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Tissue distribution of a menthyl-conjugated oligodeoxyribonucleotide antisense to PAI-1 mRNA

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The inhibitory effect of numerous analogues of PO-16, an hexadecadeoxyribonucleotide antisense to sequences –22 to –17 of PAI-1 mRNA coding for a fragment of the signal peptide, on the expression of PAI-1 in endothelial cells, and physiological consequences of the subsequently reduced PAI-1 activity tested *in vitro* and *in vivo*, were described in our previous studies. Of particular interest was PO-16 5′-O-conjugated with menthyl phosphorothioate (MPO-16R). In this work, tissue localisation of MPO-16R labelled with [^{35}S] phosphorothioate at the 3′-end, was determined. [^{35}S]MPO-16R and control [^{35}S]MPO-16R-SENSE oligonucleotides were administered intravenously into 22 rats and organ distribution of the labelled bioconjugates was assessed after 24 and 48 h. For this purpose, tissue sections were subjected to autoradiography, and quantitated by liquid scintillation after solubilisation. Overall clearance of radioactivity was already seen after 24 h, with the radioactivity recovered mainly in the kidney and liver. A smaller fraction of radioactivity was also retained in the spleen and heart. The kidney concentration of the labelled probe was higher than that of liver by 50%. The distribution of PAI-1 mRNA in untreated rat kidney, liver, spleen and heart established by two independent techniques: Ribonuclease Protection Assay and Real-Time PCR, shows the same pattern as that observed for [^{35}S]MPO-16R antisense.

Keywords: antisense oligonucleotides, tissue distribution, PAI-1

Plasminogen activator inhibitor type 1 (PAI-1) is the major physiological inhibitor of tissue plasminogen activator (tPA) and plays a crucial role in the regulation of fibrinolysis. PAI-1 expression is upregulated under pathological conditions by a number of biologically active compounds, and in consequence its concentration in blood plasma and in extracellular matrix can be dramatically increased (van Meijer & Pannekoek, 1995). For example, overexpression of PAI-1 is associated with thrombotic diseases, atherosclerosis, myocardial infarction, and sepsis (reviewed in van Meijer & Pannekoek, 1995). PAI-1 controls also the extravascular activation of plasminogen occurring on the cell surface in the process involving receptors for urokinase-type plasminogen activator (uPA). PAI-1 protects tumor stromal tissue from autoproteolytic activities, and may thus substantially promote tumor growth and metastasis formation. In addition, PAI-1 activates tumor angiogenesis (Bajou *et al.,* 1998).

Taking into consideration the importance of PAI-1 in a number of diseases, strategies aimed at its rapid inactivation may be of clinical utility. Several approaches to inhibit PAI-1 expression and activity were proposed (Levi *et al.,* 1992; Eitzman *et al.,* 1995; Friederich *et al.,* 1997). One of those being the inhibition of PAI-1 synthesis using antisense oligodeoxyribonucleotides to selected regions of PAI-1 mRNA (Sawa *et al.,* 1994; Cierniewski *et al.,* 1995). The application of antisense oligodeoxynucleotides (ODNs) to target specific mRNA sequences and to block expression of the corresponding gene products has become commonly used technique in molecular biology (Agrawal *et al.,* 1990). Upon uptake into the cells, the antisense ODNs hybridise to the target mRNA resulting in the formation of DNA–

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Abbreviations: C_t, treshold value; MPO-16R, menthyl phosphorothioate; ODN, antisense oligodeoxynucleotide; PAI-1, plasminogen activator inhibitor type 1; PS, phosphorothioate; tPA, tissue plasminogen activator; uPA, urokinase type plasminogen activator.

RNA duplexes. Translation of the target mRNA is subsequently blocked by a variety of potential mechanisms, including degradation of the mRNA by RNase H (Agrawal *et al.,* 1990) and inhibition of the initiation of translation (Dolnick, 1990). Antisense ODNs may also cause premature termination of transcription as well as prevent mRNA splicing (Agrawal *et al.,* 1990).

