Small interfering RNA urokinase silencing inhibits invasion and migration of human hepatocellular carcinoma cells

Alessandro Salvi, Bruna Arici, Giuseppina De Petro, and Sergio Barlati
Division of Biology and Genetics, Department of Biomedical Sciences and Biotechnology, IDET Centre of Excellence, University of Brescia, Brescia, Italy

Abstract
The serine protease urokinase-type plasminogen activator (u-PA) is involved in a variety of physiologic and pathologic processes; in particular, u-PA mRNA is upregulated in human hepatocellular carcinoma (HCC) biopsies and its level of expression is inversely correlated with patients' survival. To determine the role of u-PA in the invasiveness properties of HCC, we successfully downregulated u-PA by RNA interference (RNAi) technology, in an HCC-derived cell line at high level of u-PA expression. RNAi is a multistep process involving generation of small interfering RNAs (siRNA) that cause specific inhibition of the target gene. SKHep1C3 cells were transfected with a U6 promoter plasmid coding for an RNA composed of two identical 19-nucleotide sequence motifs in an inverted orientation, separated by a 9-bp spacer to form a hairpin dsRNA capable of mediating target u-PA inhibition. Stable transfectant cells showed a consistently decreased level of u-PA protein. In biological assays, siRNA u-PA–transfected cells showed a reduction of migration, invasion, and proliferation. In conclusion, u-PA downregulation by RNAi technology decreases the invasive capability of HCC cells, demonstrating that stable expression of siRNA u-PA could potentially be an experimental approach for HCC gene therapy. [Mol Cancer Ther 2004; 3(6):671–8]

Introduction
The urokinase-type plasminogen activator (u-PA) is a multifunctional serine protease that plays important roles in various physiologic and pathologic conditions. In recent decades, it has become clear that the plasminogen activation system, which mainly consists of u-PA, u-PA receptor (u-PAR), and plasminogen activator inhibitor 1 (PAI-1), plays a very important role in the aggressiveness of cancer (1-3). Such enzymatic system governs the proteolytical degradation of extracellular matrix and basement membrane, allowing cells to move across and through these barriers in the processes of tumor invasion and metastasis (4-7).

The most relevant proteolytical activity of u-PA regards plasminogen, which is converted to the active enzyme, plasmin, which is capable of degrading a broad spectrum of extracellular proteins both by directly degrading extracellular matrix components and by activating collagenases and metalloproteases (8, 9). Also, it is well known that a signal is initiated by the binding of u-PA to u-PAR and transduced to the intracellular compartment by a mechanism not involving plasmin generation. In fact, u-PA/u-PAR system is involved in activating cell signaling pathways, including diacylglycerol formation, activation of a serine kinase, focal adhesion kinase, tyrosine kinase(s), and the activation of transcription pathways (4, 10). u-PA also exerts proliferative actions (11-14) independently of its proteolytic activity. Both the inactivated form of u-PA and the amino-terminal fragment of u-PA molecule (which has no proteolytic activity) are capable of inducing a mitogenic response (15). Furthermore, the u-PA/u-PAR complex has also been shown to be involved in regulating cell migration and adhesion through its interaction with integrins and with vitronectin, an extracellular matrix component (4, 16-18).

Hepatocellular carcinoma (HCC) is an aggressive tumor with a poor prognosis. Many studies of the molecular biology of this tumor have revealed that quantitative and qualitative changes in gene expression occur during its evolution. We have previously shown that u-PA is upregulated in human tumoral liver tissue and its level of expression is inversely correlated with patients’ survival; furthermore, high levels of u-PA mRNA can be considered an unfavorable prognostic marker for HCC patients (19).

In a previous work, we down-regulated u-PA in SKHep1C3 cells, an HCC-derived cell line, by stable expression of antisense RNA technology (20). To avoid possible aspecific responses by the cells resulting in cytotoxicity and apoptosis following transfection or intracellular production of RNA duplex longer than about 30 nucleotides (nt; refs. 21-23), we successfully used a recent technological breakthrough that allows delivery of short dsRNA, small interfering RNA (siRNA), by a plasmid into eukaryotic cells to down-modulate u-PA expression (24-30).

