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Biochemical Pharmacology 70 (2005) 1277–1287
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Inhibition of Sp1 activity by a decoy PNA–DNA chimera prevents urokinase receptor expression and migration of breast cancer cells

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Received 16 June 2005; accepted 25 July 2005

Abstract

Sp1 regulates the activation of many genes involved in tumor growth, apoptosis, and angiogenesis. We have previously shown the involvement of Sp1 in the up-regulation of urokinase receptor (uPAR) expression, a key molecule in tumor invasion and metastasis. Here, we investigated whether a marked down-regulation of Sp1 activity may inhibit uPAR expression and migration ability of MDA-MB-231 breast cancer cells. To this end, we tested the decoy ability of a novel peptide nucleic acid (PNA)–DNA chimera which carries a central DNA strand, containing Sp1-binding sequence, covalently linked to two PNA fragments at both ends (PNA–DNA–PNA, PDP). The chimera was synthesized, annealed with complementary DNA (PDP–DNA), and then tested for its ability to bind Sp1 both in vitro and in living MDA-MB-231 breast cancer cells in the presence of urokinase (uPA). This PDP–DNA decoy molecule efficiently competes for the binding to endogenous Sp1 in nuclear extracts, and upon transfection with liposomal vectors, causes a marked decrease of available Sp1 in both untreated and uPA-treated MDA-MB-231 cells. Accordingly, both uPA-dependent enhancement of uPAR expression and cell migration were strongly reduced in transfected cells. Interestingly, a detectable inhibitory effect is also observed in breast cancer cells exposed to PDP–DNA in the absence of transfection reagents. Finally, the inhibitory effect of PDP–DNA appeared to be stronger than that observed with oligonucleotides carrying Sp1 consensus sequence. Our findings show that this novel PNA–DNA chimera, containing Sp1 consensus sequence, effectively inhibits Sp1 activity, uPAR expression, and motility of breast cancer cells indicating its potential therapeutic use to prevent tumor dissemination.

Keywords: Sp1 transcription factor; Urokinase receptor; Peptide nucleic acid; PNA–DNA chimera; Breast cancer

1. Introduction

Tumor invasion and metastasis is a multi-step process requiring the proteolytic degradation of the extracellular matrix constituents and modification of cell adhesion properties [1]. Although multiple classes of ECM-degrading enzymes are reported to be involved in such process, a key role has been assigned to the urokinase-type plasminogen activator (uPA) and its cognate receptor (uPAR) [2]. Consistent evidence indicates indeed that this receptor not only focuses the uPA proteolytic activity at the cell surface, but also mediates, in a ligand-dependent manner, a variety of cell responses, such as migration, adhesion, and proliferation [3,4]. The urokinase-type plasminogen activator receptor is a three-domain glycosylphosphatidylinositol-anchored protein with high affinity for uPA and its

Abbreviations: ATF, amino-terminal fragment of urokinase; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; ODN, oligodeoxynucleotide; PDP–DNA, hybrid of PDP chimera with complementary DNA; PDP, single strand PNA–DNA–PNA chimera; PNA, peptide nucleic acid; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor

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doi:10.1016/j.bcp.2005.07.024

catalytically inactive related proteins, namely single-chain uPA (pro-uPA) and amino-terminal fragment (ATF). High levels of both uPA and uPAR have been found in many human malignant tumors including breast carcinoma and they strongly correlate with a poor prognosis and unfavorable clinical outcome [5,6]. Up-regulation of uPAR gene expression in malignant cells has been reported to occur mainly at transcriptional level through activation of its promoter region by several transcription factors including Sp1, AP-1, AP-2, and NF-kB [7]. The central role of Sp1 in the regulation of uPAR gene transcription has been supported by the identification of a minimal promoter region, lacking TATA and CAAT boxes, and containing Sp1-motifs at about 100 bp upstream of the transcriptional start site [8]. Also, induction of uPAR gene expression by a constitutively active Src is reported to occur through Sp1 binding to a region spanning −152/−135 of the uPAR promoter [9].

Sp1 is a member of an extended family of transcription factors characterized by a highly conserved C-terminal DNA-binding domain containing three Cys2His2 zinc fingers [10]. It binds to GC boxes, which can be found in the promoter regions of a variety of constitutive and inducible mammalian genes. Previous studies indicated that Sp1 is responsible for recruiting TATA-binding protein and fixing the transcriptional starting site at TATA-less promoters. Traditionally considered as a constitutive transcription factor, it is becoming increasingly clear that Sp1 is a target of several signal transduction cascades, thus mediating gene-specific response to a variety of signals [11]. A number of studies indicates indeed that Sp1 is a target of multiple cellular kinases and phosphatases which directly or indirectly modulate its DNA binding and transcriptional activity [12]. Although Sp1 is usually regarded as a transcriptional activator, it has been reported that it may repress the expression of certain genes and such divergent effects appear to be promoter- and cell context-dependent [10]. Finally, Sp1 activity may be induced by many oncoproteins such as v-Rel, v-Ras, v-Src, c-Fes, and the human retinoblastoma gene product [13–15].

In a previous study, we showed that Sp1-binding activity and uPAR levels were co-ordinately elevated in breast carcinomas as compared to benign lesions [16]. In particular, we have previously observed that uPAR engagement by uPA results in a marked up-regulation of Sp1-binding activity followed by an increase of uPAR protein [16]. Moreover, in a large series of resected gastrointestinal cancers, a high Sp1-binding activity to the upstream sequence −152/−135 was found in about 60% of tumor specimens and correlated with uPAR levels in the same tumors [17]. Furthermore, several binding sites for the Sp1 family of transcription factors have been identified in the proximal promoter of uPA gene, immediately upstream of the TATA box [18,19]. These observations, taken together, raised the possibility that artificial inhibition of Sp1 transcription factor activity may prevent uPA-induced enhancement of uPAR expression, thus reducing the metastatic potential of cancer cells.