Phosphorothioate ODNs are significantly more stable than phosphodiester ODNs *in vivo* because of their resistance to degradation by serum and intracellular nucleases (Agrawal *et al.,* 1990) and were successfully applied *in vivo* to block gene expression within tumor cells, arterial smooth muscle cells and retinal cells (Carome *et al.,* 1997). Successful application of ODNs in pathophysiological and therapeutic studies requires prior demonstration that there is a significant uptake of the ODNs by target tissues. In addition, knowledge about their plasma concentrations, tissue distribution and deposition is necessary for rational approach to individualise and optimise antisense regimens for experimental studies *in vivo* (Rifai *et al.,* 1996). The distribution of ODNs in healthy tissues and body fluids have been generally well characterized in many species, but addition of any backbone modification, improving their stability or uptake by cells, can alter tissue distribution, residence time in cells, and interactions with proteins (Geary *et al.,* 2001; Bijsterbosch *et al.,* 2002). In our earlier studies we have found that PO-16 5′-O-conjugated with menthyl phosphorothioate (MPO-16R) is very effective in PAI-1 inhibition. Of particular importance was the observation of its relatively high half-life in the plasma, comparable with that of the phosphorothioate oligonucleotide PS-16R. This PO-16 derivative was found to be a strong inhibitor of PAI-1 expression and activity measured both *in vitro* and *in vivo*, and in addition it showed antithrombotic properties in experimental rat model for thrombosis (Pawlowska *et al.,* 1998; Kobylanska *et al.,* 1999). Since phosphorothioate backbone internucleoside modifications are generally considered to be responsible for deleterious side effects such as nonsequence-specific activity, MPO-16R retaining its antisense specificity and possessing only one phosphorothioate modification was selected for further evaluation as a potential therapeutic agent. The objective of this study was to examine the distribution of menthyl conjugates of ODN sense and antisense to PAI-1 mRNA in rat tissue and organs after a single intravenous injection of its ^{35}S-radiolabelled derivative.

MATERIALS AND METHODS

Animals. Twenty-two male Wistar rats weighing 270–300 g were used. Anaesthesia was obtained by pentobarbital (40 mg/kg body mass) administered intraperitoneally. The experimental protocol was approved by the Local University Ethics Committee.

Synthesis of ^{35}S-radiolabelled oligonucleotides. Oligodeoxyribonucleotide PO-16R (GAGGGCTGAAGACATC) and PO-16R SENSE (GATGTCTTCAGCCCTC) were prepared on solid support using an ABI-394 DNA Synthesiser as described before (Kobylanska *et al.,* 1999). For preparation of the conjugate (MPO-16R and SENSE MPO-16R), a menthol residue was connected *via* a phosphorothioate linkage directly to the 5′-hydroxyl group of the oligonucleotide. Radioisotope labelling of the conjugates was carried out using [α-^{35}S]dCTP and terminal deoxyribonucleotidyl transferase.

Animal experiments. A total of 0.2 mg of [^{35}S]MPO-16R or [^{35}S]MPO-16R-SENSE dissolved in 100 μl of sterile normal saline was administered into six healthy anesthetized rats by a single bolus injection *via* the tail vein. The specific activity of the dosing solutions determined immediately before administration was 6 μCi/mg. The animals were sacrificed after anaesthesia 24 h or 48 h later. Blood was collected directly from the heart. Liver, spleen, kidney and heart were isolated and processed for detection of the radiolabelled probe. Five-micrometer thick organ tissue sections were prepared by cryosectioning or by paraffin embedding of the slices obtained from organs, which were previously fixed with buffered 10% formalin solution. All sections were attached to slides and autoradiographed using PhosphoImager Typhoon (Pharmacia, Sweden). Paraffin-embedded tissue sections were stained with hematoxylin-eosin for microscopic study.