In this study, we transfected SKHep1C3 cells with a plasmid in which we cloned DNA fragments that acted as templates for the synthesis of siRNAs under the control of the U6 promoter. The resulting RNA was composed of two
identical 19-nt sequence motifs in an inverted orientation, separated by a 9-bp spacer to form a hairpin dsRNA capable of mediating target u-PA inhibition via posttranscriptional gene silencing. The results obtained confirm and demonstrate that the proliferative, invasive, and migrative capabilities of HCC cells can be reduced by u-PA target siRNA. Blocking of u-PA expression by siRNA could be a direct approach for reducing both the proteolytical and the signal transduction activity of the enzyme.

Materials and Methods

Cell Lines

SKHep1Clone3 (SKHep1C3; ref. 31), selected from human HCC-derived cells (SKHep1: ATCC HTB32) and SKHep1C3–transfected cells, were maintained in Earle’s MEM supplemented with 10% fetal calf serum (Life Technologies, Inc., Gaithersburg, MD) at 37°C in a 5% CO₂ incubator. AB5 human dermal fibroblasts (12) were also cultured in the same conditions.

Design and Preparation of Constructs

For design siRNA targeting u-PA, a DNA sequence of the type AA(N 19) was selected. This sequence (AACATTCACTGGTGCAACTGC) corresponded to nucleotides 208 to 228 of the human u-PA mRNA (National Center for Biotechnology Information access number: A35395). Synthetic sense and antisense oligonucleotides (Sigma-Genosys, The Woodlands, TX) constitute the template for generating RNA composed of two identical 19-nt sequence motifs in an inverted orientation, separated by a 9-bp spacer to form a double strand hairpin of siRNA. Two micrograms of each oligonucleotide were annealed for 3 minutes at 90°C and for 1 hour at 37°C and then ligated into 2μg of pSILENCER 1.0-U6 plasmid (containing ampicillin resistance gene and the mouse U6 RNA Polymerase III promoter; Ambion, Woodward, Austin, TX) linearized with ApaI and EcoRI (Fig. 1). The construct was cloned in TOP10 chemically competent Escherichia coli, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The sequence of the insert was confirmed by automated sequencing and by analyzing the fragments generated from digestion with HindIII.

Transfection and Selection

SKHep1C3 cells were grown to a confluency of 60% to 80% (in 6-cm–diameter Petri dishes) and then cotransfected (24) with 10μg of pSILENCER 1.0-U6 and 1μg of pEF6/V5-His TOPO vector (Invitrogen; the plasmid containing the blasticidine-selectable marker gene) using DOTAP Liposomal transfection reagent according to the manufacturer’s instructions (Invitrogen). Transfected cells were selected for blasticidine resistance (6μg/mL; pS: cells transfected with vector-alone; pS siRNA u-PA: cells transfected with siRNA u-PA construct).

DNA Extraction

Genomic DNA from parental and transfected cells was obtained with Purgene reagent (Genta system, Minneapolis, MN). To verify transfection, we amplified 50 ng of DNA from SKHep1C3, pS, and pS siRNA u-PA cells with a pair of pSILENCER 1.0-U6 specific primers, pSFOR (5’-GAGACTATAAATATCCCTTGGAG-3’) and pSREV (5’-CCGCTCTAGAACTAGTGGAT-3’), flanking the region of insert. The size of PCR products was 118 bp for mocked plasmid and 133 bp for plasmid with siRNA insert.

Reverse Transcription-PCR Analysis

Total RNA of transfected SKHep1C3 was extracted, quantified, and reverse transcribed as described (32). The following reverse transcription (RT)-PCR was done to determine the percentage of u-PA mRNA inhibition: 2μg of total RNA were reverse transcribed using u-PART (5’-ATGCTGCAGAATAAGTACATTC-3’) specific reverse primer in the 3’ UTR of u-PA mRNA, and the amplification reaction was done essentially as described previously (33). In parallel, the same amount from the same preparation of total RNA was retrotranscribed using random examers to verify the integrity of RNA and adequate cDNA synthesis using glyceraldehyde-3-phosphate dehydrogenase 1/2 (GAPDH1/2) specific primers. Quantification of the ribosomal protein L7, phosphoglycerate kinase 1 (PGK1), and β-actin expression was also done using specific primers (20).