Recently, peptide nucleic acids (PNAs) and PNAs analogs have been described as promising tools for modulation of gene expression in human diseases [20]. In the present study, we tested the decoy ability of a PNA–DNA chimera which carries a central DNA strand, containing Sp1 consensus sequence, covalently linked to two PNA fragments at both ends (PNA–DNA–PNA, PDP). This approach is aimed at inhibiting the endogenous Sp1 activity by binding available Sp1 and interfering with target gene activation in breast cancer cells. First of all, this chimera was synthesized, annealed with complementary DNA (PDP–DNA), and then tested for its ability to bind Sp1 protein in nuclear extracts and in living MDA-MB-231 breast cancer cells previously exposed to uPA or diluents. Furthermore, the effects of Sp1 PDP–DNA decoy molecule were compared to those observed with double stranded oligodeoxynucleotides (ODNs), carrying the same Sp1 consensus sequence, both in vitro and in living cells. Finally, the ability of the novel PDP–DNA molecule to modulate uPAR expression and cell migration was assessed in MDA-MB-231 human breast cancer cells.

2. Materials and methods

2.1. Cell lines and culture conditions

MDA-MB-231 human breast carcinoma and HeLa human cervical carcinoma cell lines were grown in DMEM containing 10% fetal bovine serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin in a humidified incubator in 5% CO_2 at 37 °C.

2.2. Synthesis of Sp1 PNA–DNA–PNA chimera

PNAs are DNA analogues in which the entire sugar-phosphate backbone is replaced by an *N*-aminoethyl-glycine-based polyamide structure. PNA–DNA chimeras derive by the covalent binding of a PNA oligomer to a DNA fragment in a single chain. Here, chimera synthesis proceeded on a tyrosine functionalized resin by sequential elongation of the PNA fragment, to which DNA first, and then PNA were attached as described in detail elsewhere [21]. Briefly, the DNA part of the chimera was prepared on a Pharmacia Gene Assembler and chain elongation was performed with 15 eq of methyl DNA phosphoramidites using 5-(*o*-nitrophenyl) tetrazole (8 eq) as the activator. Standard DNA capping, washing, oxidation, and detritylation cycles were used. In the last DNA elongation step, cyanoethyl 5′-amino-5′-deoxythymidine phosphoramidite was used. The PNA part of the chimera was prepared on a Perseptive Biosystems Expedite Nucleic Acid Synthesis System using standard (designed for 2 μmol scale) PNA coupling cycles and solutions. Fmoc (Bz, benzyl)/(iBu,

isobutyl)-protected PNA was used. To improve the coupling efficiency of the first PNA moiety, a double coupling cycle was employed. Upon completion of the last elongation cycle, the terminal Fmoc group was cleaved by piperidine treatment and the primary amine was acetylated. The methyl groups were removed from the phosphate functions by treatment of the resin with 0.25 ml thiophenol in 0.5 ml tetrahydrofuran and 0.5 ml triethylamine for 45 min. The resin was washed consecutively with tetrahydrofuran, methanol, acetonitrile, and water (5 × 1 ml for each solvent). The oligomers were cleaved from the support with concomitant deprotection of the remaining protective groups by treatment with 0.1 M sodium hydroxide in water/dioxane (1/1, v/v, 1.5 ml) at 55 °C for 16 h. The reaction mixtures were neutralized by the addition of acetic acid, concentrated, and redissolved in 0.15 M ammonium bicarbonate. Desalting was performed using a Sephadex G-25 (superfine, DNA grade) gel filtration column with 0.15 M ammonium bicarbonate buffers. The samples were filtered, and then purified by RP-HPLC on a LiCrosphere 100 RP-18 end capped column (4 mm × 250 mm) on a Jasco HPLC system. Gradient Elution was performed at 40 °C building up gradient starting with buffer A (50 mM triethylammonium acetate in water) and applying buffer B (50 mM triethylammonium acetate in acetonitrile/water, 1/1, v/v), with a flow rate of 1 ml/min. The molecular mass of chimera was determined by MALDI-TOF mass spectrometry.

Table 1 reports the final sequence of PNA–DNA–PNA (PDP) chimera along with the composition of the synthetic oligonucleotides used in the study. PDP contained a central DNA strand flanked by two PNA fragments at both ends (in lowercase letters): NH_2-tyr-a-5′-TCGGGGCGGGGCGA-3′-gc-Ac. Sp1 consensus sequence is underlined. The complementary DNA chain 5′-CTCGCCCCGCCCCG-AT-3′ was purchased from M-Medical, Italy. Annealing of PDP chimera and complementary DNA chain was performed by incubation at 100 °C for 5 min and the resulting double stranded PDP–DNA hybrid was used as a decoy molecule. Two double-stranded oligodeoxynucleotides containing the same Sp1 consensus sequence were used for comparison, namely a commercially available 22 mer ODN (5′-ATTCGATCGGGGCGGGGCGAGC-3′, Promega, Madison, WI) and a 16 mer ODN (5′-ATCGGGGCGGGGCGAG-3′, M-Medical). A double-stranded ODN containing a mutated Sp1-binding sequence at two positions (shown in bold) (5′-ATTCGATCGG**TT**CGGGGCGAGC-3′, M-Medical) was used as negative control.

Table 1
Decoy PNA–DNA–PNA chimera (PDP) and synthetic oligonucleotides used in the study

PNA–DNA–PNA	NH_2-tyr-a-5′-TCGGGGCGGGGCGA-3′-gc-Ac
Sp1 ODN 22 mer	5′-ATTCGATCGGGGCGGGGCGAGC-3′
Sp1 ODN 16 mer	5′-ATCGGGGCGGGGCGAG-3′
mt Sp1 ODN	5′-ATTCGATCG**TT**CGGGGCGAGC-3′

The Sp1 consensus sequence is underlined; PNA monomers are in lowercase letters; mutated Sp1 oligonucleotide (mt Sp1 ODN) carries GG → TT substitution in Sp1-binding motif (in bold).

2.3. Nuclear extracts preparation

Nuclear extracts were prepared according to the method of Dignam et al. [22]. Briefly, cells were washed in cold PBS and collected by centrifugation. The cell pellet was resuspended in 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.1 mM EGTA (homogenization buffer); homogenized by passage through a 26-gauge needle and centrifuged for 5 min at 1500 rpm. The pellet representing nuclei was resuspended in 10 mM HEPES (pH 7.9), 5% glycerol, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.4 M NaCl, and 0.1 mM EGTA (extraction buffer). The protease inhibitors leupeptin (5 mM), aprotinin (1.5 mM), phenylmethylsulfonyl fluoride (2 mM), and benzamidine (1 mM) were used throughout the extraction procedure. The suspension was rocked at 4 °C for 30 min, cleared by centrifugation for 30 min at 12,000 rpm, and stored at −80 °C. Protein concentration was determined by the method of Bradford (Bio-Rad Laboratories, Richmond CA).