All the excised organs were prepared blood free and weighed for liquid scintillation counting after solubilisation with Soluene-350 according to manufacturer (Packard Instrument B.V. Chemical Operation). Briefly, 100 mg aliquots of each organ were treated with 1 ml of Soluene-350 for 4 h at 50°C followed by the addition of 10 ml of Hionic-Fluor containing a chemiluminescence inhibitor for Soluene-350. For whole blood, 0.2 ml of a blood sample was mixed with 1 ml of Soluene-350 and then incubated for 30 min at 50°C. Afterwards, 30% H_2O_2 was added dropwise and incubation continued for 30 min at 50°C, followed by addition of 10 ml Hionic-Fluor. Blood cells were isolated by centrifugation (120 × *g*) of 1 ml whole blood. The cells were treated like whole blood and 0.5 ml of plasma was added to 10 ml of Pico-Fluor 40. The radioactivity of each organ was determined by liquid scintillation (Beckman Instruments, USA) and the values were expressed as the percentage of dose per gram of tissue normalized to the dose per gram of animal.

Ribonuclease protection assay. DNA was purified by phenol/chloroform extraction, precipitated

with ethanol and dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA) at a concentration of 1 mg/ml. Radiolabelled probe was synthesized by the procedure of Melton *et al*. (1984). Reaction conditions were as follows: 0.5 μg of linearized DNA, 10 mM dithiothreitol, 500 μM each of GTP, CTP, ATP, 8 μM UTP and 1.25 μM [α^{32}P]UTP (800 Ci /mM) in transcription buffer (40 mM Tris/HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 5 mM NaCl), 40 U RNase inhibitor (Promega Corp., Madison, WI, USA), 10 U bacteriophage Sp6 polymerase (Promega Corp., Madison, WI, USA) in a total volume of 20 μl. After 1 h incubation at 37°C RNase-free DNase was added and incubation continued for 10 min at 37°C. Single-stranded RNA was phenol/chloroform extracted, precipitated with ethanol and dissolved in hybridization buffer (20 mM Tris/HCl, pH 7.5, 20 mM EDTA, 0.6 M NaCl, 1% SDS).

Nuclei were isolated from tissues according to Srivastava and Schonfeld (1994). Pellet containing pure nuclei was resuspended in storage buffer: 20 mM Tris/HCl, pH 7.9, 75 mM NaCl, 0.5 mM DTT, 0.125 mM phenylmethylsulfonyl fluoride (PMSF), 50% glycerol. Elongation buffer (2x, 100 μl), 6 μl of 100 mM solution of nucleotide mix (GTP, ATP, CTP, UTP), 10^7 nuclei in storage buffer, 40 U of RNase inhibitor (RNasin, Promega, Madison, WI, USA), 40 ng sense or antisense oligonucleotides were added to a total volume of 200 μl. Transcription reaction proceeded for 45 min at 26°C and was stoped by addition of 100 U of DNase I (RNase free). Incubation was carried out for additional 10 min. Samples were then treated with 2 μl proteinase K (10 mg/ml) for 30 min at 56°C, extracted by phenol/chloroform and precipitated. After precipitation RNA samples were dissolved in TE buffer and used for hybridization. For each assay reaction we used 10 μg of total RNA isolated from rat tissues (three rats in each group) by the method of Chomczynski and Sacchi (1987). Radiolabelled probe (20 pg) and 10 μg t-RNA in 10 ml hybridization buffer were incubated for 17 h at 75°C. Single-stranded RNA was digested by adding 250 μl of a solution containing 50 μg/μl RNase A, 200 U/ml RNase TI (Worthington Biochemicals, Lakewood, CO, USA) and incubation for 1 h at 32°C. After phenol/chloroform extraction and ethanol precipitation radiolabelled RNA was analyzed by electrophoresis on 6% polyacrylamide gel. The gel was dried at 80°C and then exposed overnight to Kodak XAR-5 film.