Endogenous u-PA was detected by PCR analysis (conducted on cDNA specific for u-PA) with u-PAE/u-PAF; u-PAE: 5’-TCCTGACTCAACATGTACTGAC-3’ (nt 1583

![Figure 1. A, schematic presentation of U6 RNA Polymerase III promoter-based siRNA expression vector. Sequence encoding siRNA with 19 nt of homology to u-PA is inserted immediately downstream of U6 promoter. The six thymidines serve as a termination signal for Polymerase III. B, predicted second structure of the siRNA u-PA transcript from the expression vector.](image-url)
to 1605); u-PAF: 5'-ATACATTCTGGAAATATCGAGC-3' (nt 1846 to 1868). A comparative PCR method was used to determine the percentage of inhibition of u-PA, together with an image analysis system (IAS) able to scan the PCR amplified products directly from the images of the agarose gel bands, thus, obtaining a relative quantification of the u-PA products in comparison with the expression level of u-PA in SkHep1C3 untransfected cells. The relative values were expressed in pixels as integrated density (19).

Immunofluorescence

For the immunofluorescence detection of u-PA, u-PAR, and GAPDH, SkHep1C3 and SkHep1C3-transfected cells were seeded (40,000 cells/22 × 22 mm glass coverslips in 3-cm–diameter Petri dishes) and treated according to the standard protocol described previously (34). The cultures were fixed in cold methanol and then immunoreacted with the first antibodies, polyclonal antibodies anti-human u-PA (TechnoClone, Vienna, Austria), anti-GAPDH (Chemicon International, Temecula, CA), and anti-u-PA (mouse monoclonal anti-u-PAR antibodies were kindly provided by Prof. F. Blasi, DIBIT, Istituto Scientifico San Raffaele, Milan, Italy). The cells were then immunoreacted with the secondary antibodies fluorescein-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG (Calbiochem, San Diego, CA; ref. 33). The coverslips were mounted on glass slides in mounting medium and photographed with a Leitz fluorescence microscope (∗100 magnification). Ten randomly chosen fields were analyzed for each cell type with a specific analysis system (IAS) program to evaluate the integrated optical density level of each immunofluorescent cell. Analysis of variance was done considering SkHep1C3, pS and pS siRNA u-PA, u-PAR, and GAPDH integrated optical density levels. The data were considered to be significant when P ≤ 0.05.

Western Blot Analysis and Zymography

The conditioned media were prepared and analyzed by Western blotting, as described previously (34). The 24-hour serum-free conditioned media (2 mL/6-cm–diameter Petri dish) were collected from confluent cultures of parental, pS, and pS siRNA u-PA–transfected cells. Constant amounts of proteins (19.8 μg) from conditioned media of transfected and non-transfected cells were loaded in SDS-PAGE, under non-reducing conditions, on a polyacrylamide gel composed of three layers, containing different acrylamide concentrations at 4%, 6%, and 12%. Gel was blotted on nitrocellulose membranes and immunoreacted with rabbit anti-human u-PA antibodies (1:1,000 in 1% bovine serum albumin) or overlaid onto casein agar containing 2 μg/mL of human plasminogen (TechnoClone) to evaluate u-PA activity (20). Proteins (19.8 μg) from conditioned media of transfected and non-transfected cells were also loaded on an 8% polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and immunoblotted using goat polyclonal antibodies anti-PAI-1 (1:500 in 0.3% bovine serum albumin) and alkaline phosphatase-conjugated anti-goat IgG (1:7,500 v/v) to verify constant amount of loaded proteins.