2.4. Electrophoretic mobility shift assay

Nuclear proteins were subjected to electrophoretic mobility shift assay (EMSA) using standard protocols. Both the commercially available double-stranded DNA oligonucleotide containing the Sp1 consensus sequence (Promega, Madison, WI) and the PDP–DNA molecule were end-labeled with T4 polynucleotide kinase, [γ-^{32}P]ATP, and purified by gel electrophoresis. Ten micrograms of nuclear proteins were preincubated in a binding buffer containing 20 mM HEPES (pH 7.5), 40 mM KCl, 5% glycerol, and 2 μg of poly(dI-dC) for 10 min at 22 °C. The radiolabeled probe (100,000 cpm/0.1 ng) was added in the presence or absence of a molar excess of unlabeled PDP–DNA or DNA–DNA competitor and the incubation was continued for additional 20 min at 22 °C. Free and bound probe were separated on a 8% non-denaturating polyacrylamide gel followed by autoradiography. Morphodensitometric analysis of autoradiograms was performed using an image analysis system including a high-resolution CCD camera (High Technology Holland) and the Micro Computer Imaging Device (Imaging Research, Inc., Ont., Canada). For each sample, the product of optical density and the area of the band was calculated and normalized against the Sp1 signal in the untreated sample, which was taken as 100%.

2.5. PDP–DNA transfection and uPA treatment

PDP–DNA decoy molecule was transferred into cancer cells using DOTAP, *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate, as a liposomal

transfection vector (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Briefly, PDP–DNA decoy molecule was added to MDA-MB-231 cells at concentration of 0.5 μM in the presence of DOTAP (PDP–DNA:DOTAP ratio = 1:5) and cells were incubated for 18 h at 37 °C. Mock transfected cells with DOTAP alone were employed as control. When indicated, MDA-MB-231 cells were incubated for 18 h at 37 °C with PDP–DNA alone (0.5 μM), with ODNs alone (0.5 μM) and ODNs (0.5 μM) added to liposomes. Nuclear proteins were then extracted as described above and subjected to EMSA. In an additional set of experiments, PDP–DNA transfected and control MDA-MB-231 cells were treated with 10 nM uPA, or the amino-terminal fragment of uPA (ATF, amino acids 1–135) in serum-free conditioned media for 30 min and 6 h at 37 °C to induce Sp1 DNA-binding activity and uPAR levels, as previously described [16]. The ability of PDP–DNA to inhibit uPA-mediated induction of Sp1 activity was again determined by EMSA.

2.6. Cell lysates preparation and western blot analysis

After treatment MDA-MB-231 cells were lysed in 200 μl of a buffer containing 1 mM EDTA, 0.2% Triton-X, and proteases inhibitors. The suspension was homogenized by passages through a 26-gauge needle and centrifuged for 15 min at 13,000 × *g* at 4 °C. Protein concentration of the supernatant was assayed as above described. Western blot analysis of proteins from whole cell lysates was carried out using a standard procedure. Briefly, equal amount of proteins from cells (40 μg) were separated by SDS-PAGE under reducing conditions and transferred to PVDF membranes. After blocking of non-specific protein-binding sites, membranes were incubated with primary antibodies for 45 min at 22 °C. uPAR was detected using 1 μg/ml of rabbit anti-human uPAR IgG 399 from American Diagnostica (Greenwich, CT) and tubulin was detected with 1 μg/ml of monoclonal antibody (Sigma Chemical Co, St. Louis, MO). The filters were then incubated with 1:2000 peroxidase-labeled antimouse or antirabbit Ig antibodies (Amersham Biosciences Europe, Freiburg, Germany) for 1 h at 22 °C. After extensive washing, the immunoreaction was revealed by the enhanced chemiluminiscence detection system (ECL) according to the manufacturer's recommendations.

2.7. Fluorescence microscopic examination

The intracellular localization of PDP–DNA was assessed by fluorescence microscopy. Briefly, FITC-labeled complementary DNA strand (16 mer) was purchased by M-Medical, and 2.5 nmol were annealed with equimolar PDP chimera. MDA-MB-231 cells were allowed to grow and adhere on cover slips. Cells were then incubated with FITC-labeled hybrid PDP–DNA (0.5 μM) for 18 h at 37 °C in the presence or absence of DOTAP. Mock-transfected cells with empty liposomes were employed as negative control.

2.8. Cell migration assay

Cell migration assay was performed using Boyden procedure in a 48-microwell-chemotaxis chamber (Costar, Corning, NY, USA). The upper and lower wells were separated by an 8 μm pore size polycarbonate filter coated with 5 μg/ml vitronectin. Briefly, MDA-MB-231 cells (5×10^4 cells in 50 μl of serum-free medium), after transfection with PDP–DNA or ODNs were added to the upper wells whereas the lower wells contained serum-supplemented medium (FBS 10%) or uPA (10 nM). For comparison PDP–DNA, ODNs and DOTAP were used alone in parallel experiments. Cells not subjected to any treatment were used as control. The chamber was incubated for 6 h at 37 °C. At the end of the assay, non-migrating cells on the upper surface of the filters were removed with a cotton swab, whereas cells on the lower surface of the filters were fixed in ethanol, stained with haematoxylin and counted in 10 random fields/filter at 200× magnification. The absolute number of migrating cells in each well was determined and Student's *t*-test was used to compare treated and untreated cells. Results are expressed as the percentage of migrating cells considering the untreated control sample as 100%. The values of migrating cells in untreated controls were 245 ± 13 and 61 ± 8 when serum-supplemented medium and uPA were used as chemoactractans, respectively. Each experiment was performed in triplicate.

3. Results

3.1. Sp1 activity is reduced by PDP–DNA in HeLa nuclear extracts

To test whether the artificial modulation of Sp1 transcription factor activity may block uPAR expression and migration of breast cancer cells, a novel PNA–DNA chimera and several synthethic oligonucleotides containing the Sp1-binding motif were designed to be employed as in vivo decoy molecules.

