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The effect of antisense inhibition of urokinase receptor in human squamous cell carcinoma on malignancy

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Concomitant expression of urokinase type plasminogen activator (uPA) and its surface receptor (uPAR) has been shown to correlate strongly with a more invasive tumor cell phenotype. A highly malignant human epidermoid carcinoma cell line (HEp3) was transfected with a vector capable of expressing an antisense transcript complementary to 300 bases of the 5′ end of uPAR, including the ATG codon. Six stably transfected antisense (AS-2, 3, 5, 9, 10, 12) and eight control clones were characterized. All clones produced high levels of uPA activity. Examination of collagenase production and doubling time showed that all of the clones tested produced similar activities. The antisense clones showed a 20–74% reduction in the uPAR sites; the uPAR mRNA level was also reduced. A test of the invasive ability of all clones in a modified chorioallantoic membrane (CAM) showed that invasiveness of the antisense-inhibited clones was directly proportional to the density of surface uPAR. The AS-2 clone, which expressed the lowest number of uPARs showed a significantly reduced level of invasion. The invasiveness of additional AS-inhibited clones was also reduced. Seven control and four AS-inhibited clones were tested for tumorigenicity on CAMs of chick embryos. Inoculation of control cells produced large tumors, while the AS clones were non-tumorigenic. AS-2 did not produce tumors even if kept *in vivo* for up to 10 weeks. AS-2 and the Neo-1 control clone produced tumors in the majority of injected mice, but while tumors produced by control cells became palpable within the first week after inoculation, the AS-2 tumors first appeared within 4 weeks of inoculation and were less locally invasive and metastatic. Cells harvested from the AS-2 tumors grown in nude mice all expressed a high level of surface uPAR, indicating a selection for this property under *in vivo* conditions. Overall these results show that diminished expression of surface uPAR leads to a reduction in invasiveness of tumor cells and an increase in tumor latency. Diminished malignant potential early in the establishment of tumors may shift the balance in favor of the host, leading to reduced tumorigenicity and metastasis.

Key words: antisense/invasion/metastasis/plasminogen activator receptor

Introduction

Several proteases, including plasminogen activator (PA) and especially urokinase type PA (uPA), are considered to be intimately involved in the metastatic spread of tumor cells (Ossowski and Reich, 1983a,b; Dano *et al.*, 1985; Markus, 1988; Testa and Quigley, 1990; Mignatti and Rifkin, 1993). This conclusion is based on the finding of elevated levels of uPA in most malignant tumors (Sappino *et al.*, 1987; DeBruin *et al.*, 1987; Markus, 1988; Sim *et al.*, 1988; Duffy *et al.*, 1990; Janicke *et al.*, 1990), its association with a more aggressive disease in several human malignancies (DeBruin *et al.*, 1987; Sim *et al.*, 1988; Janicke *et al.*, 1990; Sappino *et al.*, 1991) and its participation in invasion (Mignatti *et al.*, 1986; Reich *et al.*, 1988; Cajot *et al.*, 1989; Schlechte *et al.*, 1989), lung colonization and 'spontaneous' metastasis in several experimental models (Ossowski and Reich, 1983a; Hearing *et al.*, 1988; Ossowski, 1988a; Axelrod *et al.*, 1989; Yu and Schultz, 1990). uPA interacts with a specific receptor (uPAR) on the surface of most mammalian cell types (Stoppelli *et al.*, 1985; Vassalli *et al.*, 1985). Cells which express uPAR, and thus can generate surface-bound uPA, are more invasive when scored in an *in vivo* stromal invasion model than cells that produce only soluble uPA (Ossowski, 1988b; Ossowski *et al.*, 1991). Similar conclusions were reached in an *in vitro* model of invasion (Quax *et al.*, 1991). Two aspects of uPAR function, its ability to localize and enhance proteolytic activity (Blasi, 1993) and its function in mediating cell migration (Gudewicz and Gilboa, 1987; Fibbi *et al.*, 1988; Odekon *et al.*, 1992; Del Rosso *et al.*, 1993; Busso *et al.*, 1994), may contribute to enhanced invasiveness and may prove to be of value when targeted therapies for malignancy are considered. In this regard, it is interesting that subcutaneous inoculation in nude mice of chloramphenicol acetyl-transferase (CAT)-expressing PC3 prostate cancer cells produced detectable CAT activity in several organs of the host 8 weeks after inoculation (Crowley *et al.*, 1993), indicating a successful spread of PC3 cells. Inoculation of cells, which in addition to expressing CAT also expressed mutated catalytically inactive uPA, led to a drastic reduction in CAT activity in all examined organs, indicating that competitive displacement of active uPA from its receptor inhibits the ability of PC3 cells to spread. In this study, however, metastasis was measured by CAT activity without a parallel histological evaluation. Also, since catalytically inactive uPA may still retain signal transduc-

