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# Inhibition of urokinase synthesis and cell surface binding alters the motile behavior of embryonic endocardial-derived mesenchymal cells in vitro

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## SUMMARY

**The expression of the serine protease urokinase is elevated during the epithelial-mesenchymal transformation of the endocardium in the developing avian heart. Elevated urokinase expression is associated with the migrating mesenchymal cells of the atrioventricular canal and bulbotruncus and not the myocardium. Treatment of isolated endocardial-derived mesenchymal cells with phosphatidyinositol-specific phospholipase C released urokinase and its receptor from the cell surface and caused significant alterations in cell morphology and motility. Likewise inhibition of urokinase synthesis by treatment of cells with antisense oligonucleotides also inhibited the migration and motility of the endocardial-derived cells. These results suggest an important role for this enzyme in cell-matrix interactions and cell migration during development.**

Key words: urokinase, mesenchyme, migration, cell surface, endocardium, cell-matrix interaction, quail

## INTRODUCTION

The serine protease urokinase plays an important role in the remodeling of the extracellular matrix by converting the inactive zymogen plasminogen to the broad spectrum protease plasmin (Quigley, 1979; Liotta et al., 1981; Sheela and Barrett, 1982; Quigley et al., 1987; McGuire and Seeds, 1990; He et al., 1989). In addition, this protease may also be involved in regulating the complex interactions of cells with the extracelluar matrix in processes such as attachment, spreading and migration. Urokinase is localized to specific regions of the cell surface. This occurs by binding to a specific receptor, which recognizes a sequence of amino acids in the N-terminal growth factor domain of the enzyme (Appella, 1987). Previous studies have demonstrated that urokinase can be localized to the basal surface of cells in the area of focal contacts thus facilitating highly localized areas of proteolytic activity (Pölläen et al., 1987, 1988; Hebert and Baker, 1988).

The urokinase receptor, like a number of other cell-surface molecules, is attached to the membrane by a distinct class of glycophospholipids called a glycosyl-phosphatidylinositol anchor. These molecules include a number of hydrolytic enzymes, antigens and cell adhesion molecules (see Low and Saltiel, 1988 for review). Possible functions for this type of attachment include increased mobility of the specific molecule in the plane of the membrane and potentially a means by which a cell releases the molecule from its surface through the action of a phosphotidylinositol-specific phospholipase.

During the early stages of heart development the endocardial cells of the atrioventricular canal (AVC) and the bulbotruncus or outflow tract (OFT) undergo an epithelial-mesenchymal cell transformation and migrate into the surrounding extracellular matrix (Markwald et al., 1977). This migration of cells is an important step in the subsequent formation of valves and septation of the heart into individual chambers. We have previously reported on an increase in urokinase activity in the heart during this transformation process (McGuire and Orkin, 1992). Here we describe experiments that examine more closely in vitro the role of urokinase in the process of mesenchyme migration and cell-matrix interactions.

## MATERIALS AND METHODS

### Embryos and cell culture

Quail (*Coturnix coturnix japonica*) embryos were used for these studies. Fertilized eggs were incubated at 37.5°C and 60% relative humidity and staged according to Zacchei (1961).

Cushion endocardial and mesenchymal cells were obtained in culture from explants of AVC and OFT from stage 17-18 embryos using a modification of a procedure previously reported (Bernanke and Markwald, 1982). Heart segments were opened longitudinally and placed endocardial-side down on a hydrated collagen gel equilibrated with Medium 199 containing 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 20% fetal calf serum. After 16 hours, an outgrowth of endocardial cells was present and the explants were removed. The cells were passaged with 0.25% collagenase in HBSS. Many of the cells in both the primary and secondary cultures assumed a fibroblastic morphology typical of mesenchymal cells in culture.

For some experiments cells were grown in serum-free media consisting of M199 containing insulin, transferrin, selenium and BSA (ITS+, Collaborative Research).

To confirm the identity of the explanted cells, cultures were treated with the QH1 antibody, followed by an FITC-labeled second antibody. Greater than 95% of the cells stained positively with QH1. This antibody has previously been shown to recognize an epitope on the surface of quail endothelial/endocardial cells (Pardanaud et al., 1987).

### Treatment of explants

Explants of heart segments were placed onto equilibrated collagen gels (see above) and incubated overnight in media containing either 1 U/ml phosphatidylinositol-specific phospholipase C (PIPLC; ICN Biomedicals, Costa Mesa, CA) or 10 μM of the appropriate oligonucleotide.

### PIPLC treatment of isolated cells

In some cases, cells were passaged onto fibronectin-coated coverslips in the presence of 1 U/ml of PIPLC in serum-free media to release the urokinase receptor and its associated enzyme. Cells were treated at 37°C for 1-2 hours and photographed. To determine the amount of surface-associated urokinase remaining following PIPLC treatment, treated and untreated cells were extracted at 4°C with Triton X-114 (Calbiochem, LaJolla, CA). Extraction of cells with the non-ionic detergent Triton X-114 followed by heat-induced separation results in the production of two separate phases. Integral membrane proteins and molecules associated with these proteins (i.e. receptor-ligand complexes) partition with the non-ionic detergent phase while free hydrophilic proteins remain in the aqueous phase (i.e. cytoplasmic compartment and the extracellular substratum; Bordier, 1981; Pryde, 1986). Cell extracts were analyzed by zymography (see below).

