effect of ODN, the highest concentrations of naked and encapsulated ODN have been employed (30 μM and 3 μM, correspondingly).

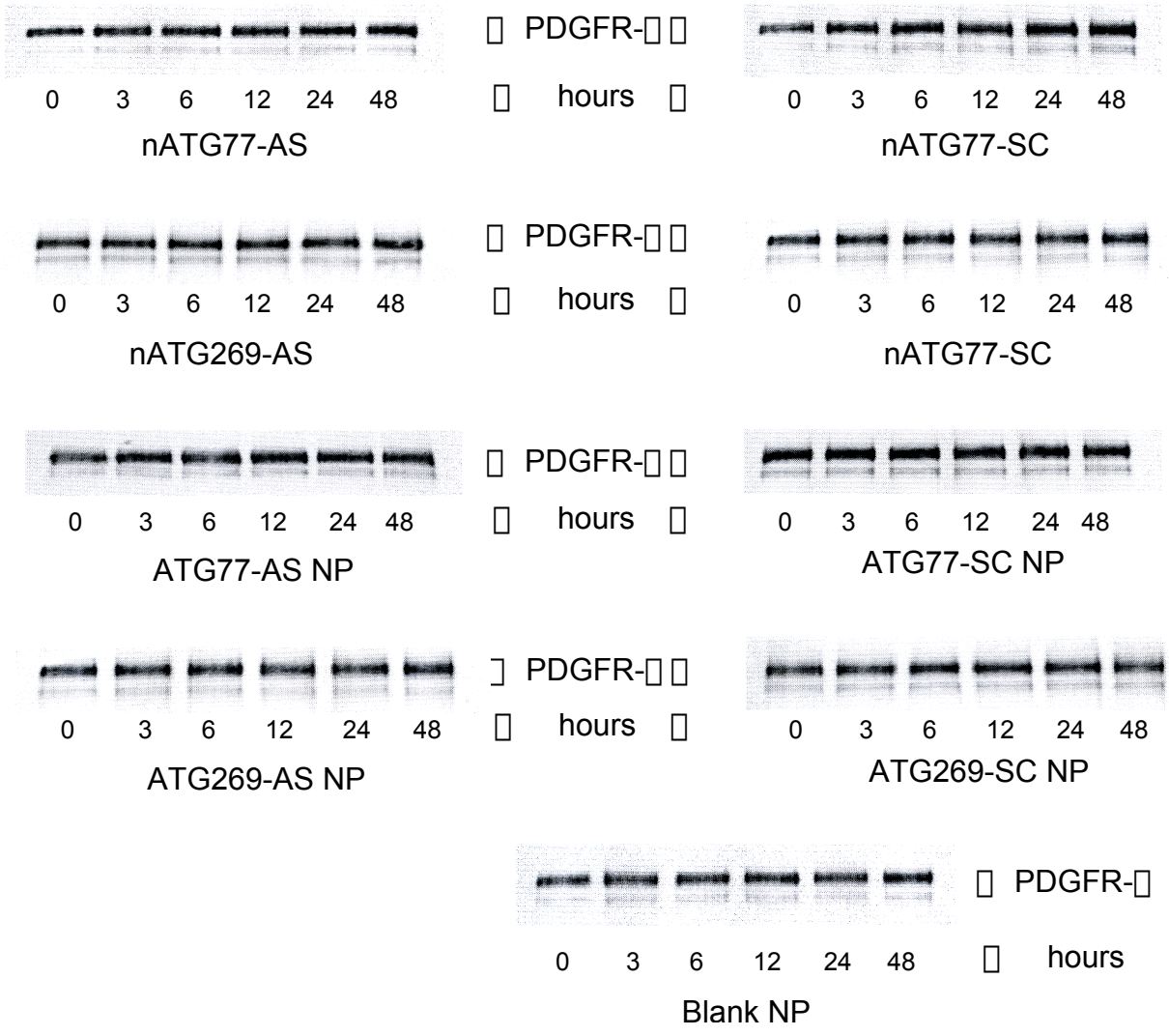

Figure 3-6. Immunoblots show the expression of PDGFR-β in RAoSMC in the presence of naked and PLGA-encapsulated ODN.
RAoSMC have been incubated with the ODN for the designated time periods prior to the cell lysis. 30 μg of crude RAoSMC lysate total protein per lane have been resolved. "0 hour" stands for untreated RAoSMC; Blank NP – NP without any ODN encapsulated. All the naked (n) ODN were used at the concentrations of 30 μM, and PLGA-encapsulated ODN-NP at the concentrations of 3 μM.