ing functions (Fibbi *et al.*, 1988; Odekon *et al.*, 1992; Rabbani *et al.*, 1992; Del Rosso *et al.*, 1993; Dumler *et al.*, 1993; Busso *et al.*, 1994), we felt that the direct blocking of uPAR expression by antisense would provide a more conclusive approach. To evaluate the role of uPAR in invasion and metastasis, we transfected cells of a highly malignant human epidermoid carcinoma with uPAR antisense constructs capable of expressing uPAR antisense RNA and isolated clones which differed only in their level of uPAR expression at the mRNA and protein levels. The malignant potential of the antisense-inhibited and control clones was characterized extensively in the chick embryo model. Two of the clones, one control and one showing the strongest reduction in uPAR sites, were also tested in nude mice.

Results

HEp3 cells, stably cotransfected with pSVneo and an expression vector (pCDM8) containing 300 bp of the 5′ portion of the uPAR cDNA which included the ATG codon, in antisense orientation downstream of a CMV promoter, were selected for G418 resistance and characterized (see Materials and methods). The same fragment was also inserted in an antisense orientation into pCDNAI/NEO and stable HEp3 transfectants were produced. Parental cells or cells transfected with pSVNeo alone, or pCDM8/pSVNeo or pCDNAI/NEO inserted with the full uPAR coding sequence, served as positive controls.

Comparison of pro-uPA binding, protease production and growth in vitro of control and uPAR-antisense clones

The number of surface uPA receptors in control clones (transfected with pSVneo alone or with pSVneo and pCDM8) and in antisense-transfected clones (20 in each group) was measured using radioactive pro-uPA as described in Materials and methods. While among the vector-transfected clones the variation in uPAR numbers was <15%, among the antisense (AS) transfected clones uPAR was reduced by as much as 74%. Two control clones pSVneo/pCDM8 and pSVneo, referred to as pCDM8 and Neo-1, and three uPAR-AS clones (AS-2, 3 and 5), which showed a 74, 37 and 33% reduction in uPAR number per cell, respectively (Table I), were selected for further characterization. The affinity of pro-uPA binding (k_D) calculated from a Scatchard plot was 5×10^{-10} to 1×10^{-9} M, and was unaffected by transfection. All clones produced high levels of uPA activity, the same types (72 and 92 kDa) and amounts of collagenases, and grew in culture with doubling times of 35–39 h (Table I). Also, an additional series of clones (three transfected with a pCDNAI/NEO containing an insert of a uPAR 5′ cDNA fragment in an antisense orientation as described for pCDM8, and six transfected with pCDNAI/NEO inserted with the entire uPAR cDNA coding region in the sense orientation; see Materials and methods) was isolated and partially characterized (see below).

Molecular characterization of the transfected clones

Construct integration was demonstrated in all three AS clones by PCR using their genomic DNA as template and sense and antisense oligonucleotides located within the CMV promoter of pCDM8 as primers. The product of this reaction (592 bp fragment) hybridized to pCDM8 DNA (Figure 1A and B) and to an oligonucleotide corresponding to the CMV promoter sequence (not shown). Judging by the intensity of the hybridization signal, the level of construct integration in the three AS clones was extremely variable (Figure 1B), with the AS-2 being very low and AS-5 showing the strongest hybridization signal (Figure 1A and B).

To determine whether uPAR antisense reduced the level of uPAR mRNA, total RNA from Neo-1 or the three AS clones was analyzed by Northern blotting using uPAR cDNA as probe. Following hybridization, the blots were stripped and rehybridized with GAPDH. uPAR mRNA was reduced by 3.3- to 7.8-fold in the AS-transfected

Table I. Protease production and uPAR numbers in control and uPAR antisense-transfected tumor cells

Clone	uPA (mU/10^6 cells)	uPAR/cell (% reduction)	Collagenases		Doubling time (h)
			72 kDa	92 kDa	
Neo-1	441	0	+++	+	39
pCDM8	564	10	+++	+	35
AS-2	436	74	+++	+	39
AS-3	666	37	+++	+	38
AS-5	370	33	+++	+	39

uPA activity was determined after 24 h of incubation (see Materials and methods). The results shown are the mean of duplicate samples and duplicate cultures. The variability between cultures was <25%, between assay duplicates <15%. The numbers represent secreted (~85% of total) and cell-associated activity. The number of sites determined by Scatchard analysis was 3×10^5 per cell in the Neo-1 clone which was taken as 100%. The experiment was repeated four times and the reduction in uPAR varied from 39 to 74, 19 to 37 and 26 to 33% for AS-2, 3 and 5, respectively. The k_D values ranged from 0.5 to 1.0×10^{-9} M. Collagenases were assayed in serum-free conditioned media by zymography as described previously (Ossowski, 1992); '+++' represents strong and '+' barely detectable lysis zones. Doubling time was determined by plating 1×10^5 cells (in duplicate) per 60 mm dish, and counting two dishes daily for 4 days.