### Antisense oligonucleotide treatment of isolated cells

Cells were treated with sense and antisense oligonucleotides. The oligonucleotides were designed to overlap the initiation codon of the urokinase mRNA (sense: 5′ ACC AAC ATG AAG TTA 3′; antisense: 5′ TAA CTT CAT GTT GGT 3′). The oligonucleotides were modified with phosphorothioate linkages rendering them nuclease resistant (Oligos etc., Guilford, CT). A search of the Gene Bank with these sequences confirmed their homology only to urokinase. To control for sequence-specific effects, two additional oligonucleotides were prepared. The FS-1 oligonucleotide (5′ TCA CTT CAT TTT AGT 3′) which differed from the anti-urokinase sequence in 3 out of 15 residues and the FS-2 oligonucleotide (5′ TAC CGT GAT GTG GCT 3′) differing in 5 out of 15 residues. Cells were incubated with 10 μM of the appropriate oligonucleotide for 16 hours in serum-free M199. The cells were subsequently passaged onto fibronectin-coated coverslips and incubated in serum-free media containing oligonucleotides. The morphology and behavior of cells was monitored for up to 2 hours. In some cases, the cells were collected and assayed for urokinase mRNA levels, urokinase activity and overall protein synthesis.

Total RNA was extracted from cells following sense or antisense oligonucleotide treatment and 30 minutes to 1 hour after passage. The relative amount of urokinase mRNA was determined by RNase protection analysis using 10-20 μg of total RNA and $2\times10^5$ counts/minute of labeled probe. $^{32}$P-labeled antisense RNA probes were synthesized from the linearized chicken urokinase cDNA plasmid pCU-1 (Leslie et al., 1990). Control samples containing the probe and tRNA were processed exactly as the samples and no protected bands were observed.

Some cells were extracted with Triton X-100 1-2 hours after passage. Protein concentration was determined by the Micro BCA assay (Pierce Chemical) and aliquots examined by zymography to confirm that antisense oligonucleotide treatment inhibited urokinase synthesis in these cells.

To determine whether the effect of antisense treatment is reversible, antisense-treated cells were passaged and cultured in the presence of endocardial-cell-conditioned media containing urokinase. Conditioned media was obtained form endocardial-derived cells grown in serum-free media for 16-24 hours. The presence of active urokinase in the conditioned media was confirmed by zymographic analysis. In some cases, the conditioned media was boiled for 10 minutes before use to inactivate the urokinase irreversibly.

In some experiments, cells were labeled with [$^{35}$S]methionine in order to quantitate levels of protein synthesis in response to treatment with oligonucleotides. Cells were labeled for 1-2 hours with [$^{35}$S]methionine (Amersham, 1000 Ci/mmol), rinsed and solubilized with PBS/Triton X-100. An aliquot of the extract was precipitated with TCA to determine the total incorporation of [$^{35}$S]methionine into protein. The total incorporation of [$^{35}$S]methionine into TCA-precipitable protein was unaffected by treatment with antisense oligonucleotide.

### Assay for urokinase activity

Aliquots of cell extracts were electrophoresed in 10% polyacrylamide minigels into which casein (1 mg/ml) and plasminogen (0.04 U/ml) had been crosslinked. Following electrophoresis, the gels were soaked in 2.5% Triton X-100, rinsed with water and incubated for 16 hours at 37°C in 100 mM Tris, pH 8.0. The zones of proteolysis corresponding to the presence of urokinase in the gel were visualized by staining the gel with 0.125% Coomassie. Controls included a comparison of identical samples electrophoresed into gels lacking plasminogen, which distinguishes between urokinase and other plasminogen-independent proteases. The relative degree of proteolysis in gels was estimated using a Zenih soft laser scanning densitometer.

### In situ hybridization

In situ hybridization was performed on frozen tissue sections of quail embryos at various stages of development with modifications of a procedure described by Simmons et al. (1989). $^{35}$S-labeled sense and antisense RNA probes were synthesized from the linearized chicken urokinase cDNA plasmid pCU-1 (Leslie et al., 1990). Sections were hybridized at 50°C for 16 hours, treated with RNase A and T1, and washed with solutions of SSC at increasingly higher stringency. Sections were coated with Kodak NTB2 nuclear track emulsion and exposed for 10-14 days.

### Quantitative analysis of cell shape and motility

Cells treated with either 1 U/ml of PIPLC, 10 μM antisense oligonucleotide or 10 M sense oligonucleotide were passaged onto fibronectin-coated coverglass chambers (Nunc, Inc.) and grown for up to 4 hours in serum-free M199 buffered with Hepes in order to maintain physiological pH. Analysis and quantitation of cell shape and motility were performed using the DynaCELL quantitative videomicroscopy system (Carl Zeiss, Inc. and JAW Associates, Baltimore). The cultures were mounted on a heated stage of a Zeiss Axiovert 35 microscope. DIC Nomarski images of the cells were enhanced and transmitted to a Compaq computer with a Hamamatsu C2400 video camera. Cell images were obtained and stored every 2 minutes for 1 hour, and computer-generated measures of cell shape and motility were obtained as described (Partin et al., 1989). Briefly, spatial and temporal Fast Fourier transforms were performed on digitized contours of individual cells yielding quantitative values expressed in arbitrary units. The values

include measurements of cell size and the frequency of activities such as pseudopod extension and membrane ruffling. These measurements were compared among individual cells and averaged for groups of cells.

## RESULTS

### Urokinase expression by migrating heart mesenchyme

Urokinase activity has been previously shown to be elevated in whole hearts of stage 18-19 embryos during the epithelial-to-mesenchymal transition of the endocardium (McGuire and Orkin, 1992). In stage 20-21 embryos, the urokinase message can be localized by in situ hybridization to the migrating mesenchymal cells which have invaded the cardiac extracellular matrix of the AVC and OFT (Fig. 1 A,B). The myocardial layer of this region is not expressing urokinase as shown by the lack of labeling. Sections incubated with the sense probes showed only background labeling (Fig. 1C,D). The mesenchymal cell population derived from the endocardium was obtained from explants of both the AVC and the OFT and was used for all subsequent experiments.

### Explant outgrowth of endocardial-derived mesenchyme

Initial studies to elucidate the role of urokinase in this system utilized inhibitors of enzyme activity. None of the inhibitors used (leupeptin, benzamidine or PAI-1) over a wide concentration range showed significant effects on cell behavior. At low concentrations, some of these inhibitors were toxic to the cells. In addition, the ability to obtaining a quantitative measurement of the decrease in urokinase activity in response to the application of these agents proved very difficult. For these reasons, we chose to utilize more specific and direct means of altering the activity of urokinase in this population of cells (release of urokinase from the cell surface by treatment with PIPLC and antisense inhibition of urokinase production).

