# Antisense Oligonucleotides to PAI-1 mRNA as Potential Agents for Therapeutic Intervention in Cardiovascular Diseases

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## 1. Thrombus as a primary cause of cardiovascular diseases

Occlusion of blood vessels by thrombi is a primary cause of cardiovascular problems (1). It directly results from activation of processes physiologically intended to arrest bleeding occurring after vessel wall damage. As a first step of the hemostatic plug formation, platelets adhere to the perivascular connective tissue which has become exposed due to a breach in the vessel wall. This platelet adhesion is followed by spreading and activation, which results in secretion from platelet organelles during aggregate formation. Platelet adhesion is crucial for thrombus formation, particularly in arteries. In this case, vessel wall damages most often caused by atherosclerosis, has led to exposure of subendothelial connective tissue to the flowing blood. Platelets contain a variety of receptors which facilitate their interaction with components of extracellular matrix and mediate their aggregation. They include integrin receptors such as $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ which interact with adhesive proteins (2-4). In addition, there are glycoprotein complex GPIb-IX and glycoprotein IV which can also specifically interact with von Willebrand factor or collagen, respectively. Several components of the connective tissue, such as von Willebrand factor, fibronectin, and, to a lesser extent, laminin and thrombospondin have been shown to be important for platelet adhesion (5,6), but collagen stands out, mainly because (a) it is a ubiquitous component of the vessel wall, (b) it provides a scaffolding on which other adhesive molecules may assemble, and (c) collagen is an unique ligand for platelet adhesion as it causes platelet activation

and aggregate formation. In parallel, despite platelet activation there is also activation of plasma factors initiated by the tissue factor derived from damaged endothelial or other cells (7). Several steps of this process occurring on the surface of aggregated platelets, finally result in the formation of thrombin which converts fibrinogen to insoluble fibrin. As a result of these interactions and aggregation of platelets, the body of thrombus grows and it can be released from the wall by flowing blood. Then, it may occlude small blood vessels.

Thrombolytics are successful for dissolving thrombi in any vessel, but clinical benefits vary according to the involved organ or limb and dramatically depend on how early they were administered (8). At present, the main indications for thrombolytic therapy are acute myocardial infarction, severe pulmonary embolism, acute peripheral arterial occlusion and deep vein thrombosis. However, the anticipated clinical benefit of thrombolytics in treating of these categories of thrombotic disease must be balanced individually against the constant risk of bleeding, especially intracranial hemorrhage. So the benefit of application of thrombolytics in acute myocardial infarction is the reduction in mortality by 20 to 50% and this is extremely dependent upon time, since 50% of mortality reduction is observed during the first hour and only 14% reduction at 6 to 12 hours (Tab. 1). That is why the first hour is called "golden hour". In pulmonary embolism, there is a rapid thrombolysis and reversal of pulmonary hypertension, in acute peripheral arterial occlusion, thrombolysis is accomplished in 70% of patients, surgery avoided in 35% and one year mortality decreased to 15%. In deep vein thrombosis there is a rapid resolution of clots and prevention of post-phlebitic syndrome in 50% of patients.

TABLE 1
CLINICAL BENEFIT OF THROMBOLYTIC TREATMENT FOR ACUTE THROMBOSIS

| Thrombotic disorder | Benefit of thrombolytics |
|---|---|
| a. Acute myocardial infarction | Mortality reduced by 20-50%<br>50% mortality reduction in the first hour<br>14% at 6 to 12 hours |
| b. Pulmonary embolism | Rapid thrombolysis<br>Reversal of pulmonary hypertension |
| c. Acute peripheral arterial occlusion | Thrombolysis in 70%<br>Surgery avoided in 35%<br>One year mortality decreased to 15% |
| d. Deep vein thrombosis | Rapid resolution in 50%<br>Prevention of post-phlebitic syndrome in 50% |

It appears that thrombolysis is now a well strategy for the treatment of these diseases and there is no doubt it has an impact on immediate survival

mostly in the case of patients who suffered acute myocardial infarction (9). However, despite a positive benefit risk ratio, in some cases this treatment is associated with undesired side effects (Tab. 2). Based on data obtained in clinical megatrials performed on more than 100 000 patients it is known that there is resistance to thrombolytics in 25%, reocclusion occurrs in 6-16% of patients and fatal hemorrhagic accidents in 0.5%. It seems that there are three approaches which should improve the results of thrombolytic therapy in acute thrombotic diseases — (a) earlier medical treatment, (b) use of more efficacious thrombolytic agents in combination with more active antithrombotic drugs, and (c) reduction of severe bleeding with safer combination of drugs.