The cell extracts were prepared as described (34, 35). Constant amounts of proteins (12.6 μg/well) were loaded on an 8% polyacrylamide gel. The separated proteins were electroblotted onto a nitrocellulose membrane, then immunoblotted using mouse monoclonal antibodies anti-GAPDH (1:300 in 1% bovine serum albumin) and alkaline phosphatase-conjugated anti-mouse IgG, rabbit polyclonal antibodies anti-protein kinase R (anti-PKR), anti-phospho-PKR (Thr 446/451), anti-elf2α (elf2α: α subunit of eukaryotic initiation factor 2), anti-phospho-elf2α (Ser51; 1:1,000 in 5% bovine serum albumin; Cell Signaling Technology, Beverly, MA), and alkaline phosphatase-conjugated anti-rabbit IgG.

The positive immunoreaction was detected with nitroblue tetrazolium and bromochloroindolyl phosphate (Promega, San Diego, CA). The bands were scanned by a digital system (IAS) and the values of integrated density were expressed in pixels (34). The amount of u-PA protein in SKHep1C3 untransfected cells was considered as 100%.

Invasion and Motility

Invasion and motility assays were done in a 24-well transwell chamber (Costar, Bodenheim, Germany). The 8-μm pore inserts were coated with 15 μg of Matrigel (Becton Dickinson Labware, Bedford, MA). Cells were added to coated filters (2 × 10⁵ cells/filter) in 100 μL of serum-free medium in triplicate wells. In the lower compartments of the chambers, a volume of 600 μL of A55 human fibroblast-serum-free-conditioned media was used as chemoattractant. After 24 hours at 37°C in a 5% CO₂ incubator, the Matrigel coating on the upper surface of the filter was wiped off using a cotton swab. Cells that migrated through the filters were fixed, stained with Hema-3, photographed, and counted.

The motility assay was conducted in a similar fashion but without coating with Matrigel. Cells (1 × 10⁵/filter) were loaded on transwell polycarbonate membrane inserts in triplicate wells. The plates were incubated for 24 hours at 37°C in a 5% CO₂ incubator, then the cells in the lower wells were fixed, stained with Hema-3, and counted. The cells that had migrated to the lower compartment of the chambers were trypsinized and counted. Each experiment was carried out in triplicate. The statistical significance of the results was calculated using the ANOVA procedure. The data were considered to be significant when P ≤ 0.05.

Proliferation Assay

SKHep1C3 and SkHep1C3-transfected cells were seeded (1.25 × 10⁴ cells/dish) in triplicate in 3-cm–diameter Petri dishes. After 24, 48, and 72 hours of incubation at 37°C, the cells were trypsinized and counted using a Burker chamber.

Results

Selection of siRNA Transfectants

SKHep1C3 cells were cotransfected with pSILENCER 1.0-U6 generating short hairpin siRNA and with pEF6/V5-His TOPO expression vector carrying selectable blasticidin
marker as described in the Materials and Methods section. Transfected cells were selected for blasticidine resistance and characterized by the presence of the insert in the genomic DNA (data not shown).

RT-PCR Analysis of u-PA Expression

Molecular characterization of parental (SKHep1C3) and transfected cells (pS and pS siRNA u-PA) was carried out by semiquantitative RT-PCR analysis of u-PA mRNA expression. Following standardization for the level of GAPDH, detected at comparable levels in all cell lines (Fig. 2B), u-PA mRNA was found to be markedly reduced in pS siRNA u-PA cells, whereas pS cells showed substantially the same levels of parental cell u-PA mRNA (Fig. 2A). Other mRNAs, such as β-actin, ribosomal protein L7, and phosphoglycerate kinase 1 (PGK1), were found to be comparable in all three cell lines (Fig. 2C-E).

Immunoblotting Analysis and Zymography

To determine whether the expression of siRNA u-PA affected u-PA protein production and secretion, constant protein amounts of conditioned media from parental, vector, and siRNA u-PA–transfected cells were used to perform Western blotting analysis with polyclonal anti-u-PA antibodies. A specific protein band ($M_r = 55,000$) was present in all the samples and was reduced by 95% in the pS siRNA u-PA conditioned media (Fig. 3A). Figure 3B shows that u-PA enzymatic activity was also found to be decreased in pS siRNA u-PA cells. To determine whether any changes in total amount of protein production and secretion had occurred, we tested constant amounts of proteins from conditioned media and from cellular extract of transfected and control cells by Western blotting analysis with polyclonal anti-PAI-1 (for conditioned media) and monoclonal anti-GAPDH (for cellular extract) antibodies. No differences in PAI-1 ($M_r = 43,000$) and GAPDH expression ($M_r = 36,000$) between parental and transfected cells were found (Fig. 3C and D, respectively).