Explants of the AVC and OFT yielded extensive outgrowths of cells after 16 hours in culture (Fig. 2A). Initially the majority of these cells migrate from the explant as a sheet of cells, which later gives rise to cells with more of a mesenchymal type of morphology that begin to invade the collagen gel. When explants are treated with 1 U/ml of PIPLC during the 16 hour incubation, the degree of outgrowth is dramatically reduced (Fig. 2B). Those cells that emerge from the explant were more fusiform in shape compared to the control explants. Likewise, treatment of

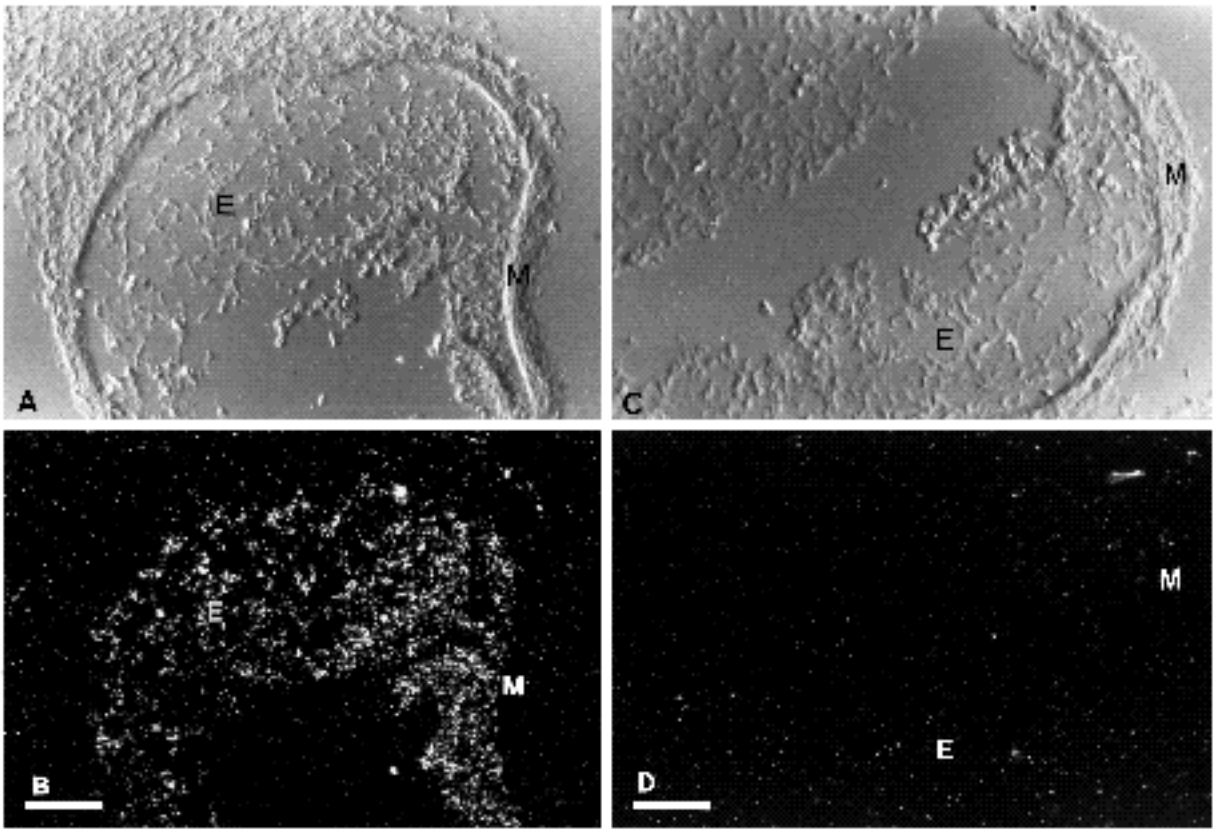


**Fig. 1.** Nomarski (A,C) and dark-field (B,D) images of in situ hybridization of urokinase riboprobe to the atrioventricular region of the stage 20-21 quail heart. In sections incubated with antisense riboprobes (A,B) the endocardial-derived mesenchyme (E), which has invaded the cardiac extracelluar matrix, is heavily labeled in comparison to the outer surrounding myocardial layer (M). Sections incubated with the sense riboprobe (C,D) showed only background labeling. Bar, 53.3 μm.

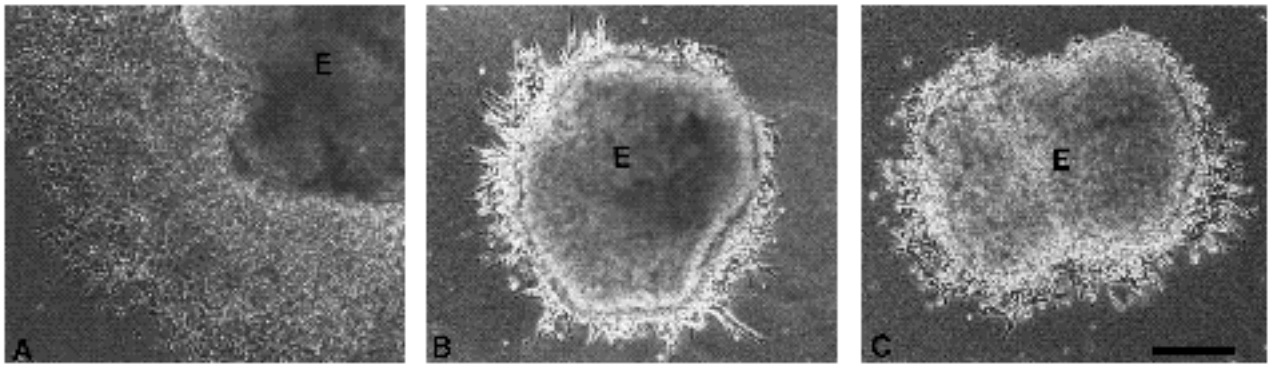


**Fig. 2.** Effects of PIPLC and antisense oligonucleotides on the outgrowth of endocardial cells from cushion tissue explants. Explants (E) of AVC and OFT were placed endocardial side down on hydrated collagen gels and cultured for 16-24 hours. Untreated explants gave rise to a significant outgrowth of cells after 16 hours (A). When explants were cultured in the presence of 1 U/ml of PIPLC (B) or 10 μM antisense oligonucleotide (C) the extent of cellular outgrowth was dramatically reduced. Bar, 182 μm.