All the sequences, ODN formulations and conditions were unable to inhibit the expression of PDGFR-β. Also, blank PLGA-NP (tested at the concentration corresponding to the amount of carrier used for 3μML ODN-NP) did not affect the receptor expression.

3.4 Effect of ODN on tyrosine phosphorylation of PDGFR-β

To study the possible interference of the tested ODN with PDGF-BB induced PDGFR-β activation, the immunoblot analysis of cell lysates from PDGF-BB-stimulated RAoSMC has been performed. The higher concentrations for all the ODN tested have been administered: 3 μM for the PLGA encapsulated ODN NP and 30 μM for the naked ODN. Neither of the ODN (all applied 24 hours prior to PDGF-BB stimulation and for the total of 48 hours) has been capable to suppress the activation of PDGFR-β.

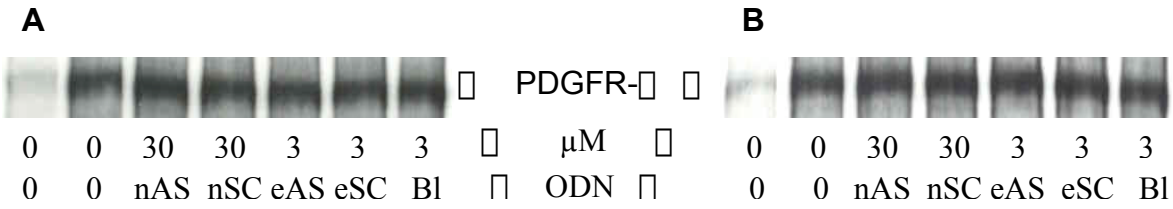

Figure 3-7. PDGF-BB-induced PDGFR-β phosphorylation in RAoSMC in the presence of naked and PLGA-encapsulated ODN.
Subconfluent RAoSMC have been serum-starved for 24 hours with parallel administration of corresponding ODN at the designated concentrations. Next, the cells have been stimulated with 10 ng/ml of PDGF-BB for another 24 hours. 30 μg of total protein of crude cell lysates per lane have been loaded. Immunoblot was developed with an antibody against phosphorylated tyrosine. A-represents immunoblot for ATG77; B-represents immunoblot for ATG269; n-naked, e-PLGA-encapsulated ODN-NP.

4 Discussion

To investigate the possibility of reduction of postangioplasty restenosis via AS approach an *in vitro* model employing RAoSMC has been established. Taking into account a key role of the PDGF/PDGFR-system in VSMC accumulation in restenotic neointima, rat PDGFR-β mRNA has been targeted. Despite the great achievements in genome sequencing for different animal species and the human being, there are still many genes for which the complete sequence has not been discovered yet. As far as AS-ODN in more than 70% proved to be effective while targeting mRNA initiation codons, it is of importance to use a standardised approach for initiation codon prediction when the mRNA of interest is incomplete. Such an approach, using available web software, has been tried. Two AS-ODN (with the corresponding SC-ODN) to PDGFR-β mRNA at nt77 and nt269 have been selected for the experimental use. They were characteristic of the highest both specificity and predictability

To the best of our knowledge the ODN sequences that have been tested in the current study have been used for the first time to target rat PDGFR-β mRNA *in vitro*. One of the limitations of AS technology, beside many attractive advantages, is high nuclease sensitivity of unmodified phosphodiester ODN. This necessitates the employment of higher concentrations of ODN required to inhibit protein expression (164). Increased nuclease degradation of unmodified ODN also leads to an increase in the abundance of ODN metabolism products (mononucleotides) that have been shown to be toxic in many ways (96, 165, 179). In order to overcome the problem of nuclease sensitivity the backbone substitution of original phosphodiester bond with phosphorothioate bond has been performed. Three nt at each end of the ODN sequences have been modified.

On top of the above mentioned, the ODN that have been used in this study had proven their higher specificity with partial phosphorothioate backbone modification as compared to fully phosphorothioate analogues (29).