TABLE 2
INTRAVENOUS THROMBOLYSIS IN ACUTE MYOCARDIAL INFARCTION CORRELATED WITH RECANALIZATION OF THE CORONARY ARTERY
(CLINICAL MEGATRIALS GUSTO, GISSI-1, GISSI-2, ISIS-2 AND HART ON ABOUT 200 000 PATIENTS)

| | |
|---|---|
| a. Mortality reduction | |
| thrombolytic alone | 27 ± 3 % |
| + heparin | 40 ± 2 % |
| b. Resistance to thrombolysis | 25 % |
| c. Reocclusion | 6 – 16 % |
| d. Fatal hemorrhagic accidents | 0.5 % |

## 2. Type-1 plasminogen activator inhibitor (PAI-1) and thrombosis

According to these lines, in our studies we focused on the development of an optimal antisense probe to plasminogen activator inhibitor (PAI-1) and demonstration whether it may be used to modulate fibrinolysis balance by selective inhibition of this protein which is known to play a critical role in the regulation of this process. Such a role of PAI-1 is supported by clinical observations indicating that PAI-1 deficiency usually leads to abnormal bleeding (10-12). On the other hand, increased concentration of PAI-1 is always associated with deep venous thrombosis and myocardial infarction (13-15). This indicates that the enhanced PAI-1 activity may predispose to vascular disease by limiting intravascular fibrinolysis.

Furthermore, PAI-1 incorporated into thrombus seems to be responsible for time-dependent thrombolytic resistance (16,17). Clinical megatrials have shown that the choice of thrombolytic agents is less important to the survival

than is the delay time between onset of symptoms and initiation of treatment. One of the major reason for such time-dependence is the fact that a 50-fold higher rt-tPA concentration is required to lyse an "old thrombus" as rapidly as a nascent platelet-rich thrombus. Immunochemical staining of thrombi formed *in vivo*, comprising a dense-platelet-rich head and a fibrin-rich tail, identified PAI-1 and α2-antiplasmin in fibrin-rich areas of both the head and the tail (Fig. 1). The mean concentration of PAI-1 in the head and tail region of fresh *in vivo* thrombus was 9.9 and 1.2 ng per mg of total protein, respectively, and it significantly increased in the aged thrombus (16). Thrombolytic resistance was most pronounced following a 2 hour aging. Permeation of a 2 hour-aged platelet rich thrombi with $R^{298}$,$E^{299}$-rt-tPA, a variant of tissue plasminogen activator resistant to inhibition by PAI-1, yielded a rapid lysis (17). These results indicate that platelet secretion of active PAI-1 is a key component of thrombolytic resistance.

Given the importance of PAI-1 in fibrinolysis, strategies aimed at its rapid inactivation may be of clinical utility. This is why the inhibition of PAI-1 was proposed by different groups and the use of several approaches was described. For example, monoclonal antibodies which block interaction of PAI-1 with tPA were developed (18). Similarly, monoclonal antibodies changing conformation of PAI-1 upon binding and thus altering its sensitivity to

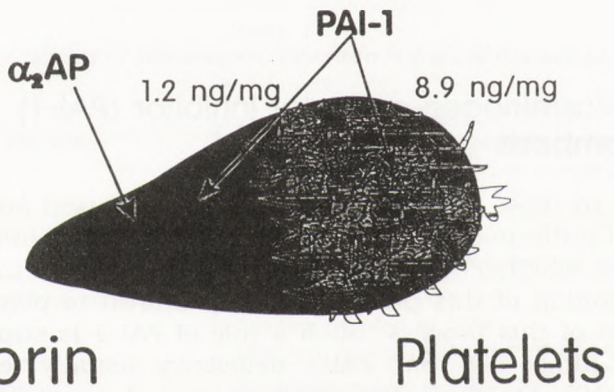



Fig. 1. Location of PAI-1 and $\alpha_2$-antiplasmin in thrombus.
Immunochemical staining of thrombi formed *in vivo*, comprising a dense-platelet-rich head and a fibrin-rich tail, identified inhibitors PAI-1 and α2-antiplasmin in fibrin-rich areas of both the head and tail.

proteases (19), low molecular inhibitors from Streptomyces, and peptide inhibitors were proposed to be used for this purpose (20).