explants with 10 μM of antisense oligonucleotide to urokinase resulted in a significant decrease in the overall outgrowth of cells (Fig. 2C). Use of sense oligonucleotides as well as base-modified antisense oligonucleotides (FS-1 and FS-2) had no effect on the ability of the explants to seed cells (not shown).

### Isolated endocardial-derived cells

Endocardial-derived mesenchymal cells obtained from explants of the AVC and OFT were passaged onto fibronectin-coated coverslips in the presence or absence of 1 U/ml of PIPLC. In the absence of this enzyme, the cells rapidly attached to this substratum and assumed a well spread and flattened morphology within 20-60 minutes (Fig. 3A). When the cells were plated in the presence of PIPLC, the cells attached but did not spread in the typical manner (Fig. 3B). Instead the cells showed many stellate or fusiform projections of the cell surface. Treatment of attached cells with PIPLC had no effect on their morphology over a 1-2 hour period (not shown).

Treatment of cells with PIPLC has been shown to cause the release of surface-associated urokinase and its receptor (Ploug et al., 1991). Endocardial-derived mesenchymal cells were examined by zymography following Triton X-114 extraction and phase separation to determine the amount of urokinase remaining on the cell surface following PIPLC treatment (Fig. 4). Densitometric analysis revealed up to 50% less surface-associated urokinase in PIPLC-treated cells compared to untreated cells.

A similar effect was seen when cells were treated with antisense oligonucleotides generated to the translation initiation region of the urokinase mRNA (see methods). Cells were treated with oligonucleotides for 16 hours, passed onto fibronectin-coated coverslips and grown for up to 4 hours in media containing the oligonucleotides. Thirty minutes after passage, the antisense-treated cells showed only minimal attachment and spreading compared to sense-treated cells on the fibronectin substratum (Fig. 5A,B). In an attempt to reverse this effect, antisense-treated cells were passaged and cultured in serum-free media which had been conditioned by endocardial-derived cells and shown

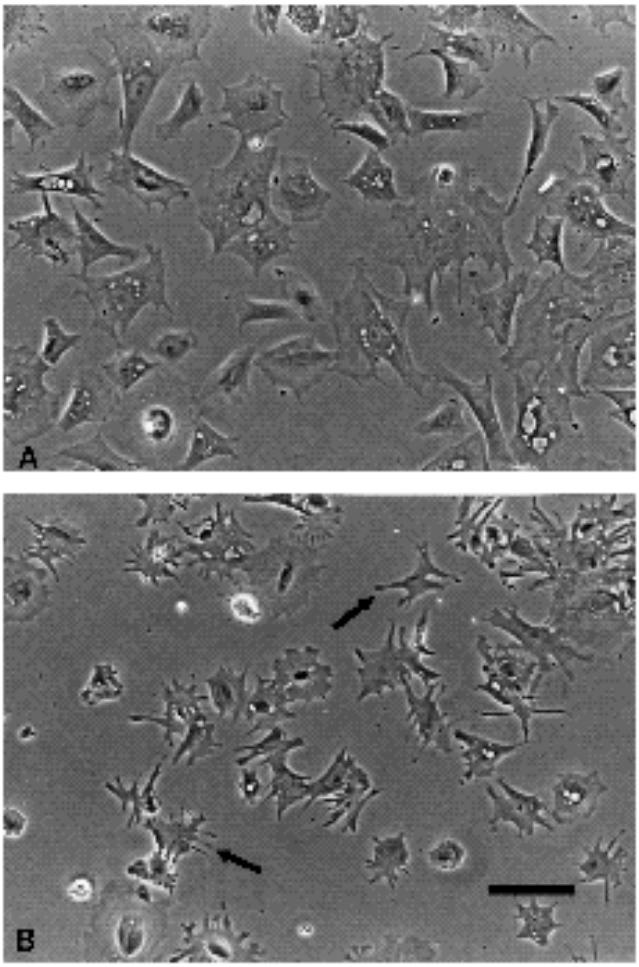


**Fig. 3.** Effect of PIPLC on endocardially derived cells in vitro. Cells obtained from AVC and OFT explants were passed onto fibronectin-coated coverslips in the absence (A) or presence (B) of 1 U/ml of PIPLC and cultured for 4 hours. Compared to the untreated cells, the cells grown in the presence of PIPLC extended many thin irregular processes and failed to spread completely. Photographs of live cells. Bar, 69 μm.