Poor cellular uptake of ODN is another major drawback for therapeutic applications. It is of importance that NP polymeric delivery system exhibited significant transfer of the ODN into the cells. Since the effect of PDGFR-β AS-ODN has been examined by others (utilizing the EVAc system matrices (159), the preliminary investigations employed PLGA-encapsulated ODN-NP that have revealed significant improvement in cellular ODN uptake (22). The sustained mode of ODN release from such NP has also been described (22).

Many studies targeting growth factors or/and their receptors using AS methodology in order to inhibit postangioplasty restenosis related events have been conducted so far (2, 132, 133). As far as most of the studies dealt with *in vivo* investigation, current study has been conducted *in vitro*.

4.1 DNA synthesis in RAoSMC in the presence of ODN

In previous reports, it has been shown that AS-ODN sequences complementary to DNA binding proteins and cell-cycle regulator genes such as c-myb, c-myc, cdc2, cdk2, nonmuscle myosin, and PCNA inhibited target protein expression, suppressed VSMC proliferation *in vitro* and *in vivo*, and inhibited neointimal formation in injured arteries of different animal species such as rat, rabbit and pig (2, 10, 132, 133, 162). So far, mostly cell cycle-related genes have been targeted such as bcl-2, c-myc, cdk2 (10, 43, 161). However, these genes are not uniquely expressed in VSMC but also are expressed in other cell types, and their use might induce side effects in tissues with high rates of proliferation. Growth factors play a central role in all phases of the vascular response to injury, yet far fewer studies have reported on the consequences of AS sequences directed against growth factors and/or their receptors (124, 137). PDGF, for example, is critical to VSMC migration and proliferation leading to intimal thickening (92) in a manner fairly selective for VSMC (136) and as a result became the focus of the present study.

The potency of the inhibitory effect on RAoSMC proliferation was observed using ODN NP. Blank NP had no deleterious effects the RAoSMC DNA synthesis as it has been assessed by means of [^{3}H]thymidine incorporation. Both ATG77 and ATG269 ODN when administered at the concentration range of 0.3-3 μM caused dose dependent inhibition of DNA synthesis with somewhat more potent in the case of ATG77 ODN. No differences between the effects of corresponding AS and SC ODN-NP have been revealed. When nODN have been used to inhibit DNA synthesis in VSMC, ATG269 ODN acted in dose-dependent and specific way, while ATG77 ODN did not.

Possible explanation for the lack of specificity in the case of the ODN-NP could be related to the release mode of the NP. While significant increase in the cellular uptake has been observed, the amount of ODN released from NP constitutes about 13% within the first 24 hours (29). This probably makes it impossible to discriminate between the effects of AS/SC sequences within such a short time

period. Another explanation could be in that the “therapeutic” window is not known for the inhibition of cellular proliferation using AS approach. It could therefore be that the in vitro effects were obtained at a dose, where the effect reached saturation and therefore we did not observe any differences between AS and SC PLGA-encapsulated ODN.

It is postulated that an inhibition of PDGF-induced VSMC activation by about 40% actually represents about 80% of the maximum inhibition that can be achieved by PDGFR-β blockade. A number of different strategies to block the action of PDGF reduce neointima formation by 50% (159, 185).

Taken together the above data show that the inhibition of DNA synthesis can be achieved with equal potency when much lower concentrations of vectorized ODN are used as compared to the corresponding ODN when used naked. In our study a maximal inhibition has been shown at the concentration of 10 μM for the naked ODN and 3 μM for the PLGA encapsulated ODN-NP. This difference in the amount of ODN required to achieve similar effect can be explained by the improved cellular uptake, spatial and temporal activity, and increased stability against nuclease digestion rendered by PLGA-NP delivery system (39).

In an earlier report on PDGFR-β AS for suppression of intimal thickening EVAc polymer delivery system has been used (159). Although EVAc demonstrated the feasibility and benefits in this study it is advantageous to use to utilize a biodegradable polymer, since long-term administration of non-degradable material may cause an inflammatory cellular response (119, 187).