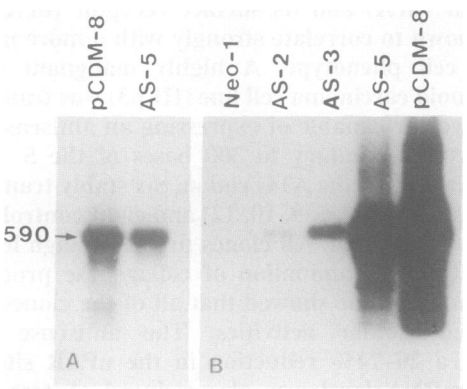

Fig. 1. Detection of construct integration by PCR analysis. Genomic DNA (1 μg per reaction) extracted from cultures of Neo-1 and AS-2, 3 and 5 cells, and 10 ng of pCDM8 DNA were used as templates in PCR to amplify a 592 bp fragment of the CMV promoter. The PCR products (26 cycles) were analyzed by hybridization with pCDM8 DNA. Exposures: (**A**) 30 min, (**B**) 6 h. The amplified products were also hybridized with a CMV oligonucleotide as a probe with similar results (not shown). This experiment was repeated three times with virtually identical results.

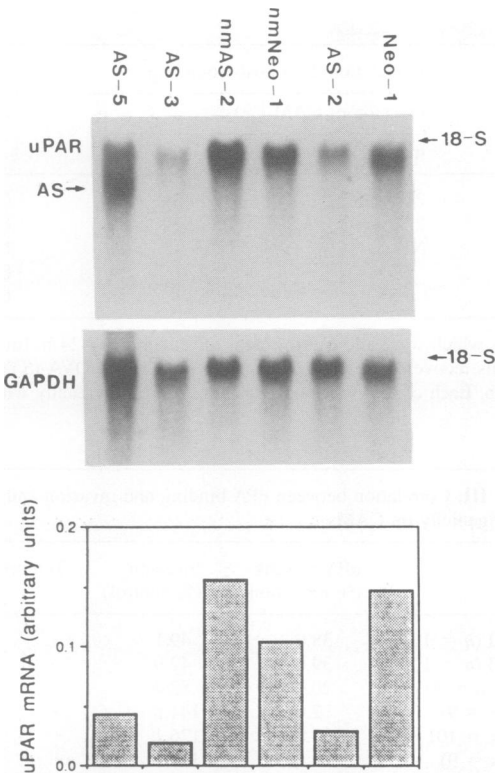

Fig. 2. Effect of antisense on the uPAR mRNA level in transfected cells and in cells obtained from nude mouse tumors. 30 μg of total RNA from control or antisense-transfected cells were hybridized with ^{32}P-labeled uPAR cDNA. The blots were stripped and rehybridized with mouse GAPDH cDNA. AS → indicates the position of the 300 bp uPAR antisense transcript. nmNeo-1 and nmAS-2 are RNAs isolated from primary cultures of tumors obtained by inoculation of Neo-1 and AS-2 cells into nude mice. Exposure for uPAR, 2 days; for GAPDH, 22 h. The film was scanned and the values are expressed as units of uPAR mRNA per unit of GAPDH.

clones as compared with the Neo-1 control (Figure 2). Similar results were observed in four independent experiments (results not shown). A band of ~300 bp, corresponding to the AS uPAR RNA, was detected in each of four tests using RNA from clone AS-5 (Figure 2, arrow, and results not shown). In one experiment (results not shown) a band of similar molecular weight was also detected in RNA from the AS-3 clone when a sense oligonucleotide (see Materials and methods) was used as probe; no uPAR-AS RNA was detected in the AS-2 clone.

To test whether the reduction in uPAR mRNA was paralleled by a reduction in uPAR protein, which could explain the reduced pro-uPA binding, cell lysates of Neo-1 clone and AS-2, the clone showing the most pronounced reduction in uPAR numbers, were analyzed by Western blotting with specific anti-uPAR mAbs (Figure 3); purified soluble uPAR served as a positive control. Scanning of the uPAR bands, which migrated with an approximate molecular weight of 45 kDa (slightly larger than the pure uPAR), showed a 78% reduction in uPAR protein in AS-2 cells. A reduced level of uPAR protein was found in all three AS clones (AS-2, 3 and 5) as compared with controls when lysates from metabolically labeled cells were immunoprecipitated with the specific anti-uPAR mAb and analyzed by autoradiography (results not shown).