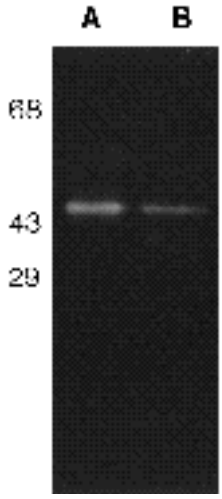


**Fig. 4.** Zymographic analysis of remaining cell surface urokinase following 1 hour of PIPLC treatment. Cells were passaged onto fibronectin and incubated in serum-free media containing 1 U/ml of PIPLC for 1 hour. The cells were rinsed and extracted with Triton X-114 as described in methods. Control cells were grown in serum-free media alone. The detergent phase of the extract containing the urokinase and its receptor was analyzed by zymography. Lane A, −PIPLC; lane B, +PIPLC. A 50% decrease in the surface-associated urokinase was seen in the PIPLC-treated cultures. Molecular weight markers are to the left.

to contain urokinase. After 30 minutes, a large number of the antisense-treated cells in the presence of urokinase-containing media had attached and spread significantly more than did antisense-treated cells in serum-free medium alone (compare Fig. 5C and B). Antisense-treated cells grown in boiled conditioned medium, which irreversibly destroys the urokinase activity, showed little attachment and spreading (compare Fig. 5D and C). Cells treated with the base-modified oligonucleotides FS-1 and FS-2, which would be expected to exhibit decreased binding to the urokinase mRNA, showed normal attachment and spreading (Fig. 6A and B). By four hours after passage, the antisense-treated cells that had attached exhibited a stellate and fusiform morphology but never completely spread as compared to cells treated with the sense oligonucleotide (Fig. 7). These results confirm the specificity of the

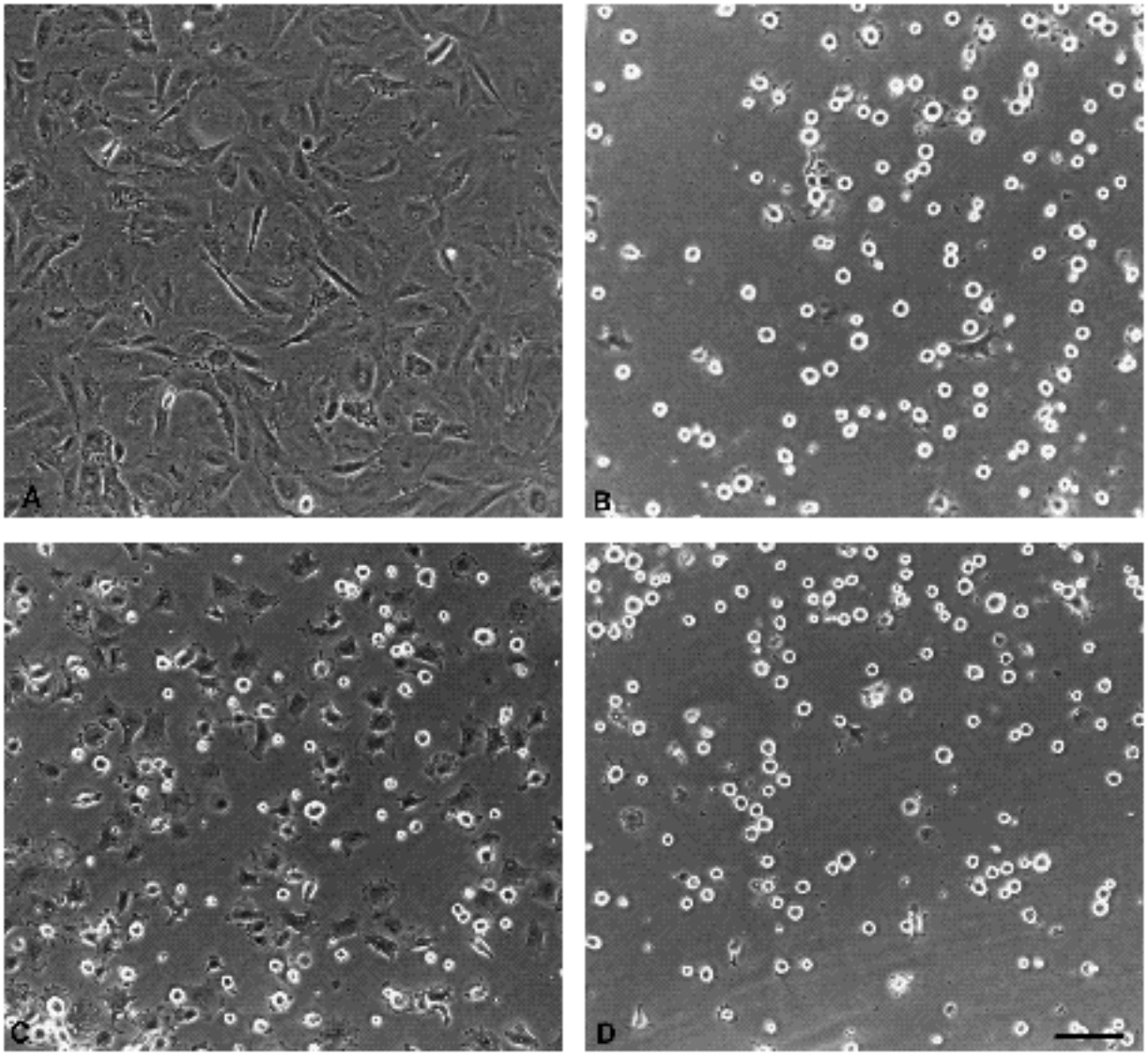


**Fig. 5.** Treatment of cells with 10 μM of sense oligonucleotide (control) did not affect their ability to attach and spread on fibronectin after 30 minutes (A). After overnight treatment with 10 μM antisense oligonucleotide and 30 minutes after passage, the majority of cells remained loosely attached and unspread (B). When antisense-treated cells were passaged and grown in the presence of conditioned media containing urokinase a large number of the cells were able to attach and spread within 30 minutes (C). When antisense-treated cells were grown in boiled conditioned media (denatured urokinase) most cells remained unspread at 30 minutes (D). Photographs of live cells. Bar, 105 μm.