4.2 The impact of ODN on cellular viability

The importance of studying the cellular viability under ODN treatment stems from the observations on the sensitivity and rapid degradation of ODN by intracellular endonucleases and exonucleases usually via 3’→ 5’ activity (3, 44, 190). Consequently, the degradation products of the sensitive ODN, dNMP mononucleotides, may be toxic and also exert antiproliferative effects (179). It became more complicated, when it has been demonstrated that the toxic effects of dNMP (in particular dGMP) can be correlated with mononucleotide dephosphorylation by the cell-surface enzyme ecto-5’-nucleotidase. The latter has been shown in several cell lines, such as human umbilical endothelial cells, HeLa cells, to be capable of inhibiting the function of other critical proteins, such as

thymidine kinase (96) or ribonucleotidase reductase (179), the events that can result in inhibition of cell growth. The issue of ODN toxicity is further supported by the observations of cell type dependent ODN tolerance. For instance, it has been shown that 50 μM of 28-bp ODN is not toxic to HeLa S3 cells (53) but exert marked toxicity when injected into *Xenopus* oocytes at a 100-fold lower concentration (136).

In our study we used end-protected ODN by the means of phosphorothioate backbone modification (3 at each end) that was proved to be defensive against exonucleases (164), while the possibility of endonuclease attack still remains. ODN encapsulation into PLGA-NP provides the controlled release delivery mode (22, 28) that further offers increased resistance to nuclease degradation.

For the above-mentioned reasons we tested the ODN to discriminate possible toxic effects for the following proliferation studies. Naked ODN at the highest concentration of 30 μM did not affect viability of RASMC during the time frame of 48 hours. It is well in the concentration range employed in other *in vitro* studies (159).

Much lower ODN concentrations could appear toxic, when a delivery system is used predominantly due to the improved cellular uptake and therefore increased cytoplasmic concentration as it has been shown earlier for PLGA-encapsulated ODN-NP (28). PLGA-encapsulated ODN-NP did not induce apoptosis as they have been administered to RASMC in culture at the highest concentration of 3μM for the period of 48 hours.

That has been shown in cellular DNA study with PI staining followed by FACS analysis. Critically, as far as PLGA-NP are getting increasingly attractive as a drug delivery system (52, 55) this technique could be used as a method of choice to test the drug toxicity *in vitro*. That is because the most popular ways of studying cellular viability are colorimetric techniques that cannot be applied while PLGA is used as a carrier due to the affected sample transparency.

4.3 The effect of the ODN on PDGFR-β expression and on PDGF-BB-induced activation

In order to investigate whether the observed inhibition of RAoSMC proliferation was specific, experiments to study the expression of PDGFR-β and its activation have been conducted. It is agreed that biological end-points cannot be used as a proof of AS efficacy, because of the intrinsic backbone activity. So, it is important to remember that the “gold standard” of AS efficacy is down-regulation of

protein expression. Several reports have raised concern that the antiproliferative activity of AS-ODN to c-myb and c-myc, for example, arose from aptameric rather than from hybridization-dependent AS mechanism (13, 71).

Previous studies demonstrated the need to match the kinetics of ODN action to the kinetics of AS target gene expression. When gene expression is prolonged, as it in the case of c-myc, a more sustained ODN release device was required to demonstrate the biological effect (43). On the other hand, it has been demonstrated that intramural delivery of advanced c-myc neutrally charged AS morpholino compound completely inhibits c-myc expression already after 2 hour sustained administration (10). These observations highlight the importance of both initial and sustained inhibition of protein expression. Viewed in this light, the PLGA-NP system used in this study for ODN delivery becomes very attractive. Earlier, the double stage release mode has been shown (28, 29) with a burst effect of 30% release within the first 3 days and further sustained release reaching 80% of total amount of ODN released within 1 month.

It has been shown (145) that half-life of PDGFR-□ protein is about 3 hours, which provides apparent potency for down-regulation of expression by 50% at maximum that could be achieved already after 3 hour ODN treatment. In our study we treated the cells with high concentrations of the ODN for a time period of 3 up to 48 hours. We did not observe any inhibitory effect of the ODN tested on the protein expression.