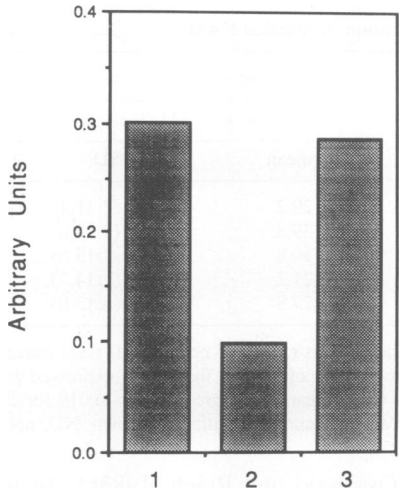

Fig. 3. Quantitation of uPAR protein by Western blotting. Detergent lysates of 1×10^6 cells of either Neo-1 or AS-2 clones were analyzed by Western blotting using anti-uPAR mAb. The values were obtained by scanning of the blot. Lane 1, Neo-1; lane 2, AS-2; lane 3, 10 ng of purified recombinant uPAR. In separate experiments, metabolically labeled cell lysates immunoprecipitated with the same antibodies gave essentially identical results.

The effect of uPAR reduction on invasion and tumorigenicity in chorioallantoic membranes of chick embryos

The invasive ability of the two control and three AS clones was tested in the modified CAM assay using wounded CAMs that were allowed to reseal *in vivo*. We showed previously that the degree of wounded CAM resistance to tumor cell invasion was directly proportional to the recovery period between wounding and cell inoculation (Ossowski, 1988b). Control and antisense-transfected cells were labeled with [^{125}I]UdR for 24 h and inoculated onto CAMs which were allowed to reseal for either 18 or 24 h. Control clones (Neo-1 and pCDM8) resulted in a baseline invasion of 30–40% on CAMs resealed for 18 h, and 22% on CAMs resealed for 24 h (Table II), highlighting the increase in CAM resistance with increased resealing time. At 18 h only the AS-2 clone, in which the number of uPARs was <30% of control, showed a strong (P = 0.011) reduction in invasion (Table II). When the conditions of the assay were made more stringent by incubating the CAMs for 24 h prior to tumor cell inoculation, the invasion by the two additional antisense clones, AS-3 and 5, in which uPAR numbers were greater than in the AS-2 clone (Table I), was also reduced (Table II). Similar results were observed when nine additional clones (three AS and six transfected with uPAR in the sense orientation) were tested for invasion in CAMs resealed for 18 h. The two AS clones which bound ~40% less uPA than control cells also showed a >50% reduction in invasiveness (Table III). All of the sense-transfected cells (except for S-12, which showed slightly reduced invasiveness) were as or more invasive than the untransfected HEp3 cells (Table III).

We have shown previously that HEp3 cells kept in culture reversibly lose their malignant potential, first losing their ability to form metastases and then their ability to form tumors. Re-exposure of these cells to *in vivo* conditions (chick embryos or nude mice) restores their malignant

Table II. Invasion of resealed CAMs

Clone	Inoculation 18 h post-wounding			Inoculation 24 h post-wounding		
	invasion of CAM (%)		*P*	invasion of CAM (%)		*P*
	mean	SD		mean	SD	
Neo-1	29.2	(±11.4)	0.019	21.7	(±14.3)	0.002
pCDM8	39.8	(±18.6)	0.124	22.8	(±22.3)	0.875
AS-2	10.8	(±13.6)	0.011	ND	NA	NA
AS-3	21.2	(±14.7)	0.318	7.3	(±4.3)	0.001
AS-5	27.3	(±13.0)	0.822	6.5	(±5.7)	0.000

[^{125}I]UdR-labeled cells (3.0×10^5 cells/CAM) were inoculated onto wounded CAMs which were allowed to reseal for either 18 or 24 h. Invasion represents the number of cells in the CAMs expressed as the percentage of total cells recovered (see Materials and methods). ANOVA (SYSTAT) was used for the comparison of groups: $P = 0.019$ for 18 h and $P = 0.002$ for 24 h. Each of the AS clones was compared individually with Neo-1 by Post-Hoc analysis and the results are shown. ND, not done; NA, not applicable.

potential (Ossowski and Reich, 1983b). To test whether decreased uPAR expression leads to diminished tumorigenic potential, Neo-1 and AS-2 cells were inoculated onto six CAMs each, and passaged weekly until large tumors were formed. Neo-1 cells produced large tumors within 4 weeks on the CAM (Figure 4). Such a lag period is expected of cells passaged *in vitro* for a prolonged period of time (Ossowski and Reich, 1983b). On the other hand, AS-2 cells treated in an identical way and kept in culture prior to inoculation onto CAMs for the same period of time did not recover their malignant potential even after 10 weeks on the CAM (Figure 4), suggesting that a low number of uPAR extended the period of readaptation to *in vivo* conditions. The test of nine additional clones for CAM tumorigenicity showed similar results: all six sense-transfected clones formed tumors >2×2 mm in the first week of *in vivo* growth, while all three AS clones (with the exception of one CAM inoculated with AS-9) formed masses <2×2 mm. The ability of control cells in this set of experiments to form tumors in the first week of *in vivo* growth may be the result of an increased susceptibility of these CAMs to invasion (see legend to Table III and Materials and methods).