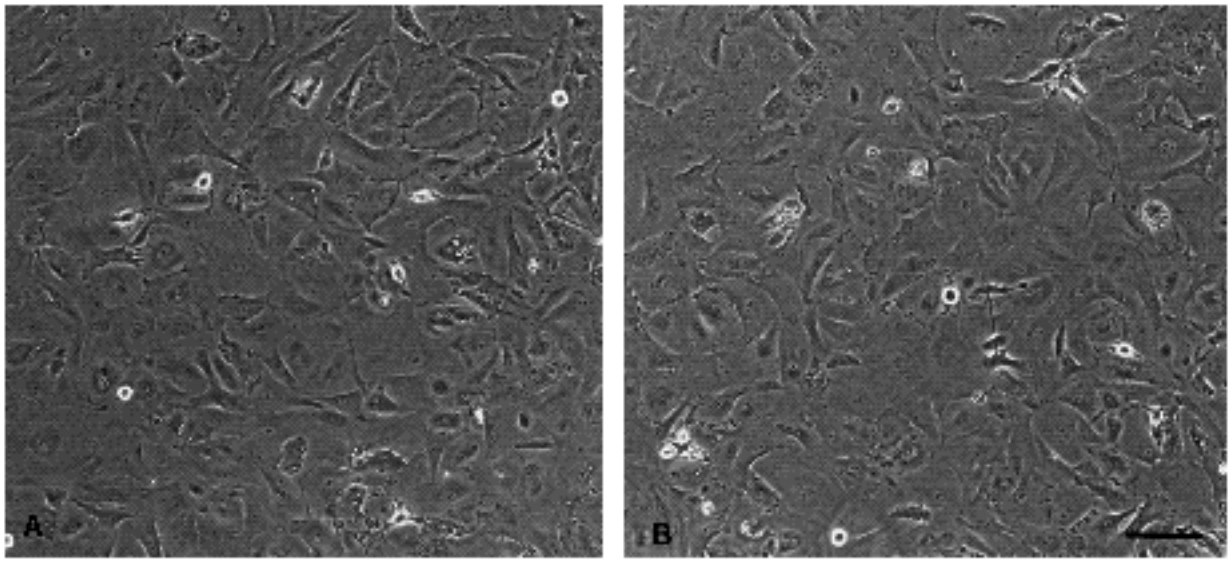


**Fig. 6.** Treatment of cells with the base-modified antisense oligonucleotides FS-1 and FS-2. Cells were incubated overnight with 10 μM of each oligonucleotide and 30 minutes after passage the cells were attached and spread on the fibronectin substrata. Bar, 105 μm.

antisense effects and further suggest an important role for surface-associated urokinase in regulating cell-substratum interactions.

The extent of inhibition of urokinase synthesis due to treatment of cells with the antisense oligonucleotides was examined by RNA analysis and zymography. Cells were treated for 16 hours with oligonucleotides and passed onto new substrata. Cells were extracted one hour after passage with Triton X-100 to obtain total cellular urokinase. Analysis of extracts by zymography revealed that treatment of cells with the antisense oligonucleotide caused a significant decrease in urokinase production (25.6% of control cells). In addition, the level of urokinase mRNA in sense and antisense-treated cells was determined by RNase protection assay. A 61.5% decrease in the steady-state level of urokinase mRNA was found in antisense-treated cells (Fig. 8). Cells treated with the base-modified antisense oligonucleotides had levels of urokinase activity comparable to sense-treated control cells based on zymographic analysis of the cell layer (FS-1, 95% of control; FS-2, 98% of control).

## Quantitative measurement of cell motility

Analyses of the extent of motility of representative cells in PIPLC and sense and antisense-treated cultures were performed on cells between 1 and 4 hours after plating using the DynaCell quantitative video microscopy system as described above. Spatial and temporal Fourier transforms were performed on digitized images of individual cells and values were derived which represent, in arbitrary units, the frequency of activities such as pseudopod extension, undulation and membrane ruffling. The measurements were compared among individual cells and averaged for groups of cells. Treatment of cells with either antisense oligonucleotides at 10 μM or PIPLC at 1 U/ml was shown to significantly inhibit the overall motility of cells (Fig. 9). This

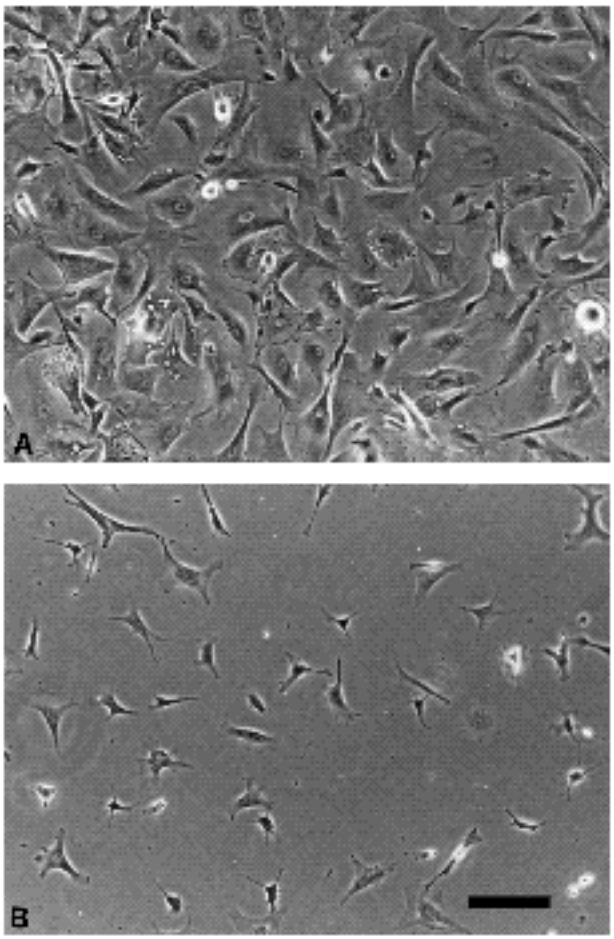


**Fig. 7.** Cells treated with antisense oligonucleotides (B) and examined 4 hours after passage continued to exhibit the fusiform and stellate morphology compared to the sense-treated cells (A). Photographs of fixed cells. Cells were plated at equal densities. Fewer cells are present in B as many of the antisense-treated cells were only loosely attached and washed off during the fixation steps. Bar, 125 μm.