Quantitative real-time PCR analysis. Total RNA (1 μg) was extracted from the liver, spleen, heart and kidney tissues using Trizol reagent (Life Technologies, Inc.), and was used directly for cDNA synthesis using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. PAI-1 and β-actin expression was quantified by real-time RT-PCR using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol. Briefly, 2.5, 2.0, 1.5, 1.0, 0.5 and 0.25 μl of synthesized cDNA was amplified in triplicate for both β-actin and each of the target genes to create a standard curve. Likewise, 2 μl of cDNA was amplified in triplicate for all isolated samples for each primer/probe combination, and for β-actin. Each sample was supplemented with appropriate forward and reverse primers at 0.3 μM, fluorescent probe, and made up to 50 μl using qPCR^{TM}Mastermix for SYBR Green I (Eurogentec, Seraing, Belgium). All the following PCR primers were designed using PrimerExpress software (Applied Biosystems): forward 5′ TGCTGGTGAATGCCCTCTACT 3′, reverse 5′ CGG TCATTCCCAGGTTCTCTA 3′, forward 5′ CGTACCACTGGCATCGTGAT 3′, reverse 5′ GTGTTGGCGTACAGGTCT TTG 3′, specific for mRNA of PAI-1 and β-actin, respectively. Each target probe was amplified in a separate 96-well plate. All samples were incubated at 50°C for 2 min and at 95°C for 10 min and then cycled at 95°C for 30 s, 56°C for 1 min and 72°C for 1 min for 40 cycles. SYBR Green I fluorescence emission data were measured and mRNA levels were quantified using the critical threshold (C_t) value. Analyses were performed with ABI Prism 7000 (SDS Software). Controls without reverse transcription and with no template cDNA were performed with each assay. To compesate for variations in input RNA amounts and efficiency of reverse transcription, β-actin mRNA was quantified and the results were normalized to these values. Relative gene expression levels were obtained using the $\Delta\Delta C_t$ method (Winer *et al*., 1999). Amplification-specific transcripts were further confirmed by obtaining melting curve profiles.

RESULTS AND DISCUSSION

Several factors may affect the inhibitory efficiency of antisense ODNs in controlling gene expression *in vivo*. These include the physicochemical properties of the ODNs determining their partition between the liquid phase and the lipid bilayer of the cell membranes. They also include structural features of cell membranes that control permeability to ODNs and limit the kinetics of their transport into various cells. ODNs distribution studies published so far have shown that radiolabelled antisense oligonucleotides administered intravenously are usually distributed evently throughout the body tissues and that the intracellular fate of the oligonucleotides strongly depends on backbone modification and their sequence-dependent ability to form tetraplex structures (Szklarczyk & Kaczmarek, 1997; Manoharan, 2002; Maszewska *et al*., 2002). Our present studies were focused on describing the organ location of the

strong anti-PAI-1 ODN MPO-16R, a phosphorothioate analogue of a hexadecadeoxyribonucleotide complementary to rat PAI-1 mRNA. It blocked PAI-1 expression in endothelial cells and caused a systemic inhibition of PAI-1 expression in rats as measured in blood plasma, in platelets, and after thrombus formation (Cierniewski *et al.*, 1995; Pawlowska *et al.*, 1998; Kobylanska *et al.*, 1999). Since the optimal period of time for detection of its inhibitory effect on PAI-1 activity was 24 h after bolus injection, in the present study, organ distribution of [^{35}S]MPO-16R was tested in rats 24 and 48 h after intravenous administration. For this purpose, animals were sacrificed and different organ tissues were processed for detection of radioactivity content. Figure 1A shows tissue levels of [^{35}S]MPO-16R retained in 1 g of the different organs of rat, expressed as the percentage of the dose detected per 1 g of the whole animal. Kidney appeared to be the main target for the antisense [^{35}S]ODN. The accumulation of the radioactive probe in the kidney measured after 24 h was 21%. After 48 h the probe was still detectable at 16%. Liver and spleen showed significantly lower accumulation, 11% and 10% after 24 h and 4% and 7% after 48 h, respectively. Smaller fractions were accumulated in the heart, about 4% after 24 h and 2% after 48 h. Taking into consideration the total mass of each organ, kidney appears as the main target organ for ^{35}S-MPO-16R. The sense probe showed notably lower percentages of accumulation (Fig. 1B). After 24 h the accumulation was 5.8, 3.8, 2.5 and 2.1% in kidney, liver, spleen and heart, respectiveley, but after 48 h very low fractions of the sense probe persisted (1.7, 0.8, 1 and 0.7% in kidney, liver, spleen and heart, respectively). The high retention level of radioactivity in specific rat tissues after 24 h since the administration of MPO-16R correlates well with the time necessary to obtain the highest reduction in PAI-1 activity in the plasma, determined in our previous study (Pawlowska *et al.*, 1998). Furthermore, it appears that the menthyl-modified PO-16R does not undergo rapid cellular exocytosis and the persisting radioactivity derived from the labelled probe is substantial after 24 h or even after 48 h since the administration.