Firstly, the binding ability of PDP–DNA to Sp1 protein was assessed in nuclear extracts from Hela cells. Equal amounts of proteins were incubated with the radiolabeled commercially available 22 mer ODNs containing Sp1-binding motif in the presence or absence of a large molar excess of unlabeled PDP–DNA molecule (100-, 300- and 1000-fold) or unlabeled 22 mer (100-, 300- and 1000-fold) and 16 mer (300-fold) ODNs. The resulting complexes were separated by EMSA and revealed by autoradiography. As shown in Fig. 1A, PDP–DNA efficiently competes with radiolabeled ODNs for the binding to endogenous Sp1. Conversely mutated ODNs are not able

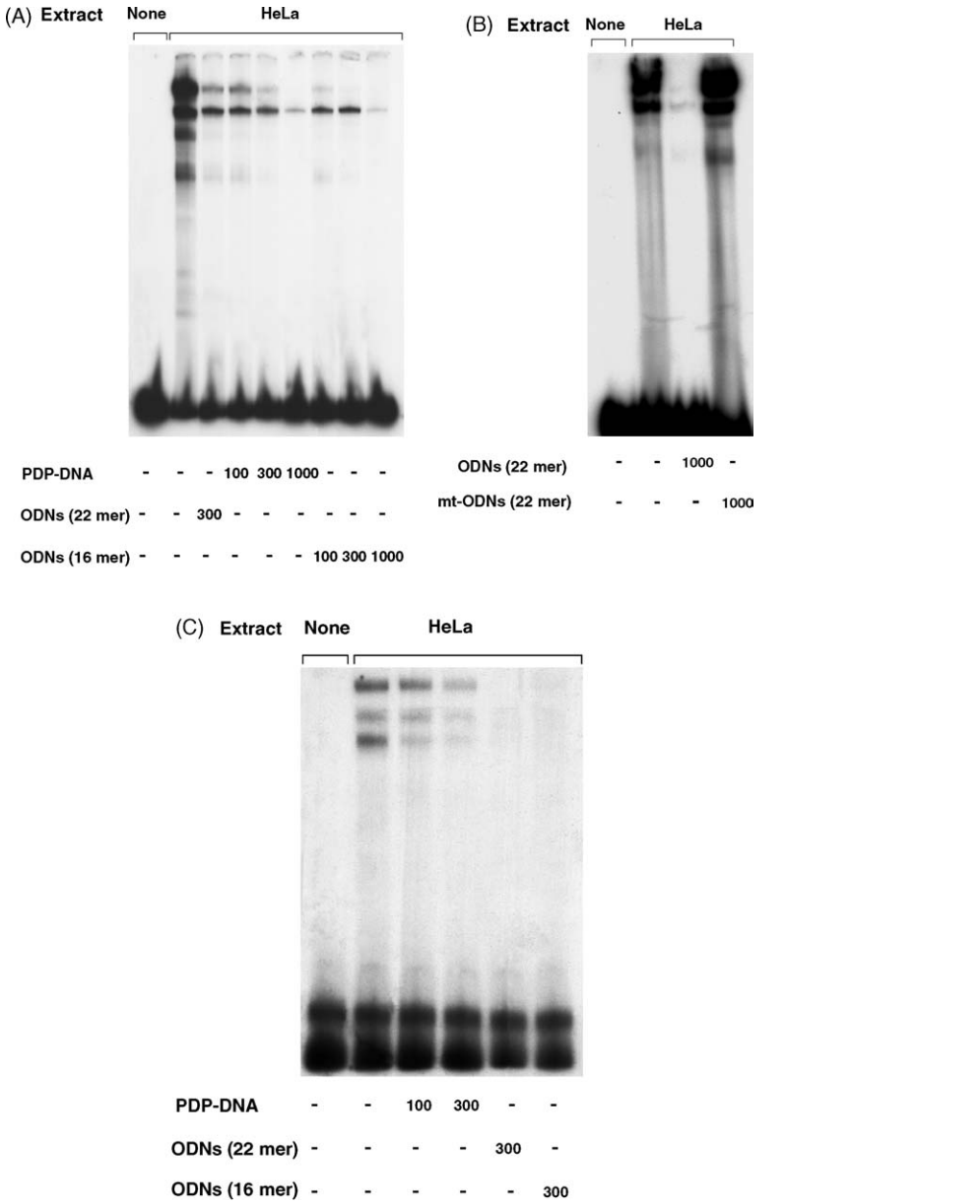

Fig. 1. Representative EMSA showing the binding of PDP–DNA decoy molecule to Sp1 in nuclear extracts. (A) Nuclear extracts (10 μg) from HeLa cells or diluents (none) were incubated with ^{32}P-labeled 22 mer oligonucleotide containing the consensus sequence for Sp1, in the presence or absence of a large molar excess of unlabeled PDP–DNA (100-, 300-, 1000-fold) or unlabeled 22 mer ODNs (300-fold) and 16 mer ODNs (100-, 300-, 1000-fold), all containing the same Sp1 consensus sequence. The resulting complexes were analyzed by 8% gel electrophoresis and revealed by autoradiography. Sp1 bands were abolished by large molar excess of unlabeled PDP–DNA and ODNs. (B) Specificity of the binding was confirmed using Sp1 ODNs and mutated ODNs as unlabeled competitors (1000×). (C) EMSA was performed with ^{32}P-labeled PDP–DNA hybrid as probe, in the presence or absence of a large molar excess of unlabeled PDP–DNA (100- and 300-fold) or unlabeled 22 and 16 mer ODNs (300-fold), all containing the same Sp1 consensus sequence. Again unlabeled PDP–DNA efficiently competed with the homologous probe for the binding to Sp1.

to compete with radiolabeld probe for the binding to endogeneous Sp1 (Fig. 1B) indicating the specificity of the bands. When PDP–DNA was radiolabeled and employed as probe in the EMSA, it confirmed an efficient binding to endogeneous transcription factor (Fig. 1C). It is noteworthy that the resulting complexes had an electrophoretic pattern similar to that obtained with radiolabeled ODNs and were competed for by a large molar excess of unlabeled PDP–DNA hybrid (100- and 300-fold) or Sp1 ODNs (300-fold).