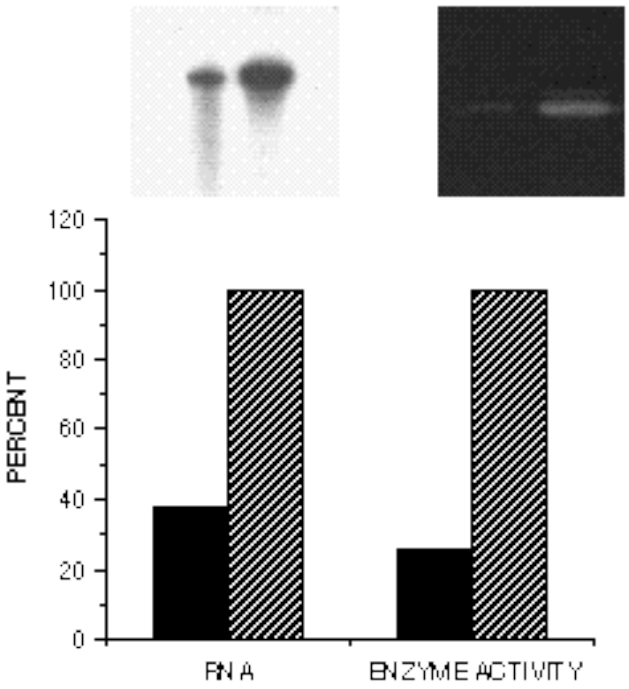


**Fig. 8.** Treatment of cells with antisense oligonucleotides inhibits the synthesis of urokinase. Cells were treated with sense and antisense oligonucleotides and analyzed by RNase protection assay and zymography. RNase protection assay of 10 μg of total RNA demonstrated a nearly 3-fold decrease in the level of urokinase mRNA in antisense-treated cells. Lane 1, antisense; lane 2, sense. Extraction of cells with Triton X-100 followed by zymography also showed a significant decrease in total urokinase in antisense-treated cells. Each sample, 2.12 μg total protein; lane 3, antisense; lane 4, sense. Values for antisense-treated cells are expressed as a percent of sense-treated cells (control) at 100%.

measure of cell activity is corrected for differences in cell size and incorporates the specific activities of pseudopod extension, membrane ruffling and undulation. All of the above measures of motility were significantly decreased in cells treated with either PIPLC or antisense oligonucleotides compared to untreated cells on fibronectin substrata.

## DISCUSSION

Previously we have demonstrated that urokinase activity is elevated in developing systems that undergo an epithelial-mesenchymal transformation (McGuire and Orkin, 1992; McGuire and Alexander, 1992). This process occurs in the developing heart in the endocardial cushions of the atrioventricular canal and the bulbotruncus and also in the somites and sclerotome during morphogenesis of the axial skeleton. In both of these systems, increased urokinase expression is associated with a migratory mesenchymal cell population. In the present study, urokinase transcripts were localized in the heart by in situ hybridization to only the endocardially derived mesenchymal cells that populated the cardiac jelly of the cushion tissue. The myocardial layer of the heart did not express urokinase. These data confirm our previous studies, which localized urokinase protein by immunostaining to this same cell type (McGuire and Orkin, 1992). In this study, we have begun to investigate the possible role of this enzyme in cell migration and cell-matrix interactions in this developing system.

Urokinase can be released from the cell surface by treatment with phosphotidylinositol-specific phospholipase C (PIPLC). An attempt was made to use this as a means of decreasing the proteolytic activity associated with the cell surface in the endocardial-derived mesenchymal cells. Treatment of explants with PIPLC inhibited the outgrowth of endocardial cells. In addition, cells in culture treated with PIPLC extended many processes that did not appear to readily detach from the substratum. When cells were viewed and images were collected over a 1-2 hour period of time, the extensions of the cell membrane appeared to be virtually 'stuck' to the substratum. This was quantitatively confirmed

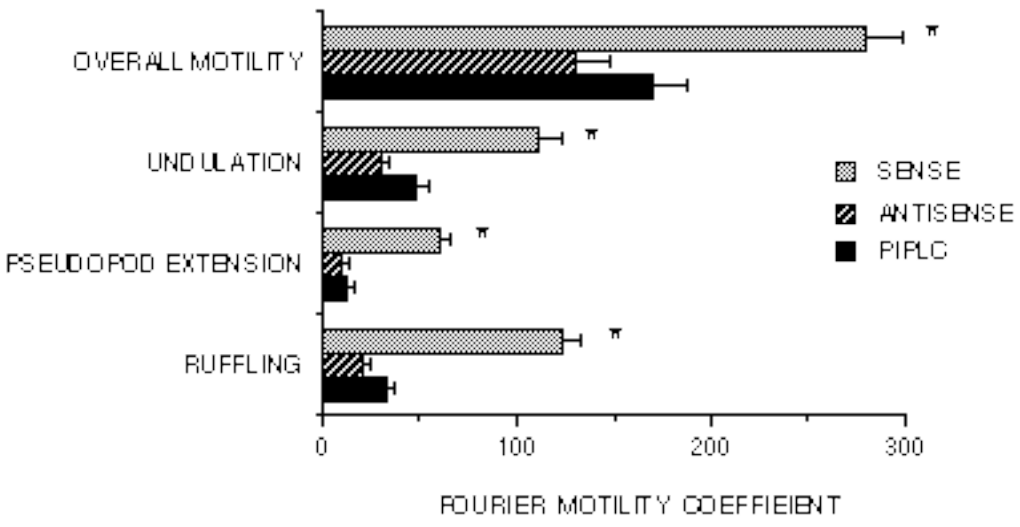


**Fig. 9.** Antisense inhibition of urokinase synthesis decreased the spreading and motility of cells on fibronectin. Cells treated with 10 μM sense and antisense oligonucleotides were passaged onto fibronectin and analyzed up to 4 hours with the Zeiss Dynacell videomicroscopy system to quantitate aspects of cell motility. The graphical data of Fourier motility coefficients for the various parameters of cell motility are shown. Data represent the mean and s.e.m. $n$=15 cells from 3 different experiments. *significantly greater than the PIPLC and antisense-treated cells at the $P \leq 0.05$ level.