## 3. Antisense approach to control fibrinolysis

By inhibiting the expression of specific gene products, antisense oligonucleotides provide a theoretically simple and precise tool for identifying the mechanisms responsible for various cellular processes. To date, the widest application of antisense technology has been addressed toward the controlling of smooth muscle cell proliferation (21-25), a process involved in a number of pathological processes including post-angioplasty restenosis, bypass graft failure, post-transplant arteriopathy, and chronic hypertension. Smooth muscle proliferation is associated with expression of a number of cell cycle specific genes and it is these genes that have been the target of antisense approach. Thus, *in vitro* cell culture studies have demonstrated that administration of antisense oligonucleotides to protooncogene c-myb (a DNA binding protein) leads to a reduction in the targeted mRNA level, decrease in protein expression, and ultimately, dose-dependent suppression of cell proliferation (22). Similar results have been obtained with antisense oligonucleotides targeting protooncogene c-myc (21), proliferating cell nuclear antigen (PCNA, a subunit of DNA polymerase) (23), and nonmuscle myosin heavy chain (22), a gene thought to be involved in mitosis.

To assess the capability that antisense oligodeoxynucleotides may possess to modulate PAI-1 mediated fibrinolysis we designed site-specific 23-24-residue oligodeoxynucleotides targeted at mRNA sequences for the human PAI-1 (26). We targeted at three regions of the PAI-1 mRNA, two within the coding sequence of the message, namely nucleotide regions corresponding to PAI-1 sequences 265-272, 342-349, and the third one which is located upstream of the start codon (-24)-(-17). Unmodified phosphodiester oligodeoxynucleotides and their phosphorothioate analogues, complementary to these regions, were synthesized. In addition, we constructed several controls, an oligomer with random sequence, with two mismatches in the central section, and oligonucleotides with sense sequence (Tab. 3).

In order to analyze the effect of antisense oligonucleotides on PAI-1 expression in human endothelial cells, concentrations of PAI-1 in conditioned media were determined by the activity test and ELISA. In the first approach, the ability of PAI-1 to inhibit t-PA activity with the use of fibrin as a substrate for plasmin, before and after reactivation of the inhibitor by SDS-treatment was tested.

TABLE 3
INHIBITION OF PAI-1 RELEASE FROM ENDOTHELIAL CELLS BY ANTISENSE OLIGONUCLEOTIDES

| Designation | n | PAI-1 sequence | Inhibition [%] | | Concentration for 50% inhibition [μM] |
|---|---|---|---|---|---|
| | | | Cell extract | Conditioned media | |
| PO-1 | 24 | 265-272 | 38.9 | 0 | n.d. |
| PS-2 | 24 | 265-272 | 56.4 | 95.5 | 4.0 |
| PO-3 | 24 | -24 to -17 | 24.8 | 0 | n.d. |
| PS-4 | 24 | -24 to -17 | 93.7 | 98.3 | 5.6 |
| PO-5 | 23 | 342-349 | 2.0 | 6.4 | n.d. |
| PS-6 | 23 | 342-349 | 84.5 | 64.1 | n.d. |
| PS-9H | 16 | -20 to -17 | n.d. | n.d | 2.0 |
| PS-16H | 16 | -24 to -20 | n.d. | n.d. | 0.9 |
| PS-11H | 16 | -23 to -18 | n.d. | n.d. | 4.0 |
| PO-7 | 24 | random | 0 | 19.8 | — |
| PS-8 | 24 | random | 0 | 31.3 | — |
| PS-4(M) | 24 | mismatch | 0 | 11.0 | — |
| PS-16(M) | 16 | mismatch | n.d. | n.d. | — |
| PS-16NON | 16 | random | n.d. | n.d. | — |
| PS-16 S | 16 | sense | n.d. | n.d. | — |
| PS-20 | 21 | 265-271 | n.d. | n.d. | 21.6 |
| PS-21 | 21 | 264-272 | n.d. | n.d. | 16.6 |