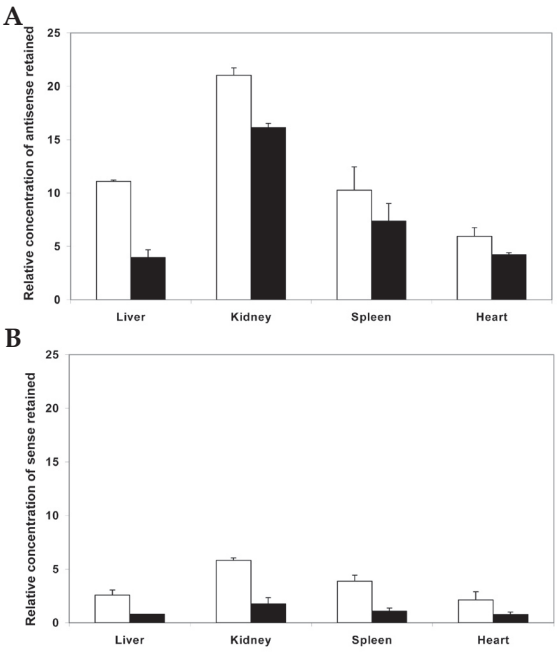

Figure 1. Tissue distribution of antisense (A) and sense (B) ^{35}S-labelled MPO-16R conjugates.
Each probe was administered into two groups of three animals each. After 24 h (empty bars) or 48 h (filled bars) organs were collected, solubilised with Soluene-350 and radioactivity was determined by liquid scintillation. Values are the radioactivity content per gram of tissue expressed as the percentage of total dose applied per gram of whole animal, ±S.D.

The radioactivity detected within the tested samples is equally distributed as evidenced by autoradiography of the frozen sections (Fig. 2).

Figure 3 shows the percentage of radioactivity recovered in 1 ml of whole blood, blood cells and blood plasma after 24 and 48 h for the both tested probes.

Ribonuclease protection assay (RPA) was performed to measure PAI-1 mRNA level in organs, from animals treated with either oligonucleotide. The

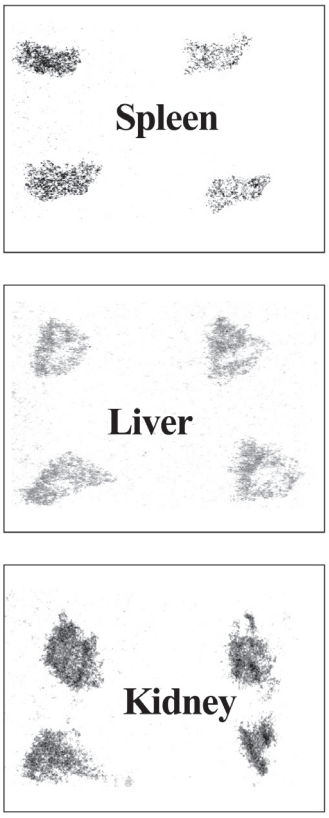

Figure 2. Distribution of radioactivity in sections of rat spleen, liver, and kidney 24 h after antisense ^{35}S-labelled MPO-16R administration.
Autoradiography was done using Phosphoimager Typhoon (Pharmacia, Sweden). Representative images are shown.