Several explanations could be given for the inhibition of cellular proliferation by ODN in spite of unaffected receptor expression. Despite the fact that PS backbone modification is the most widely used, it has many properties that render them sub-optimal AS effector molecules. The phosphorothioate backbone is known to induce sequence-dependent effects attributable to its length-dependent high affinity for various cellular proteins, especially heparin-binding growth factors, such as acidic FGF, PDGF, and VEGF (164). It was hypothesized that ODN with four sequential guanosines might be capable of the binding to the growth factors while non-G ODN have also precipitated non-specific activity (35). Nonetheless, other studies have shown specific in vivo and/or in vitro effects of AS-ODN lacking multiple sequential guanosines on these and other genes involved in cell-cycle progression such as cdc2, cdk2, nonmuscle myosin, and PCNA (2, 132, 133, 161).

To test the possibility of ODN binding to either PDGF-BB or PDGFR-β that could potentially be the reason for the inhibition of cellular proliferation we studied PDGF-BB-induced PDGFR-β activation under administration of the ODN at the highest concentrations. None of the sequences has been capable to down-regulate PDGFR-β phosphorylation when applied for 24 hours.

The data on PDGF-BB-induced PDGFR-β phosphorylation in RAoSMC in the presence of the ODN show that in our study we could not obtain any evidence concerning the possibility of sequence-independent and direct interference of the tested ODN with either the growth factor or its receptor.

Another possible reason for the inhibitory effect of ODN on DNA-synthesis in RAoSMC, observed in the present study, could be sequence-independent ODN interaction with cellular proteins playing a critical role in cellular proliferation. Recently, one of such specific cellular proteins has been shown to bind to ODN and the biological activity of the ODN correlated with binding to this protein (7). The protein, named nucleolin, has been detected in both nuclear and cytoplasmic extracts and in proteins derived from the plasma membrane of cells that strongly shows the ubiquitous accessibility for ODN. This protein has been implicated, directly or indirectly, in many cellular functions (182) and its levels are related to the rate of cell proliferation (62).

Although we have been unable to show a specific action for the tested ODN, there is growing concern that sequence-independent biological activities of PS-ODN may actually contribute to observed *in vitro* and *in vivo* responses by synergizing with the down-regulation of the specific target (39, 164). Viewed in this light, AS methodology remains attractive and will continue to be extensively used while the concentrations, time mode, and proper delivery systems have to be adjusted (71, 163).

4.4 Limitations of the present study

The present study has also potential limitations that should be considered. The time period of ODN administration (the longer of 48 hours) could be a possible explanation for the lack of specificity in the effects of AS/SC PLGA-encapsulated sequences as far as they are characteristic of double-stage release mode with 30% burst release within the first 3 days and total 80% within 1 month. The saturation of

the effects could be another explanation for the lack of difference in the action of AS and SC ODN-NP.

Having studied the effect of the ODN on PDGFR-β expression level within 48 hours of the ODN administration, we did not observe any down-regulation of the receptor in RAoSMC. The PDGF-BB-induced activation of the receptor was not affected either. This might lead to the conclusion that the other but unrevealed ODN interactions could be responsible for the observed biological effect. Although the receptor level was not affected, this does exclude possible down-regulation of the mRNA level. To the best of our knowledge there are no data on the effect of partially phosphorothioate ODN on mRNA level as compared to fully phosphorothioate ODN. This could not be the explanations as to the mechanism of the ODN action but might definitely make the picture more clear as to the ODN pharmacology.

All in all, to clarify the above-mentioned limitations the studies with modified time and dose modalities should be conducted and, probably, new sequences should be tested.

4.5 Conclusions

1. The tested ODN showed strong inhibition of VSMC proliferation. A novel PLGA-NP delivery system facilitated a decrease in the ODN concentration as compared to the naked ODN formulation with the same effect observed.
2. The ODN concentrations that have been sufficient to inhibit DNA synthesis in VSMC did not reveal any cytotoxic effects.
3. The lack of PDGFR-β expression down-regulation in the presence of the tested ODN strongly suggests other intracellular ODN interactions to underlie the observed biological effects.
4. Our findings strongly suggest AS methodology to be a potent tool to inhibit VSMC proliferation, which is one of crucial events in the development of postangioplasty restenosis.

5 Summary

The main complication of percutaneous transluminal coronary angioplasty is restenosis. The proliferation of medial vascular smooth muscle cells (VSMC) is thought to be a key component in restenotic neointima formation. Signal transduction through the platelet-derived growth factor receptor (PDGFR) system is implicated in the process of post-angioplasty restenosis. A sustained inhibition of this pathway is a promising strategy, which has gained considerable research attention. Antisense (AS) methodology has been utilized in many studies in an attempt to inhibit growth-regulatory or cell cycle genes. However, all the AS oligonucleotides (ODN) used were fully phosphorothioated analogues that possess some non-specific properties besides many advantageous features.