3.2. Sp1 activity is reduced by PDP–DNA in MDA-MB-231 cells

The ability of PDP–DNA molecule to reduce the extent of available Sp1 was then tested into living MDA-MB-231 cells by treatment with 0.5 μM Sp1 decoy hybrid for 18 h, in the presence or absence of a liposomal vector. Nuclear extracts from untreated and treated cells were then subjected to EMSA using both ^{32}P-labeled 22 mer Sp1 oligonucleotide (Fig. 2A and B) and ^{32}P-labeled PDP–DNA (Fig. 2C). The

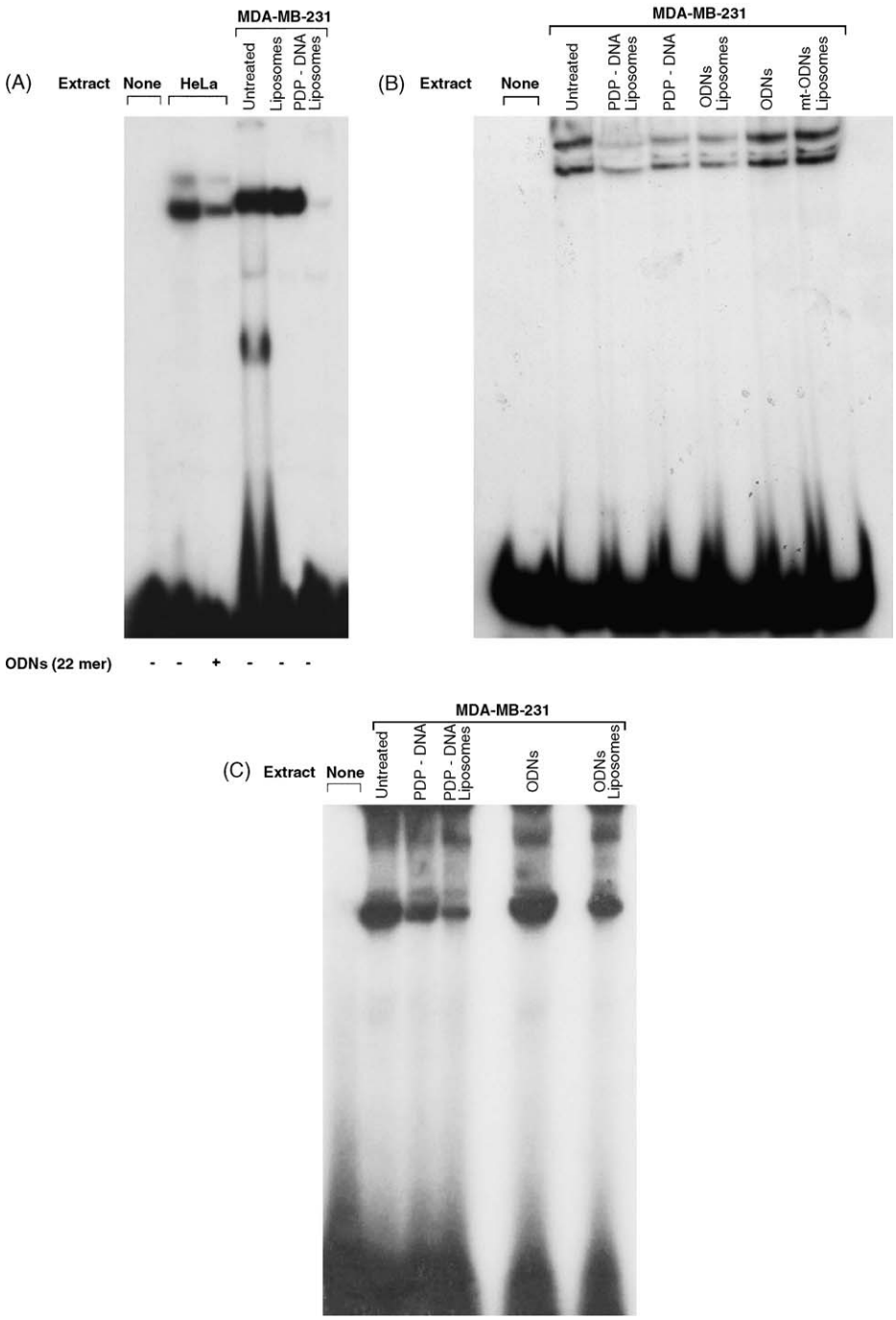

Fig. 2. Representative EMSA showing the binding of PDP–DNA hybrid to endogenous Sp1 in living MDA-MB-231 cells. (A) Breast cancer cells were incubated with PDP–DNA (0.5 μM) in the presence of liposomes (DOTAP) for 18 h at 37 °C. Nuclear proteins were then extracted and 10 μg samples were incubated with ^{32}P-labeled 22 mer oligonucleotide containing the consensus sequence for Sp1. The resulting complexes were analyzed by 8% gel electrophoresis and revealed by autoradiography. Untreated and mock-transfected cells with empty liposomes were employed as negative control whereas nuclear extracts from HeLa cells were used as positive control. Sp1 signal was strongly reduced by cell transfection with PDP–DNA. (B) MDA-MB-231 cells were incubated with PDP–DNA (0.5 μM), 16 mer Sp1 ODNs (0.5 μM) and mutated Sp1 ODNs (mt-ODNs, 0.5 μM) in the presence or absence of liposomes (DOTAP) for 18 h at 37 °C. EMSA was performed as described using ^{32}P-labeled 22 mer oligonucleotide as probe. Note the reduction of Sp1 signal after treatment with PDP–DNA hybrid alone and with Sp1 ODNs added to liposomes. No significant changes were observed in samples treated with Sp1 ODNs alone or mutated Sp1 ODNs. (C) EMSA was performed with ^{32}P-labeled PDP–DNA and nuclear extracts from breast cancer cells treated with PDP–DNA (0.5 μM) and 16 mer Sp1 ODNs (0.5 μM) added to liposomes or alone. Again the stronger decoy effect was obtained after cell transfection with PDP–DNA followed by cell transfection with Sp1 ODNs and treatment with PDP–DNA alone. No significant changes were obtained with treatment with ODNs alone.