In addition, the inhibition of PAI-1 expression in endothelial cells was tested by immunoprecipitation. After incubation of endothelial cells with [$^{35}$S]methionine for 4 hours, PAI-1 was immunoprecipitated from media with monoclonal antibody, analyzed by SDS-PAGE, autoradiographed and subjected for densitometric scanning. Immunoprecipitates of untreated control cultures analyzed by electrophoresis in SDS polyacrylamide gels under non-reducing conditions showed a strong band at $M_r$ 50 kDa, visualized after exposure of the fluorograms. Based on the intensity of this band, % of inhibition of PAI-1 concentration in conditioned media and cell extracts was calculated. As we can see in Table 3, antisense oligonucleotide PS-4 and its shorter version PS-16H were the most efficient inhibitors of PAI-1 synthesis in endothelial cells. They produce 50% inhibition of PAI-1 expression at the concentrations of 5.6 and 0.9 μM, respectively. Control oligonucleotides produced only insignificant effect on PAI-1 synthesis in endothelial cells, proving specificity of the analyzed inhibitory effect.

In another set of experiments, we attempted to improve the properties of PS-16 oligonucleotide as antisense agent by modification of its structure, which could facilitate its transport to the target cell, stability *in vivo*, and

specificity of its interaction with cellular targets (Tab. 3). For this purpose, we used diastereomers [All-Sp] and [All-Rp]-PS-16, synthesized by oxathiaphospholane method (27). They were purified and used in the same assay system for expression of PAI-1 in endothelial cells. To improve the cellular uptake of antisense oligonucleotides, cholesteryl-phosphorothioate residue was attached to 5'-end of PO-16 and PS-16 (Tab. 3, Chol-PO-16 and Chol-PS-16) by means of 2-cholesteryl-2-thiono-1.3.2-oxathiaphospholane (28). In addition, bulky dithymidine 3',5'-(4,4'-dimethoxytriphenylmethanephosphonate) (29) lipophilic groups were attached *via* phosphorothioate linker to 5'-ends and 3'-ends of PO-16 and PS-16, respectively (Tab. 3, Bl-PO-16, Bl-PS-16, PO-16-Bl, PS-16-Bl), for protection against cellular nucleases.

As we can see in Figure 2, after 4 hours of incubation with endothelial cells, the strongest inhibitor of PAI-1 synthesis appeared to be PS-16. Attachment of bulky group to 5'-end of PO-16 (Tab. 3, Bl-PO-16) significantly enhanced its inhibitory activity. Diastereomer [All-Sp]-PS-16 possesses significantly stronger inhibitory effect than [All-Rp]-PS-16 construct, however, they both were not much better inhibitors than PS-16 constituting the so called "random mixture" of all possible diastereomers (31).

In order to evaluate if PS-16 oligonucleotide is able to reduce PAI-1 concentration *in vivo* this phosphorothioate oligonucleotide was administered to rats through single intravenous injections. Bolus intravenous dose was administered via the tail vein without anesthesia. When rats were injected with 3 mg of the phosphorothioate oligonucleotide there was a time-dependent reduction in PAI-1 activity detectable in their blood plasma. Concentration of PAI-1 in blood plasma samples was analyzed based on the ability of PAI-1 to inhibit t-PA activity with the use of fibrin as a substrate for plasmin, after reactivation of the inhibitor by SDS-treatment. In parallel, rats were injected with two control oligonucleotides, i.e. PS-16 containing double mismatch (Tab. 3, PS-2MIS) and an oligonucleotide with a scrambled sequence (Tab. 3, PS-NON). Animals injected with control oligodeoxynucleotides did not show significant changes in their blood PAI-1 activity (Fig. 3). The same effect was seen when 1.5 mg of antisense oligodeoxynucleotide was administered. There was essentially no effect of control oligonucleotides on PAI-1 activity in rat plasma. It thus demonstrates that this antisense oligonucleotide inhibits PAI-1 expression both *in vitro* and *in vivo*.