Growth, invasion and metastasis of Neo-1 and AS-2 cells in nude mice

The relatively short gestation of chick embryos necessitates weekly passage of the inoculated cells, even if no growth of the inoculum is observed. The possibility exists, therefore, that cells may be lost during this procedure. This is not the case in nude mice where cells do not have to be disturbed following inoculation. We therefore injected 1.5×10^6 Neo-1 or AS-2 cells subcutaneously into nude mice. The mice were palpated every 2 days, and when detectable, tumor length and width were measured with a calliper every 3–4 days. The median number of days required post-inoculation to produce palpable tumors by the Neo-1 cells was 6, while for AS-2 cells it was 24.5 days. Once palpable, however, the growth rates of both were similar (Figure 5). As evident from the results summarized in Table IV, the prolonged tumor latency was the most prominent of the effects associated with the reduced number of uPA receptors. A lesser effect on local microinvasion and on metastasis to the lungs by the AS-2 clone, determined by histopathological examination, was also observed. The ability to form metastases by the AS-2-induced tumors was not correlated with the retention of

Table III. Correlation between uPA binding and invasion and tumorigenicity on CAMs

Clones	uPA binding (% reduction)	Invasion (% control)	Tumorigenicity
AS-10 (n = 10)	38.0	49.1	−
AS-12 (n = 10)	39.0	42.9	−
AS-9 (n = 5)	20.0	82.4	−
S-2 (n = 9)	2.0	141.8	+
S-3 (n = 10)	−132.0	126.1	+
S-7 (n = 9)	−26.0	101.7	+
S-10 (n = 8)	18.0	106.3	+
S-12 (n = 9)	20.0	94.5	+
S-13 (n = 9)	−6.0	116.9	+

Transfection was performed by electroporation with AS-pCDNAI/NEO (5′ fragment of uPAR-cDNA) or S-pCDNAI/NEO (full uPAR cDNA coding region in the sense orientation). Binding of uPA was carried out with [^{125}I]uPA at a single saturating dose; a negative number indicates binding of uPA greater than in the control. Invasion was carried out as described. The mean percentage invasion by control cells, which ranged between 41.4 and 53.7 in individual experiments, was considered as 100%. For tumorigenicity, 5×10^5 cells per CAM (three CAMs per clone) were inoculated and the embryos incubated for 7 days. Cells forming a mass >2×2 mm were deemed tumorigenic.

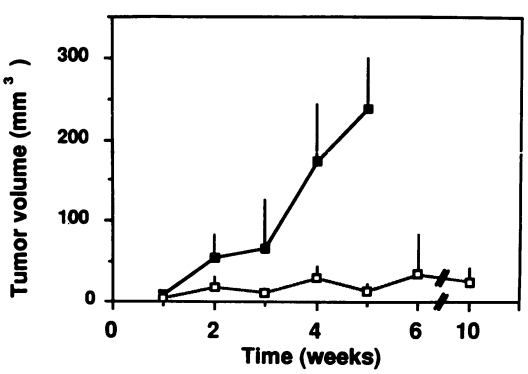

Fig. 4. Growth of Neo-1 and AS-2 on CAMs. Neo-1 and AS-2 cells (5×10^5 per CAM) were each inoculated on six CAMs of 10 day-old chick embryos, incubated for 1 week at 37°C, the tumors excised and their lengths and widths measured and volumes calculated. The tumors were combined, minced and reinoculated on fresh CAMs. The results shown are the mean of tumor volumes from four to six CAMs. The vertical lines show standard deviations. ■, Neo-1; □, AS-2.

G418 resistance; of the three tumor cells isolated from nude mice deemed to be positive for lung metastasis, two were G418-sensitive and one was resistant.

The prolonged latency period of AS-2 cells followed

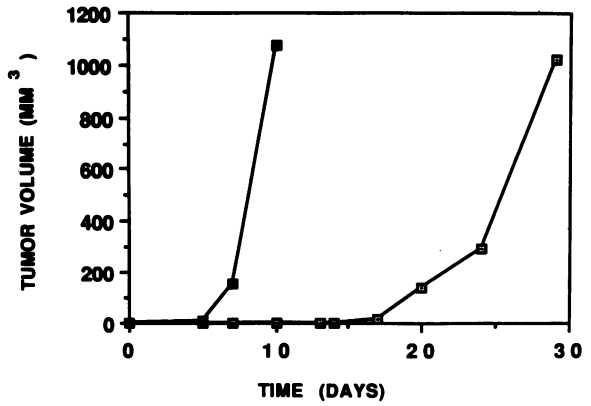

Fig. 5. Growth of Neo-1 and AS-2 in nude mice. Cell suspensions (2×10^6 per mouse) in 50 µl of PBS were injected subcutaneously in the interscapular region of 6 week-old nude mice. The site of injection was examined daily for palpable nodules, at which time the tumors were measured with a calliper every 2–4 days. ■, Neo-1; ⊡, AS-2.