The objective of the present study was to study the possible impact of partially phosphorothioated AS-ODN to PDGFR-β mRNA on VSMC proliferation *in vitro*. The backbone modification has been performed to possibly decrease the sequence-independent effects of the ODN and make them more stable against enzymatic degradation. Two AS-ODN, 18 bp long, to the predicted initiation codons have been designed at nucleotides 77 and (ATG 77) and 269 (ATG269) with the corresponding scrambled (SC) sequences. We utilized both naked (n) ODN and controlled release poly(DL-lactide-co-glycolide) nanoparticulate (NP) system to improve cellular ODN delivery. To discriminate between the cytotoxic effects of ODN and their pure anti-proliferative action the cellular viability has been studied in XTT conversion assay for the nODN and in propidium iodide deoxyribonucleic acid staining method (for the ODN NP) with the help of flow cytometry. Cellular DNA synthesis has been tested in bromo-deoxy-uridine labelling (for the nODN) and [^{3}H]thymidine incorporation (for the ODN NP) assays. To further elucidate the underlying mechanism of the ODN action we studied the expression of PDGFR-β and its PDGF-BB-induced activation by means of polyacrylamide gel electrophoresis protein separation with the following immunoblotting.

In the range of non-toxic concentrations (the highest 30 μM for the nODN and 3 μM for the ODN NP) ODN potently inhibited DNA synthesis in rat aortic smooth muscle cells. Namely, ATG269 ODN revealed strong inhibition in a dose-dependent manner for both nODN and ODN-NP, while nAS ODN acted rather specifically resulting in a higher DNA synthesis suppression as compared to a corresponding

nSC ODN. ATG77 ODN acted unspecifically with dose-independent inhibition in the case of nODN and clear dose-dependency in the case of ODN-NP.

We did not observe any effect of the tested ODN on the expression of PRGFR-β when they have been delivered to the cells at different time points up to 48 hours. PDGF-BB-induced PDGFR-β phosphorylation has not also been affected by the ODN administration within the period of 24 hours. These observations suggest that the DNA synthesis has been down-regulated via some other mechanisms that still have to be elucidated.

Taken together our results support the effectiveness of AS technology in inhibition of cellular proliferation. The tested ODN were potent in inhibition of DNA synthesis in rat aortic VSMC at non-toxic concentrations. Employment of poly(DL-lactide-co-glycolide) ODN-NP allowed using lower ODN concentrations to achieve strong inhibition of VSMC proliferation and could be used as an effective ODN delivery system. New AS sequences should be designed and tested in order to achieve specific action.

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7 Acknowledgments

I would like to thank Prof. Dr. Vinzenz Hombach for the opportunity to work in his department.

I am deeply indebted to PD Dr. Johannes Waltenberger from the Department of Internal Medicine II, Cardiology, Ulm University Medical Centre, Chief of the Laboratory of Molecular Cardiology for his critical and helpful discussions and comments during preparation of this MD thesis. Moreover, I am very obliged to him for the excellent opportunity to make my thesis in his laboratory and his persistent support during all my stay in Germany.

I would like to express my special gratitude to Mrs. Ulrike Mayr for introducing me to the SDS-PAGE, Western Blot, BrdU labelling and [^{3}H]-thymidine incorporation techniques, for excellent laboratory collaboration and expert advice as well as for her friendly assistance.

I extend my sincere gratitude to Dr. Stefanie Kliche, Dr. Guido Fellbrich, and Dr. Marios Loukas for the friendly working atmosphere and their cooperation. I am also indebted to Dr. Guido Fellbrich for critical reading of this manuscript.

I wish to thank Dr. Ingo Melzner from the Department of Pathology, Ulm University Medical Centre for his introduction to the laser scanning microscopy and FACS analysis, Dr. Gita Pezeshki from the Department of Anatomy and Cell Biology for her full cooperation on animal work.

This MD thesis work has been supported by grant Wa732/4-1 from the Deutsche Forschungsgemeinschaft.

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