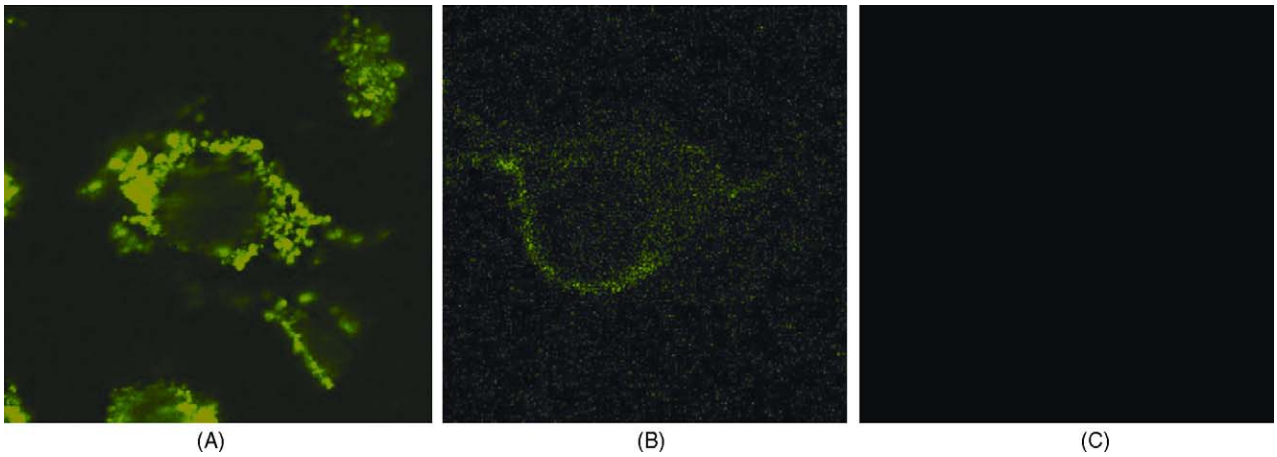

Fig. 3. Intracellular localization of FITC-labeled PDP–DNA by fluorescence confocal microscopy. MDA-MB-231 cells were incubated with FITC-labeled PDP–DNA (0.5 μM) for 18 h at 37 °C in the presence (A) or absence (B) of liposomes (DOTAP) and then examined by fluorescence microscopy. (A) PDP–DNA is efficiently transfected into cells by liposomes. (B) A detectable although weak signal could be observed also in breast cancer cells treated with PDP–DNA alone. (C) Mock transfected cells with empty liposomes were used as negative control.

extent of available Sp1 was dramatically reduced by cell transfection of PDP–DNA with liposomes independently of the probe used (Fig. 2A and C). A reduction of available Sp1, although to a lesser extent, was also observed after treatment of cells with PDP–DNA hybrid alone and with Sp1 ODNs added to liposomes (Fig. 2B and C). No significant changes of Sp1 signal could be observed in cells exposed to Sp1 ODNs alone (Fig. 2B and C) or to mutated Sp1 ODNs and liposomes (Fig. 2B). The average estimated reduction of Sp1 signal was 72 ± 14% after treatment with PDP–DNA and liposomes. Interestingly, in the absence of liposomes, PDP–DNA still caused a consistent decrease of Sp1 activity (42 ± 8%). When cells were treated with ODNs added to liposomes, ODNs alone and mutated ODNs, reduction of Sp1 signal was 46 ± 5, 7 ± 2, and 0.5 ± 0.5% as compared to untreated cells, respectively. These findings indicate that after transfection PDP–DNA is able to reach the intracellular target Sp1 and to function as a decoy molecule strongly inhibiting its binding to labeled probe in the EMSA. Moreover, a significant amount of PDP–DNA can enter cells without the use of liposomal vectors showing approximately the same decoy activity of Sp1 ODNs delivered via liposomes.

The intracellular localization of PDP–DNA in MDA-MB-231 cells exposed to FITC-labeled PDP–DNA and liposomes for 18 h was confirmed by confocal microscopy. In Fig. 3, a representative image shows that PDP–DNA was efficiently transfected into cells by the liposomal vector. Although weak, a detectable signal could be observed also in cells treated with PDP–DNA in the absence of transfection reagents.

3.3. Inhibition of uPA-induced up-regulation of Sp1-binding activity and uPAR levels by PDP–DNA

To test the ability of PDP–DNA to inhibit the uPA-induced up-regulation of Sp1-binding activity and uPAR levels, MDA-MB-231 cells were incubated with 0.5 μM Sp1 decoy hybrid loaded on liposomes or alone for 18 h, and then treated with uPA (10 nM) or ATF (10 nM) for 30 min. Nuclear proteins were then extracted and subjected to EMSA (Fig. 4). In agreement with our previous findings [16], cell treatment with ATF strongly enhanced Sp1-binding activity, which was prevented by transfection of MDA-MB-231 cells with PDP–DNA. A detectable, although limited, reduction of Sp1 activity was also observed in response to treatment with PDP–DNA alone in uPA-stimulated cells. Morphodensitometric analysis showed a 77 ± 15 and 28 ± 13% reduction of Sp1 complexes when uPA-stimulated cells were pre-treated with PDP–DNA in the presence or absence of liposomes, respectively. To investigate whether the reduced Sp1 activity may result in a reduction of uPAR expression, MDA-MB-231 cells were transfected with PDP–DNA hybrid and exposed to uPA for 6 h. Whole cell lysates were then obtained and analyzed for their uPAR content by Western blotting (Fig. 5). The uPA-induced up-regulation of uPAR expression was inhibited by transfection of cells with the PDP–DNA decoy hybrid. Quantitation of α-tubulin ensured equal loading.

3.4. Inhibition of tumor cell migration activity by PDP–DNA

The finding that the novel PDP–DNA molecule effectively reduces Sp1 activity and uPAR levels raises the possibility that also cell migration may be impaired. To test this possibility, MDA-MB-231 cells were incubated with 0.5 μM Sp1 decoy hybrid loaded on liposomes or alone, and then subjected to a migration assay using vitronectin-coated filters and serum-supplemented medium (10%) (Fig. 6A) or uPA (10 nM) (Fig. 6B) as chemoactractant. In the presence of serum, a 69 ± 4% reduction of cell migration was observed in breast cancer