by the data that showed a significant decrease in the overall motility of the PIPLC-treated cells as well as decreases in pseudopod extension, undulation and ruffling. A similar effect of PIPLC has been shown in other systems as well. A recent study by Chang et al. (1992) demonstrated that removal of PI-anchored proteins from the surface of cells in the developing grasshopper limb disrupted the characteristic and stereotyped migration of axons. In this case, it may have been the removal of a cell adhesion molecule such as NCAM, which has been shown to be attached to the membrane by a PIPLC-sensitive anchor, which caused the disruption of normal growth cone motility. Further studies are in progress to determine if the administration of PIPLC to the developing heart in ovo will cause similar disruptions in the migration of the endocardially derived mesenchymal cells.

Treatment of cells with PIPLC effectively removed some but not all of the surface-associated urokinase as indicated by zymographic analysis. Extraction of cells with Triton X-114 following PIPLC treatment revealed that up to 50% of the membrane-associated urokinase was still present. This may be urokinase bound to receptor that was not cleaved from the surface of the cells due to inaccessibility or the specific concentration of PIPLC used. In addition, it may also represent another form of the urokinase receptor that is not anchored to the membrane by a phosphatidylinositol moiety or urokinase associated with other cell surface molecules.

The treatment of cells with PIPLC has been shown to release a number of different proteins from the cell surface in addition to urokinase and its receptor. For this reason, we attempted to use antisense oligonucleotides as a more specific and direct inhibitor of urokinase function. A quantitative decrease in the actual production of urokinase by the use of antisense oligonucleotides directed at the urokinase mRNA dramatically affected cell attachment, spreading and motility. Differences in cell morphology were observed for cells treated with PIPLC compared to those treated with antisense oligonucleotides. This may be due to the fact that different amounts of urokinase were present on the cell surface depending on the type of treatment employed.

The antisense technology has been well characterized recently and has been used to inhibit the expression of a number of proteins in a variety of cell types (Potts et al., 1991; Kronmiller et al., 1991; Caceres et al., 1991). In the in vitro studies reported here, cells were pretreated with antisense oligonucleotides followed by passage to 'trigger' an increase in the production of urokinase and simulate a migratory behavior in these cells. During the course of the experiments, we had observed that longer incubation times (16-24 hours) with the antisense oligonucleotides prior to passing the cells appeared to result in more dramatic and uniform effects on cell morphology and behavior. This may reflect the amount of time necessary for cells to incorporate a sufficient amount of the oligonucleotides. Others have shown that incubation time and the size of the oligonucleotides are critical factors in determining the amount of oligonucleotide that becomes cell associated (Marcus-Sekura, 1988; Loke et al., 1989). Incubation times up to 24 hours and as short as 4 hours have been shown to produce significant decreases in the expression of a variety of different proteins (Marcus-Sekura, 1988; Potts et al., 1991; Caceres et al., 1991; Loke et al., 1989). In this study, the mechanism of antisense inhibition appeared to be due to a turnover of the RNA/DNA hybrids as a nearly 3-fold decrease in the level of urokinase mRNA was detected in antisense-treated cells by RNase protection assay.

Results from the present studies led us to question more closely the role of urokinase in the process of cell spreading and migration, which has been addressed by a number of other authors for both normal and malignant cell types (Erickson and Isseroff, 1989; Mignatti et al., 1986; Krystosek and Seeds, 1981). Migratory cells form transiently stable interactions with the extracellular matrix by means of focal contacts or focal adhesions (for review see Woods and Couchman, 1988). Studies by Duband et al. (1991) suggest that a disruption of the interaction of integrins with the extracellular matrix is essential for cell locomotion. The extracellular protease urokinase is in a position at the cell surface to be involved in the release of cell contact from the substratum during spreading and migration. Urokinase may act directly on the extracellular matrix (Quigley et al., 1987) or it may function through the activation of the broad spectrum protease plasmin. Since the present studies were all done in the absence of serum and therefore plasminogen, the former hypothesis seems possible provided that these cells are not producing plasminogen themselves. Additional targets for the action of urokinase other than specific matrix molecules may exist and may include various membrane receptors involved in cell-matrix interactions. Cleavage of the integrin receptor for example would likely result in release of cells from the substratum. It has been suggested that the β integrin receptor may be sensitive to proteolysis under certain conditions (Giancotti et al., 1985). In addition, studies by Miskin et al. (1978) and Hatzfeld et al. (1982) have shown that increased plasminogen activator and plasmin activity degrade specific receptors associated with the cell surface and significantly affect the composition and metabolism of the cell membrane.

In summary, these results have shown that decreasing the synthesis or activity of urokinase dramatically affects the motile behavior of cells in culture suggesting an important role for urokinase in regulating cell-matrix adhesion and migration. These observations are contradictory to what has been generally reported, that increasing urokinase, as in transformed cells, tends to decrease adhesion and cell spreading. We have in fact reported that an increase in urokinase production by the cell type used in this study in response to a fragment of fibronectin decreased the cells' ability to spread as the cells presumably could not form stable cell-matrix contacts due to the increased proteolytic activity (McGuire and Alexander, 1993). This suggests to us that a critical amount of cell surface urokinase is necessary for 'normal' migratory behavior and that too little or too much urokinase can alter this behavior by either preventing the detachment of cells from the substratum or inhibiting the formation and/or stabilization of new cell-matrix contacts. Further studies are in progress to define more precisely the targets and substrata for urokinase other than plasminogen which may be involved in these important cell behaviors.

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