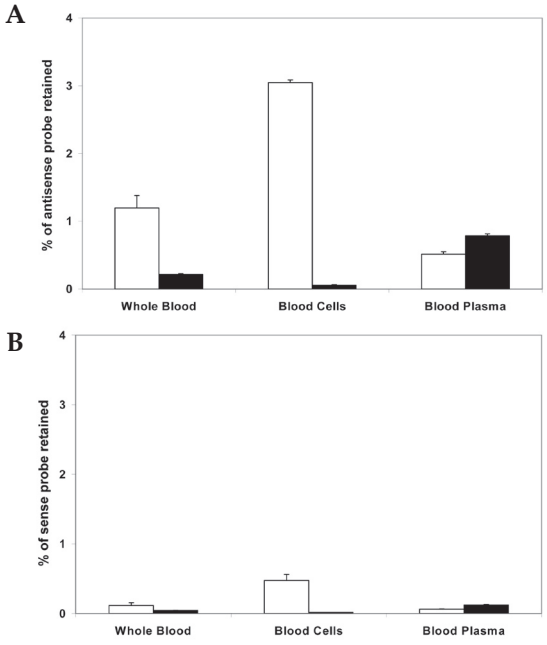

Figure 3. Distribution of antisense (A) and sense (B) ^{35}S-labelled MPO-16R probes in blood.
Probe was injected in two groups of three animals each. Animals were sacrificed after 24 h (empty bars) or after 48 h (filled bars). Blood was collected from each animal and fractionated for cells and plasma, lysed with Soluene-350 and radioctivity was determined by liquid scintillation. Values are expressed as a percentage of a dose per gram of tissue in one ml of each blood component, ±S.D.

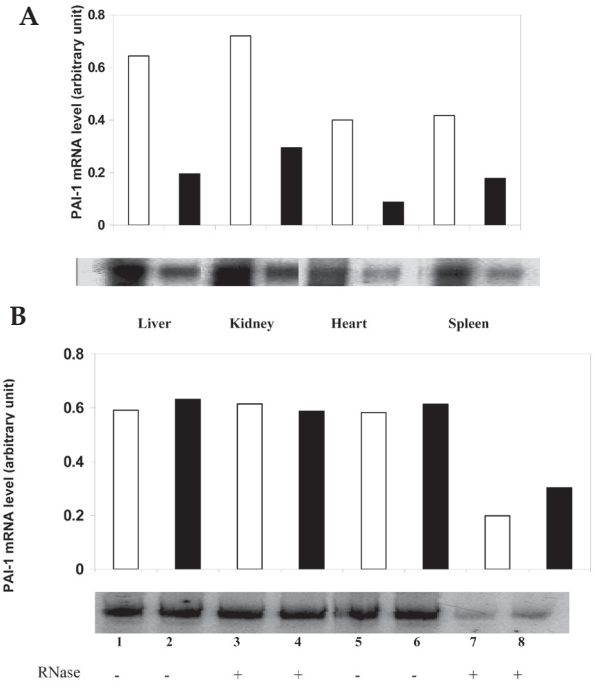

Figure 4. Ribonuclease protection assay
(A) Tissue specific transcription of PAI-1 gene in the presence of antisense probe in liver, kidney, heart, and spleen without (empty bars) or with (filled bars) RNase treatment, 24 h after antisense administration (B). The effect of sense probe (lanes 1–4) and antisense probe (lanes 5–8) without treatment with RNase (lanes 1, 2, 5, 6) or with RNase (lanes 3, 4, 7, 8). Tissue specific transcription level of PAI-1 gene in liver (empty bars) and kidney (filled bars). Typical autoradiograms and their densitograms from three experiments are shown.

antisense oligonucleotide decreased the cellular level of specific mRNA in all tested organs (Fig. 4A).