To evaluate the possible activation of PKR and of α subunit of eukaryotic initiation factor 2 (eIF2α), the levels of phosphorylated and unphosphorylated forms of PKR and of eIF2α were examined in transfected and in non-transfected cells. Western blot analysis of PKR ($M_r = 74,000$) and eIF2α ($M_r = 40,000$) showed similar levels in non-transfected and transfected cells; the lack of phosphorylation of PKR together with the lack of phosphorylation of eIF2α excludes the activation of PKR cell signaling pathway in siRNA u-PA–transfected cells (Fig. 4).

Immunofluorescence Analysis of GAPDH, u-PA, and u-PAR Protein Expression

Immunofluorescence analysis with polyclonal anti-u-PA, monoclonal anti-u-PAR, and monoclonal anti-GAPDH antibodies showed that u-PA and u-PAR fluorescent protein signal of pS siRNA u-PA cells was reduced by 89% and 74%, respectively, compared with parental and vector-alone cell lines ($P < 0.01$) (Fig. 5). GAPDH immunofluorescent signal was similar in non-transfected and transfected cells (Fig. 5).

Effect of siRNA u-PA on Cell Invasion, Motility, and Proliferation

The in vitro invasion assay was designed to test whether transfection of siRNA u-PA altered the invasive potential of tumor cells in Matrigel-coated transwell chamber. Considering as 100% the number of SKHep1C3 cells able to invade to the filters in 24 hours, the percentage of vector-alone–transfected cells was 83% and that of siRNA u-PA–transfected cells 27% (Fig. 6A). The invasive potential of pS siRNA u-PA cells appeared significantly reduced ($P < 0.05$).
To study the effect of siRNA u-PA transfection on migration, parental and transfected cells were seeded on transwell chambers with uncoated filters. After 24 hours of incubation, the motility potential of pS siRNA u-PA cells was strongly inhibited (Fig. 6B; \( P < 0.05 \)).

To determine changes in the growth pattern of SKHep1C3 cells stably transfected with siRNA u-PA, a growth curve was done on them along with parental cells and the cells transfected with the empty vector. u-PA inhibition by siRNA u-PA transfection modulated the proliferation of hepatocellular carcinoma cells; in fact, the proliferation of pS siRNA u-PA cells was markedly decreased compared with parental and vector-alone–transfected cells (\( P < 0.05 \)) (Fig. 6C), thus, indicating u-PA involvement in the proliferative potential of hepatocarcinoma cells.

**Discussion**

The u-PA converts the proenzyme plasminogen to enzymatic active plasmin. It is controlled by PAIs, of which PAI-1 seems to be the main physiologic inhibitor. u-PA has its receptor in a membrane-anchored glycoprotein (u-PAR/CD87), which has been implicated in pericellular proteolysis during cell migration or tissue reorganization (4, 6). The u-PA/u-PAR may be a multifunctional system, not only promoting extracellular matrix proteolysis, but also involved in integrin-mediated cell adhesion and migration by interaction with vitronectin (16). Furthermore, u-PA/u-PAR system has been implicated in intracellular signaling, cellular differentiation, growth, and chemotaxis via a mechanism independent of u-PA enzymatic activity (19).

There is considerable evidence of an association between u-PA expression and the mitogenic activity of the cells (36, 37). In vitro studies have shown that u-PA acts as an autocrine mitogen in human melanoma cells (11). In addition, exogenous u-PA induces the proliferation and growth of cells of various origins (12, 13).

We have previously shown that u-PA gene transcription is up-regulated in human HCC tissues and that high levels of u-PA mRNA can be considered an unfavorable prognostic marker for HCC patients (19, 38). RNA duplexes of 19 to 23 nt (called siRNAs) have recently been shown to mediate the inhibition of gene expression: this mechanism is called RNA interference (RNAi; refs. 24, 29, 30). RNAi has rapidly become a functional genomics tool in a broad range of species; it has been used for transient or stable knock down of gene expression in cell lines and very recently in experimental animal models. Several genes have been successfully knocked down by RNAi in mammalian cells due to improved siRNA delivery systems, and attention is now turning to verification of the potential of RNAi as a tool for gene therapy (39, 40). RNAi has the greatest impact as an experimental therapeutic approach in two clinical areas: cancer and infective diseases.