In summary, the conceptual simplicity of antisense approaches to inhibit gene expression has led to numerous attempts to inhibit specific gene products and to apply antisense concept to various therapeutic uses. We think that it also can be applied to modulate fibrinolysis activity by decreasing of PAI-1 activity. However, while there is a considerable body of knowledge regarding the *in vitro* use of antisense oligonucleotides, there is little information regarding their *in vivo* usage. Antisense oligonucleotides can be delivered to the desired segment of arterial wall in a variety of ways (Fig. 4). A local extravascular delivery can be accomplished by placing oligonucleotide-containing polymer release device in the close proximity of the vessel wall, ensuring high local concentration of oligonucleotides and minimizing

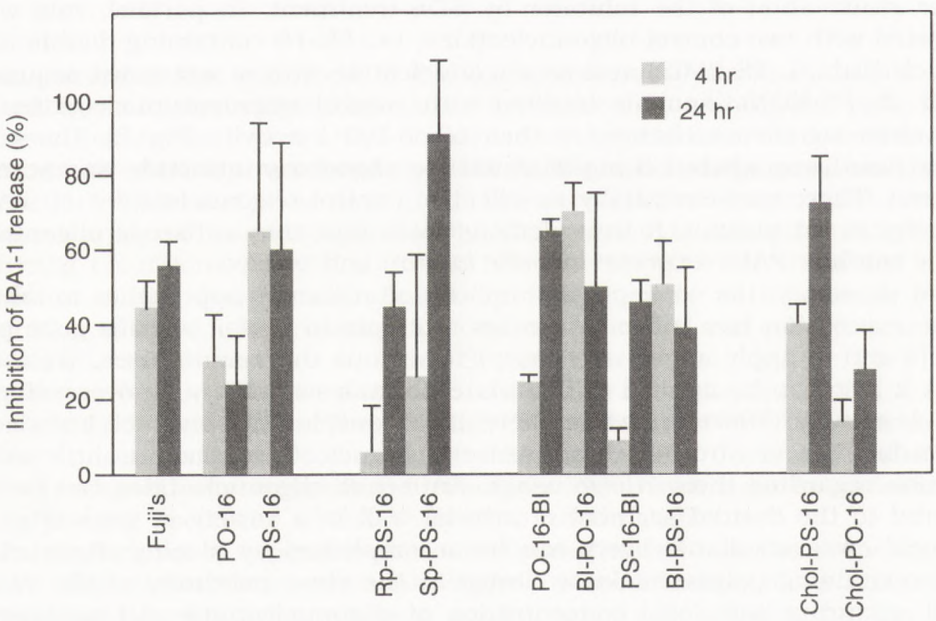
## ANALOGES OF PO-16 OLIGODEOXYNUCLEOTIDE ANTISENSE TO PAI-1 mRNA

ANTISENSE SEQUENCE: Signal peptide coding sequence of mRNA

d(GAG GGC TGA AGA CAT CTG CAT CCT) rat

d(GAG GGC TGG AGA CAT CTG CAT CCT) human

| | |
|---|---|
| PO-16 | d(GpApGp GpGpCp TpGpGp ApGpAp CpApTp C) |
| PS-16 | d(GsAsGs GsGsCs TsGsGs AsGsAs CsAsTs C) |
| PS-2MIS | d(GsAsGs CsGsCs TsGsGs TsGsAs CsAsTs C) |
| PS-NON | d(TsGsAs GsAsGs CsGsTs AsGsTs CsGsGs C) |
| PS-SENSE | d(GsAsTs GsTsCs TsTsCs AsGsCs CsCsTs C) |
| Rp-PS-16 | [all-Rp]-d(GsAsGs GsGsCs TsGsGs AsGsAs CsGsTs C) |
| Sp-PS-16 | [all-Sp]-d(GsAsGs GsGsCs TsGsGs AsGsAs CsGsTs C) |
| BI-PO-16 | $T_{PDMIT}$-d(GpApGp GpGpCp TpGpGp ApGpAp CpApTp C) |
| BI-PS-16 | $T_{PDMIT}$-d(GsAsGs GsGsCs TsGsGs AsGsAs CsAsTs C) |
| PO-16-BI | d(GpApGp GpGpCp TpGpGp ApGpAp CpApTp C)p$T_{PDMIT}$ |
| PS-16-BI | d(GsAsGs GsGsCs TsGsGs AsGsAs CsAsTs C)s$T_{PDMIT}$ |
| Chol-PO-16 | Chol-O-P(S)-d(GpApGp GpGpCp TpGpGp ApGpAp CpApTp C) |
| Chol-PS-16 | Chol-O-P(S)-d(GsAsGs GsGsCs TsGsGs AsGsAs CsAsTs C) |