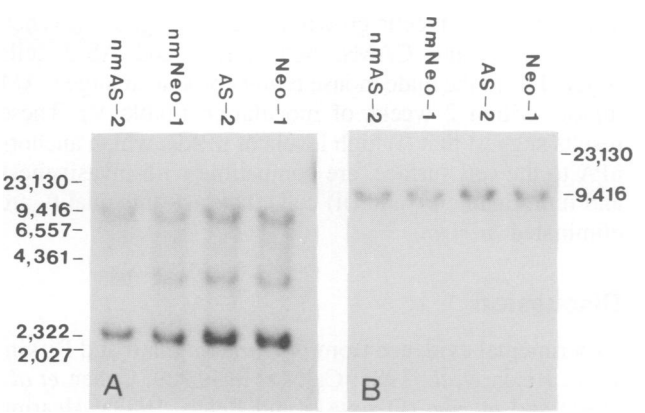

Fig. 6. Southern blot hybridization analysis of DNAs from Neo-1 and AS-2 before and after passage in nude mice. *Hin*dIII digests of genomic DNAs (10 µg per sample) were hybridized with uPAR cDNA (**A**) or uPA cDNA fragment (see Materials and methods for details) (**B**). Exposure = 3 days.

Table IV. Properties of Neo-1 and AS-2 tumors in nude mice

Clone	Tumor take (positive/total)	Latency (days) median	Local invasion (microscopic)	Lung metastases
Neo-1	9/10	6.0	7/9 (77%)	6/9 (66%)
AS-2	7/10	24.5	3/7 (43%)	3[a]/7 (43%)

[a]In histological sections of two out of the three metastasis-positive lungs, only a single metastatic focus was identified per section, one with a few and one with ~30 cells. The lungs of mice inoculated with Neo-1 and one AS-2 mouse had large, multiple metastatic foci both in the pleura and in the parenchyma of the lungs.

Table V. Properties of cells isolated from tumors grown in nude mice

Clones	Resistance to G418 (resistant/total)	uPAR sites per cell ($\times 10^{-5}$)	Growth on CAM at week 2 (tumor weight; mg)
Nm*Neo-1	9/9	3.0 (±0.2)	110 (±60)
Nm*AS-2	4/7	3.2 (±0.17)	117 (±54)

Nm, nude mice. To determine G418 resistance, cells isolated from nude mouse tumors were grown in the antibiotic for 2 weeks. The proliferation rate of G418-resistant cells was indistinguishable from that of cells which had not been grown *in vivo* (Table I), while the G418-sensitive cells died within the first week of culture. uPAR number and tumorigenicity on CAMs were determined as described in Materials and methods. The uPAR number is the mean of four determinations.

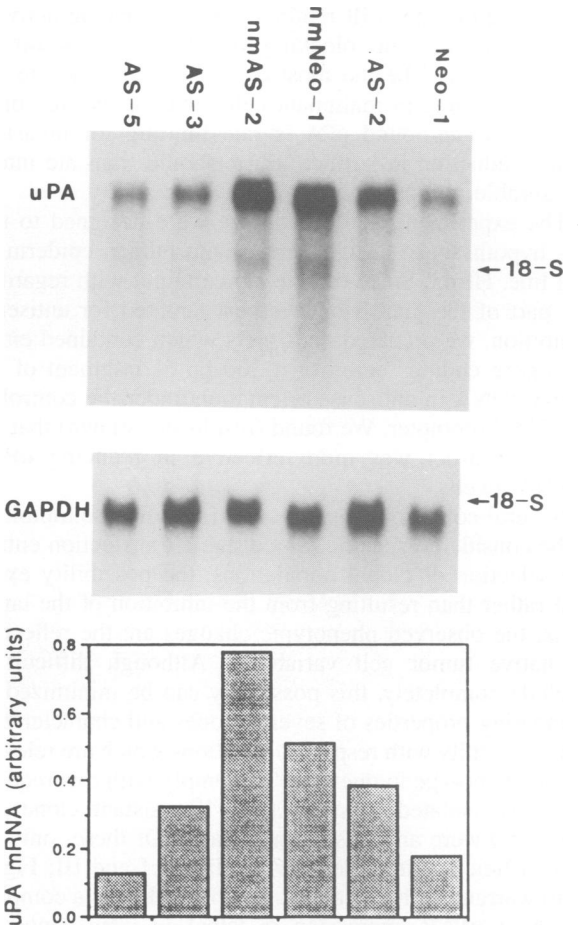

Fig. 7. uPA mRNA in cells prior to and after passage in nude mice. All the conditions were exactly as in Figure 2, except that a labeled fragment (see Materials and methods for details) of uPA cDNA was used as a probe. Exposure for uPA, 23 h; for GAPDH, 10 h.