**Figure 4.** Detection of PKR, phospho-PKR, eIF2\(\alpha\), and phospho-eIF2\(\alpha\) in cellular extract of SKHep1C3, pS, and pS siRNA u-PA cells, verified by immunoblotting.
In this work, we down-modulated u-PA via siRNA stable expression strategy in SKHep1C3 hepatocarcinoma-derived cell line, characterized by high levels of u-PA mRNA expression. This approach may provide important evidence of the role of u-PA in specific malignant properties of HCC-derived cells. The production and the secretion of u-PA were greatly reduced in siRNA u-PA–transfected cells compared with control cells. Significant inhibition of u-PA protein production was found despite reduced inhibition of mRNA u-PA expression.

In our system, the lack of phosphorylation of PKR and the lack of phosphorylation of eIF2α in siRNA u-PA–transfected cells, verified by immunoblotting analysis, excludes activation of the IFN pathway. Reduction of u-PA protein was not due to a non-specific inhibition of translation via the PKR pathway. At the mRNA level, RT-PCR expression of four housekeeping genes has been found to be constant in transfected and in non-transfected cells. All these data indicate that the decrease of target u-PA seems to be specific. The mechanism of action of siRNA in mammalian cells has as yet been poorly investigated. Some authors suggest that siRNAs might work as microRNAs (41), which knock down gene expression not only via mRNA degradation but also/or via translation arrest. This is probably due to imperfect pairing between...
antisense strand of microRNA/siRNA and mRNA that does not allow mRNA cleavage; the result is a block in the translation process (41). Additional mechanisms such as changes in DNA methylation or chromatin structure may also be involved (42). For all these reasons, RNAi studies are now proceeding in two directions: to understand the mechanism of action of siRNA in mammalian cells and to test more therapeutic applications.

In our system, the amount of u-PAR protein, detected by immunofluorescence analysis, was also shown to be lower in siRNA u-PA–transfected cells. Our results are in line with what is known about the ability of u-PA to up-regulate the expression of its cellular receptor at a transcriptional level, by increasing the activity of the transcriptional factor Sp1, and also at a posttranscriptional level, by promoting u-PAR mRNA stability (43, 44). There is considerable evidence that the u-PA/u-PAR system plays a role in tumor cell proliferation (45, 46). In our study, we observed that siRNA u-PA–transfected cells were characterized by reduced proliferation.

In summary, we have shown that inhibition of endogenous u-PA using siRNA technology reduced the motility, invasion, and proliferation of HCC cells. Many authors have shown that the u-PA system is a therapeutic target for a variety of human tumors, not only for HCC (1, 47), and our results obtained using RNAi technology provide further support. Some authors have used RNAi in liver disease, but as yet only one group has directly transfected siRNA oligos in HCC-derived cells targeting cyclin E, which controls the progression from G1 to S phase and when overexpressed is involved in carcinogenesis of HCC (48). Other authors have investigated the effect of RNAi activity on the replication of HCV (49) and HBV (50) viruses, which are responsible for hepatitis C and B, respectively. The results showed a sharp reduction in the levels of replicative intermediates and viral protein. To our knowledge, this is the first report of inhibition of u-PA in HCC tumorigenic cells using RNAi technology. Although we obtained comparable u-PA inhibition with plasmid expressing antisense u-PA mRNA, the advantages provided by siRNA technology should be relevant for in vivo studies avoiding aspecific defense responses against duplex RNA longer than 30 nt (21-23).

In conclusion, our findings confirm that down-modulation of u-PA may be an effective way of achieving a significant reduction in the malignant properties of HCC-derived cells and the results obtained from siRNA u-PA technologies may be of help in designing and testing in vivo anti-invasive therapeutic strategies.

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References


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