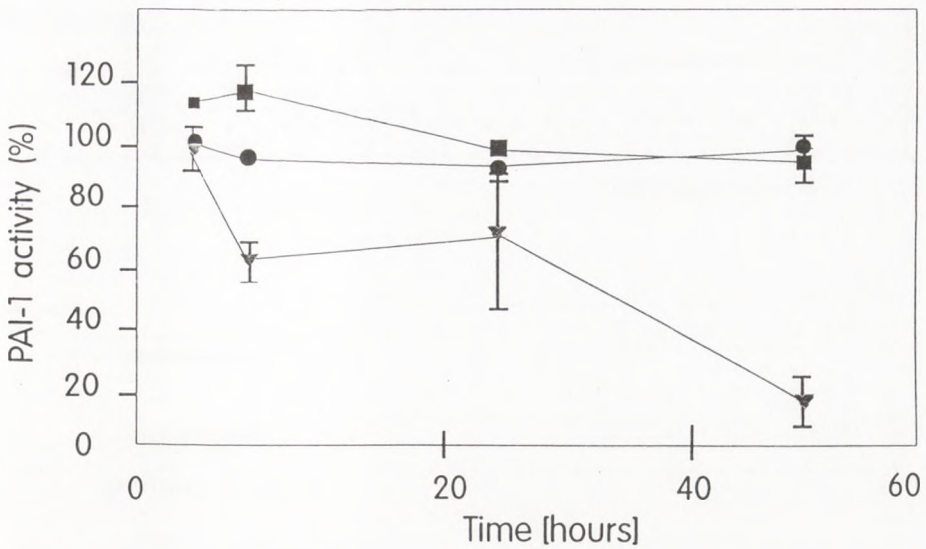



Fig. 3. PAI-1 activity in rat plasma after single injections of antisense PS-16.
This phosphorothioate oligonucleotide was administered to rats through single intravenous injections. Bolus intravenous dose was administered via the tail vein without anesthesia. Concentration of PAI-1 in samples of blood plasma was analyzed based on the ability of PAI-1 to inhibit t-PA activity with the use of fibrin as a substrate for plasmin, after reactivation of the inhibitor by SDS-treatment. In parallel, rats were injected with two control oligonucleotides, i.e. the one containing double mismatches and oligonucleotide with scrambled sequence.

blood flow washout (A). Alternatively, local intravascular delivery can be attempted by direct injection of oligonucleotides into the arterial wall using a perfusion balloon catheter (B), bathing (C) or coating (D) an arterial wall segment with an oligonucleotide-containing solution or gel either in naked form or at the presence of liposome particles that would facilitate cell absorption. The same catheter designs can be used to deliver oligonucleotides by viral-mediated infection. In addition, stents impregnated with the antisense compound or local iontophoretic infusions may be attempted. Finally, systemic intravenous administration of antisense oligonucleotides has been tried as well. The last approach has an obvious drawback of increased

Fig. 2. Inhibiton of PAI-1 release from HUVEC by different analogs of PO-16 used at the concentration of 2.5 μM.
All phosphodiester and phosphorothioate oligodeoxynucleotides were tested to establish their effect on PAI-1 concentration in human endothelial cell cultures. Concentration of PAI-1 in conditioned media was analyzed based on the ability of PAI-1 to inhibit t-PA activity before and after reactivation of the inhibitor by SDS-treatment. Fuji's antisense oligodeoxynucleotide to PAI-1 mRNA with sequence 5'-CCCCTCCCCACCAAGAGATT-3' described by Sawa et al. (30) was used as a positive control.