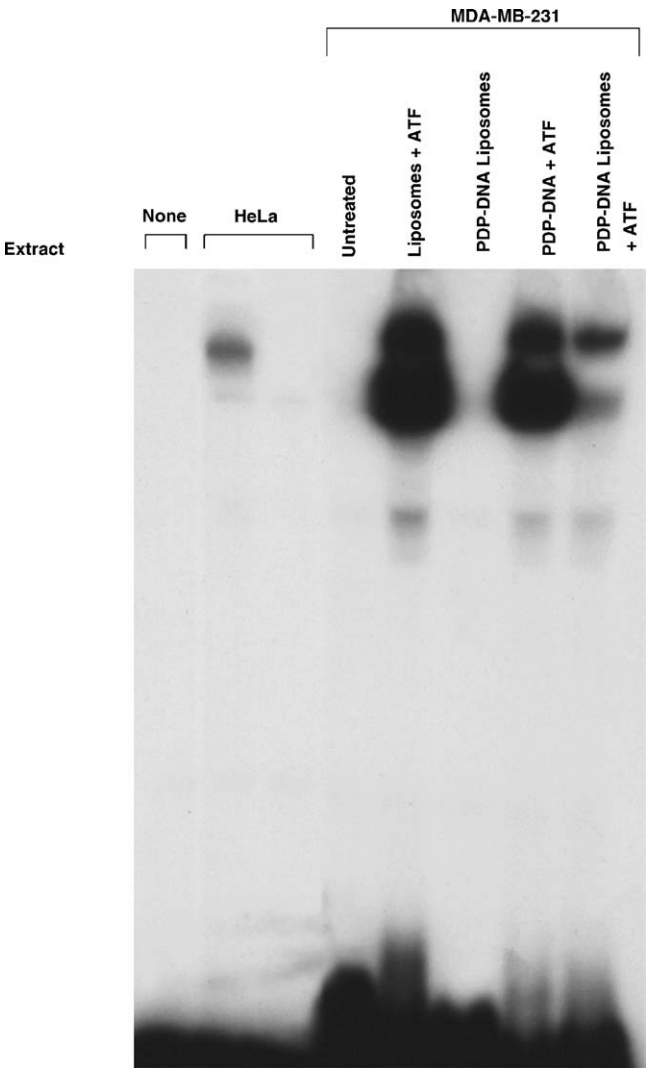

Fig. 4. Inhibition of uPA-induced Sp1-binding activity by PDP–DNA. MDA-MB-231 cells were incubated with PDP–DNA (0.5 μM) for 18 h at 37 °C added to liposomes (DOTAP) or alone, and then treated with 10 nM amino-terminal fragment of uPA (ATF) for 30 min. Nuclear proteins were extracted and subjected to EMSA with ^{32}P-labeled 22 mer oligonucleotide containing the consensus sequence for Sp1. Treatment with uPA strongly enhanced Sp1-binding activity in MDA-MB-231 cells. A dramatic reduction of the uPA-dependent enhancement of Sp1-binding activity is observed after cell transfection with PDP–DNA. A detectable, although limited, reduction of Sp1 signal was also observed in response to treatment with PDP–DNA alone in uPA-stimulated cells.

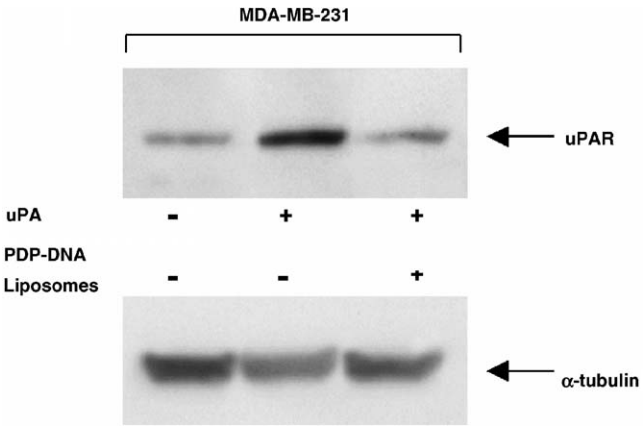

Fig. 5. Down-regulation of uPA-induced uPAR overexpression. MDA-MB-231 cells were transfected with PDP–DNA and DOTAP as described in materials and methods and then incubated with uPA (10 nM) for 6 h at 37 °C. Western blot analysis was performed on equal amount of proteins (40 μg) from whole cell lysates. uPAR was detected using polyclonal anti-human uPAR IgG 399 and equal loading was confirmed by anti-α-tubulin. The uPA-dependent enhancement of uPAR expression was blocked by cell transfection with PDP–DNA.

cells transfected with the PDP–DNA decoy hybrid (Fig. 6A). A considerable reduction (48 ± 3%) of migrating cells was also observed following exposure to the PDP–DNA hybrid alone confirming that the amount of hybrid molecule entering the cells in the absence of carrier, is sufficient to inhibit cell migration to an extent equivalent to that observed for ODNs loaded on liposomes (45 ± 1%). No significant changes in cell migration were observed after treatment of cells with Sp1 ODNs alone (13 ± 5%) or mutated Sp1 ODNs (0 ± 2%) loaded on liposomes. Similar effects were observed when cells were allowed to migrate toward uPA (Fig. 6B) after treatment with PDP–DNA added to liposomes or alone (57 ± 10% and 30 ± 7% reduction, respectively).

4. Discussion

The present study provides evidence that cell transfection with a decoy PNA–DNA chimera containing Sp1-binding motif down-regulates both Sp1 activity and uPAR levels in breast cancer cells. Accordingly, cell migration is strongly impaired in these cells. Interestingly, the PNA–DNA chimera can efficiently enter cells in the absence of transfection reagents producing a detectable inhibitory effect.

Recently, many efforts have been focused on the inhibition of transcription factor activity as a potential innovative tool for effective anti-cancer therapy [23,24]. In particular, CRE-decoy ODNs have been reported to alter the expression of clusters of different genes and to inhibit growth in breast cancer cell lines [25]. Synthetic ODNs bearing the consensus sequence for NF-kB have been successfully employed to down-regulate the expression of a number of genes including IL-6 and adhesion molecule-1 [26] as well as to induce apoptosis in human osteoclasts [27]. Similarly, a decoy strategy has been applied to the estrogen receptor by disrupting its binding to the cognate estrogen responsive element (ERE) [28]. A decoy molecule based on a PNA–DNA chimera mimicking Sp1-binding sites of HIV-1 has been extensively tested in nuclear extracts of human erythroleukemia K562 cells [29]. In agreement with these observations, here we show that the decoy molecule based on a PNA–DNA chimera has a strong in vivo inhibitory effect on Sp1 activity and uPAR expression and provides considerable advantages over decoy ODNs.