by a growth rate similar to that of the control suggested that the exposure to *in vivo* conditions selected a population of cells better adapted to survive in the host. The examination of cells isolated from each of the nine Neo-1-initiated tumors showed that they maintained resistance to neomycin (Table V), while only four of the seven AS-2 tumors were resistant. Southern blot analysis of genomic DNA from naive and nude mouse-grown Neo-1 and AS-2 cells yielded very similar hybridization patterns with uPAR or uPA cDNA, suggesting that no major rearrangement of these two genes occurred during growth *in vivo* (Figure 6). Northern blot analysis showed, however, that the level of uPAR mRNA in AS-2 cells isolated from a tumor was no longer reduced (Figure 2). This change was paralleled by an increase in uPAR sites which was observed in all AS-2 tumors, regardless of whether they maintained or lost neomycin resistance (Table IV). The nude mouse-grown AS-2 cells also had increased levels of uPA mRNA,

as compared with cells grown in culture (Figure 7). When reinoculated onto CAMs, both Neo-1 and AS-2 cells isolated from the nude mouse tumors produced large CAM tumors within 2 weeks of inoculation (Table V). These results suggest that (i) high levels of uPAR, which anchors uPA to the cell surface, are compatible with invasiveness and tumorigenicity, and (ii) cells that lack this quality are eliminated *in vivo*.

Discussion

Experimental evidence from *in vitro* (Mignatti and Rifkin, 1986; Reich *et al.*, 1988; Cajot *et al.*, 1989; Cohen *et al.*, 1991) and *in vivo* (Ossowski and Reich, 1983a; Hearing *et al.*, 1988) models, and the correlation of uPAR expression levels and cancer aggressiveness (Schlechte *et al.*, 1989; Hollas *et al.*, 1991; Bianchi *et al.*, 1994) suggest that uPAR-bound urokinase is an important component of the invasive phenotype. Since uPAR-bound urokinase rendered catalytically inactive by either mutation or antibody binding may still retain signal transducing activity, we argued that the blocking of uPAR expression by antisense would be the most conclusive approach to the study of its role in malignant cell behavior. We reasoned that if surface-bound uPA is rate limiting for invasion, then a reduction in surface uPAR should translate into a measurable reduction in tumor invasive ability.

The experiments described here were designed to test this hypothesis in a highly malignant human epidermoid cell line, HEp3. Since there is no certainty with regard to the part of the gene which is best targeted for antisense inhibition, we prepared constructs which contained either the entire coding region or a 300 bp 5′ fragment of the uPAR cDNA in antisense orientation under the control of the CMV promoter. We found (results not shown) that the latter construct was more effective in reducing uPAR mRNA levels.

Several conditions must be met for antisense inhibition to be considered specific. Since stable transfection entails the selection of clonal populations, the possibility exists that rather than resulting from the inhibition of the target gene, the observed phenotypic changes are the reflection of native tumor cell variability. Although difficult to exclude completely, this possibility can be minimized by comparing properties of several clones and characterizing them carefully with respect to functions which are relevant to the phenotype in question. To comply with this requirement we isolated nearly 100 G418-resistant clones, of which 50 were antisense transfectants. Of these, only six had sufficiently inhibited uPAR (Tables I and III; Figure 2) to warrant further analysis. It is possible that a complete block in uPAR expression is lethal *in vitro*, since we noticed (only in dishes transfected with the antisense construct and not with pSVneo or pCDM8 alone) clones made of 12–20 very large cells that did not divide even when transferred to medium without G418 (results not shown). In this respect it would be interesting to determine the phenotype of cells in which the uPAR gene was inactivated by homologous recombination. The characterization of uPA binding activity revealed only minor (<15%) interclonal variation in uPAR numbers. A similar lack of interclonal variation was observed in a previous attempt to identify HEp3 clones with low uPAR numbers in which repeated selection of survivors from anti-uPAR antibody treatment, followed by complement killing, was performed (results not shown). The observation that all six of the uPAR-reduced clones arose within the antisense-transfected population, and that a very large number of control or untransfected clones had constant (or greater for some sense-transfected clones) uPAR numbers, indicates that the antisense RNA is responsible for the reduction in receptor numbers.

Since we have shown previously that uPA and collagenases were involved in invasion by HEp3 cells (Ossowski, 1992), we tested whether transfection and clonal selection had an effect on these properties. All of the clones used for biological experiments produced levels of uPA similar to that of parental cells (Table I and results not shown). Neo-1, pCDM8 and AS-2, 3 and 5 were tested and found to produce similar collagenase activities (Table I) and to divide *in vitro* as rapidly as the parental cells, suggesting that any differences in tumor growth *in vivo* could not be attributed to intrinsic differences in their growth rate.