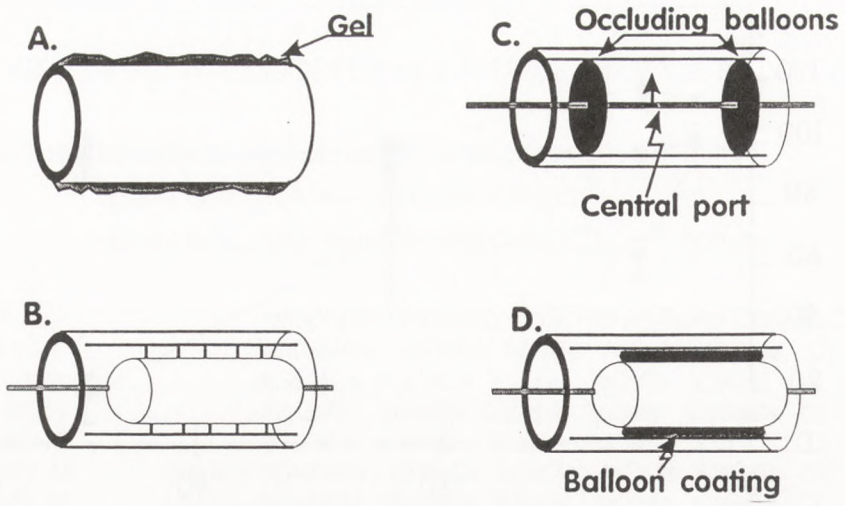



Fig. 4. Different ways of local delivery of antisense oligonucleotides.
Oligonucleotides can be delivered locally, by placing oligonucleotide-containing polymer release device in a close proximity of the vessel wall, ensuring high local concentration of oligonucleotides and minimizing blood flow washout (A). Alternatively, local intravascular delivery can be attempted by direct injection of oligonucleotides into the arterial wall using a perfusion balloon catheter (B), bathing (C) or coating (D) an arterial wall segment with an oligonucleotide-containing solution or gel either in naked form or at the presence of liposome particles that would facilitate cell absorption.

cost due to high oligonucleotide dose requirement and the potential of systemic toxicity. The first report of *in vivo* antisense study used a polaxamer (Pluronic F 127) gel to extravascularir deliver antisense c-myb oligonucleotides immediately after a ballon injury in a rat carotid injury model. This gel compound has a unique temperature inversion property existing in a soluble form at 4°C and in a polymer form at body temperature. Once applied to tissue, the gel is rapidly resorbed providing relatively short (< 3 hours) bolus delivery of oligonucleotides. However, even this short arterial wall exposure to antisense oligonucleotide results in a prolonged (2 weeks) biological effect with antisense treated arteries demonstrating little neointimal formation (25). Subsequently the same approach was used to deliver antisense c-myc (32), cdc2 and cdk2 (33) oligonucleotides achieving similar results. However, as encouraging as these studies are in rat model, the success of antisense approach to the treatment of restenosis will ultimately depend on its successful demonstration in large animal models, and, eventually, in humans.

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## Antisense Oligonucleotides to PAI-1 mRNA as Potential Agents for Therapeutic Intervention in Cardiovascular Diseases

Summary

Thrombolytic therapy provides clinical benefit in patients with vascular occlusions, depending upon the organ or limb that is threated. The impact of therapeutic intervention varies from the quiet alteration of the course of deep vein thrombosis, for which non-life threatening post-phlebitic syndrome can largely be avoided, to the striking reversal of pulmonary hypertension and possible life-saving benefit in massive pulmonary embolism, the immediate alteration of clinical course in acute peripheral arterial occlusion by reducing the need for surgical intervention, cardiopulmonary complication and one year mortality, and finally to the dramatic and life-saving potential when applied in patients with acute myocardial infarction. At least three alternative and complementary approaches to improve thrombolytic therapy can be distinguished: a) earlier and accelerated treatment in order to reduce the duration of ischemia, b) development of alterative or engineered plasminogen activators with increased thrombolytic potency and/or specific thrombolytic activity, in order to enhance coronary thrombolysis, and c) the use of more specific and potent anticoagulant and antiplatelet agents for conjunctive use with thrombolytic agents, with the aim to accelerate recanalization and prevent reocclusion. In this report we discuss the possibility of using antisense oligonucleotides specific to PAI-1 mRNA to reversibly decrease PAI-1 level in blood plasma and thus prolong a half-life time of endogenous plasminogen activators in circulation.

**Key words:**
cardiovascular diseases, recombinant proteins, peptide analogues, antisense oligonucleotides.

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