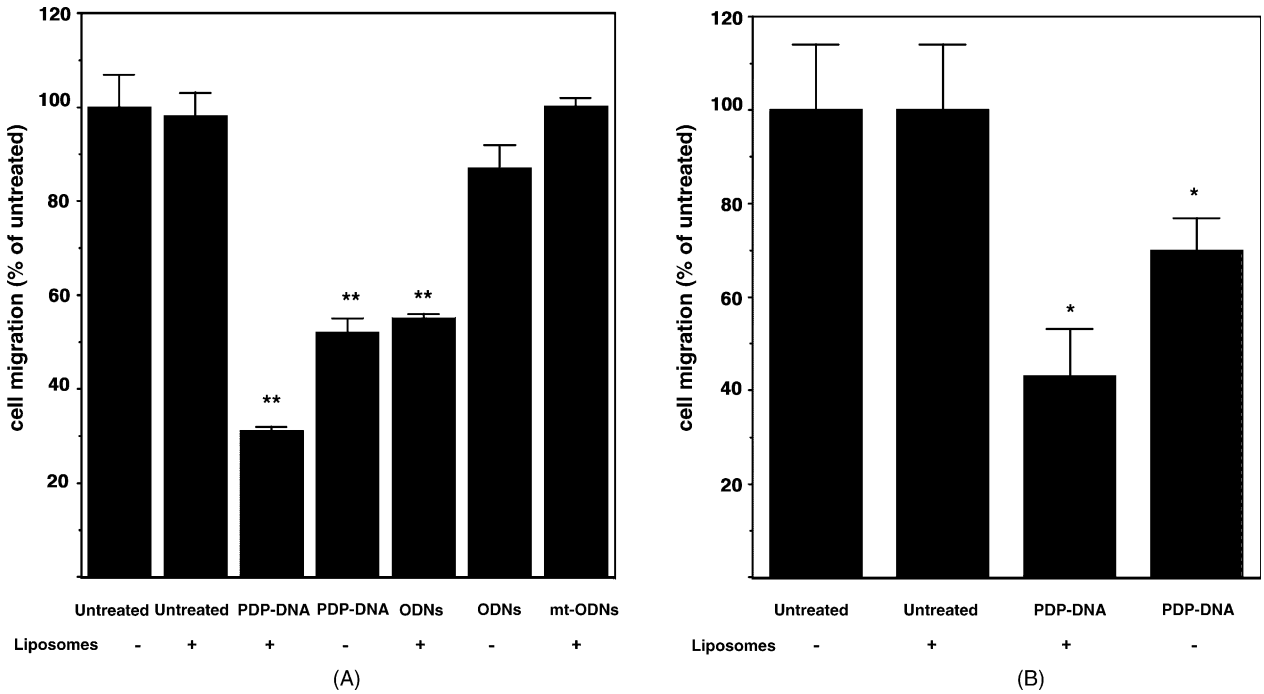

Fig. 6. Effect of PDP–DNA on migration activity of MDA-MB-231 cells. Breast cancer cells were incubated with PDP–DNA (0.5 μM), with 16 mer Sp1 ODNs (0.5 μM) and with mutated Sp1 ODNs (mt-ODNs, 0.5 μM) in the presence or absence of liposomal vector (DOTAP). Cell migration assay was performed using Boyden procedure in a 48-microwell-chemotaxis chamber with vitronectin-coated filters. Both serum-supplemented medium (10%) and uPA (10 nM) were used as chemoactractans. Cells were allowed to migrate for 6 h at 37 °C, and then counted on the lower surface of the filter in 10 random fields. Cells not subjected to any treatment or transfection was used as control. The results are expressed as the percentage of migrating cells considering the untreated control sample as 100%. Each experimental procedure was performed in triplicate. Statistical significance: $^{**}p < 0.001$; $^{*}p < 0.05$. (A) When serum-supplemented medium (10%) was used as chemoactractant, the stronger inhibitory effect on cell migration was obtained after cell transfection with PDP–DNA followed by cell transfection with ODNs and treatment with PDP–DNA alone. No significant changes of cell migration occurred after treatment with ODNs alone and mt-ODNs added to liposomes. (B) Similar results were obtained using uPA as chemoactractant.

The resistance of PNA–DNA chimeras to enzymatic degradation in cellular extracts and serum reported by several authors [20,30] may account for the enhanced inhibitory effect of the hybrid molecules compared to ODNs. It is reported indeed that PNA–DNA chimeras with only one PNA unit at the 5′- and 3′-ends are 25 times more stable in human serum than the corresponding unmodified ODNs [31]. Another favorable property of PNA–DNA chimeras shown in this study is the ability to produce a detectable functional effect at relatively low concentration (0.5 μM) even in the absence of liposomal complexation. The improved aqueous solubility and cellular uptake of chimeras compared to pure PNA [31] may account for such favorable property.

Despite the fact that both ODNs and PDP–DNA hybrids exert their decoy effect in a sequence specific manner as shown by comparison with mutated ODNs, selective localization to target cells remains a major limitation for therapeutic application of transcription factor decoy strategies. To overcome such limitation, ex vivo gene therapy of human vascular bypass grafts with E2F decoy has been performed in patients enrolled in Phase I/II clinical trials [32]. Also, intratumoral or local administration of decoy Sp1 ODNs has been used to down-regulate TNF-α, VEGF, and angiotensin type 1 receptor expression in animal models [33,34]. Interestingly, intravenous injection of NF-kB decoy ODNs reduced liver metastasis of reticulosarcoma cells in mice [35]. Further studies are needed to test whether the higher stability of PNA–DNA chimeras in biological fluids may allow their direct use against tumor or vascular cellular targets and whether their selective localization may be improved by conjugation with appropriately designed homing molecules.

In the last decades many efforts have been focused on the development of selective protease inhibitors including matrix metalloproteinase and uPA inhibitors. However, despite the extensive rationale for their therapeutic application, an effective in vivo inhibition of matrix degradation was rather difficult to be accomplished in pre-clinical and clinical settings [36,37]. The decoy strategy used in this study is able to interfere with the signal transduction pathway triggered by uPA and converging on Sp1, thus reducing the migration activity of breast cancer cells. Many other signal transduction pathways converge on Sp1 that in turn regulate the expression of a variety of genes involved in different cellular processes including proliferation and angiogenesis [10]. An extensive analysis of gene expression by microarray technology would identify the clusters of genes that are down-regulated or eventually up-regulated by Sp1 decoy strategy, thus

providing an insight of the potential synergies or side effects of this therapeutic approach. Further studies are also needed to test whether indirect mechanisms of down-regulation occur upon transfection of PDP–DNA. The simultaneous inhibition of different cellular processes involved in the formation of metastases may provide indeed considerable advantages over therapeutic strategies directed against single cellular function by maintaining metastatic cells in an inactive non-invasive sub-clinical state.

Acknowledgments

This work was partly supported by CNR Agenzia 2000, FIRB-MIUR, EU grants European Molecular Imaging Laboratories Network (EMIL) contract no. 503569 and Integrated Project LSHC-CT-2003-503297.

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