Figure 4B shows that the sense probe had no effect on the transcription level. The antisense probe inhibited transcription in both liver and kidney, with a higher degree of inhibition in the liver than in the kidney. According to decreased inhibition by antisense oligonucleotide detected in studied tissue, the order was as follows: heart, spleen, liver and kidney, which means that the amount of PAI-1 mRNA was on the opposite direction by means of kidney, liver, spleen, and heart. The measurements of radioactivity level in organs, autoradiograms of tissue sections

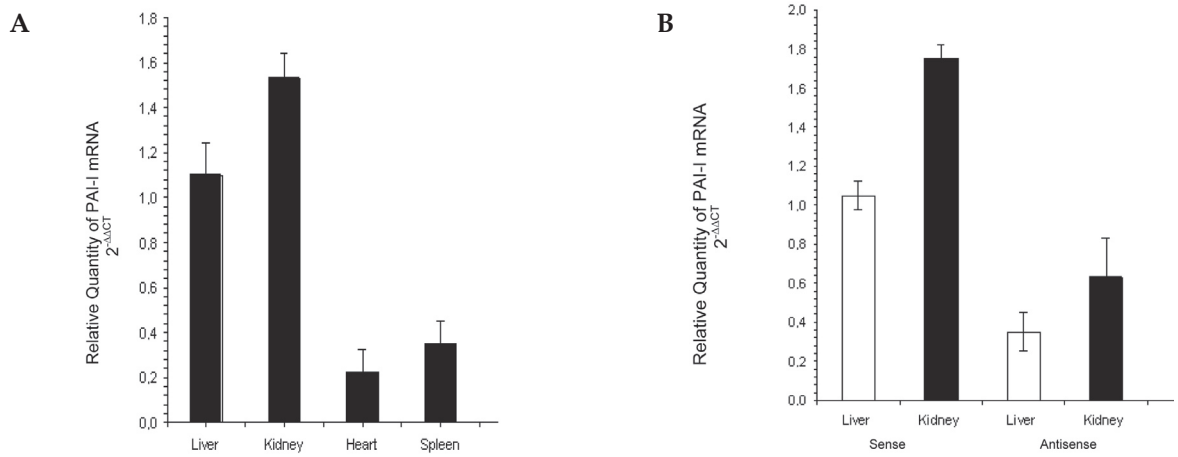

Figure 5. Quantitative real-time PCR analysis of tissue specific expression of PAI-1 mRNA.
(A) PAI-1 mRNA level after antisense probe application in liver, kidney, heart and spleen. Organ tissue sections came from animals sacrificed after 24 h. (B) PAI-1 mRNA level in the presence of sense and antisense probe in liver and kidney cells. Tissue specific transcription of PAI-I gene in liver (empty bars) and kidney (filled bars). The graphs show the average ±S.D. of three experiments.

and the ribonuclease protection assay showed clear compatibility of the transcription level of PAI-1 gene and the accumulation of the MPO-16R antisense in the studied organs. Our observations on organ distribution of MPO-16R in rat after systemic administration are consistent with earlier data describing the location of PAI-1 mRNA in rat organs. PAI-1 mRNA was found in many rat tissues and organs, i.e. in the liver, kidney, heart, spleen and testis (Nargolwalla *et al.,* 1990; Quax *et al.,* 1990; Konkle *et al.,* 1992; Keeton *et al.,* 1993; van Meijer & Pannekoek, 1995; Oikawa *et al.,* 1997). The most significant regulation of PAI-1 mRNA was observed throughout the kidney (Oikawa *et al.,* 1997). In order to confirm that the tissue concentration of PAI-1 mRNA and MPO-16R are similar, the mRNA abundance was determined by quantitative real-time PCR analysis. (Fig. 5A, B) The level of PAI-1 mRNA determined in tissues correlated positively with the degree of antisense probe retention. MPO-16R sense had no effect on the level of PAI-1 gene transcription.

Thus, the similarities in the distribution of MPO-16R and PAI-1 mRNA, particularly the highest concentration of the antisense ODN in kidney, the organ showing the highest level of PAI-1 mRNA, indicate that this accumulation of [^{35}S]ODN may result from the sequence-specific interaction with PAI-1 mRNA.

We also provide evidence that by systemic application of antisense oligonucleotides it is possible to cause an effective inhibition of PAI-1 expression in all organs, with the highest degree of inhibition in kidney and liver. These data strongly suggest that the MPO-16R oligonucleotide antisense to PAI-1 mRNA should be considered as a new potential agent for targeted fibrinolytic and cancer treatment therapy, especially at sites of the highest PAI-1 expression.

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