Ideally the targeted mRNA and protein should be most effectively inhibited in clones with the highest extent of construct integration and antisense RNA expression. In one of the three examined clones (AS-2), we found a substantial (>70%) inhibition of uPAR mRNA levels and protein expression in the absence of detectable antisense RNA or a high number of integrated constructs (Figures 1–3). In contrast, in AS-5 in spite of an extremely high copy number of integrated construct and easily detectable antisense RNA, the uPAR mRNA was reduced to a lesser degree. Thus, it appears that a high copy number of integrated constructs or the presence of detectable levels of antisense RNA did not predetermine an inhibition of uPAR mRNA. Neither the inability to detect the AS RNA, nor the lack of correspondence between the level of construct integration and the degree of reduction in uPAR mRNA, are totally unexpected and were observed by others (van der Krol *et al.*, 1988; Kasid *et al.*, 1989; Neckers *et al.*, 1992). Although no sound explanations exist for these discrepancies, it is believed that (i) positional effects may influence the level of *trans*-gene transcription (van der Krol *et al.*, 1988), (ii) the RNA duplexes may be highly unstable, and (iii) the antisense RNA is more unstable than mRNA and thus less easily detectable. In fact, in Northern blots of RNA from clones transfected with pCDNAI/NEO inserted with the full coding uPAR cDNA in the sense orientation, an additional band corresponding in size to the inserted transcript was observed (results not shown). Most importantly, a reduction in uPA binding (Table I) always correlated with a reduction in uPAR mRNA levels (Figure 2), suggesting that, by whatever mechanisms, the effect on RNA had biochemical and biological consequences. Similar observations were reported by others in c-raf-1 or EGFR antisense inhibition (Kasid *et al.*, 1989; Moroni *et al.*, 1992).

To exclude the possibility that the antisense effect is mediated by inhibition of another homologous mRNA species, we searched the nucleotide sequence database using the NCBI BLAST program. This search did not reveal relevant similarities with the portion of uPAR cDNA coding for the antisense RNA, with the possible exception of HEK2, a newly cloned tyrosine kinase

receptor (Bohme *et al.*, 1993) of unknown function, which showed three short stretches of homology. Although expression of HEK2 in HEp3 cells will have to be examined, our conclusion is that the observed phenotypic effects were mediated via uPAR rather than HEK2 inhibition. For instance, reduced expression of tyrosine kinase receptors is usually associated with growth inhibition, but the antisense-inhibited clones exhibited unaltered growth rates (Table I).

In view of the above considerations, we believe our results directly demonstrate that reduced surface uPAR expression causes a notable diminution in HEp3 cell invasiveness. This conclusion is reinforced by the observation that the invasive ability of the AS-transfected cells is directly proportional to the density of uPAR displayed on the surface of the six clones (Tables I–III). All of the tested AS-transfected clones were found to be non-tumorigenic in CAMs of chick embryos (Figure 4 and Table III). In nude mice, while Neo-1 cells produced palpable tumors within 6 days after inoculation, the AS-2 cells remained 'latent' for almost 4 weeks (Figure 5 and Table IV). These results suggest that an intact invasive ability, inclusive of surface-bound uPA, is essential for early stages of tumor establishment and growth. The precise function of surface-bound uPA in the establishment of tumors remains to be determined, but a role in angiogenesis, the processing of pro-hormones or growth factors, or the generation of chemotactic proteolytic products, can easily be envisioned. Also, a reduction in their surface proteolytic activity may render tumor cells more susceptible to host defense mechanisms.

The results in nude mice may be considered preliminary since, in spite of the multitude of control experiments performed, the reduced malignancy of the AS-2 clone may simply represent clonal variation within the HEp3 cell population. Although possible, the following arguments make it unlikely. HEp3 cells were subjected earlier to a very detailed clonal analysis under conditions of 60% cloning efficiency. The conclusion from this analysis stated that 'there was no evidence that HEp3 populations were heterogenous with respect to tumorigenicity' (Ossowski and Reich, 1983b). Also, it would have to be postulated that in each of the AS-2 tumors produced in seven individual nude mice the uPAR inhibition was eliminated (Figure 2) at the same time as the cells underwent an independent additional event that produced a consistently malignant variant.

The fact that of the seven AS-2 tumors only three lost their G418 resistance indicates a random elimination of an exogenous gene. In contrast, a full complement of surface uPAR was found in cells from all seven AS-2 tumors (Table V) and the steady-state level of uPAR mRNA was indistinguishable from that of control, suggesting a sort of purposeful selection. The expression of another malignancy-linked gene, the uPA, in cells isolated from nude mouse tumors, as compared with naive cells, was increased in individual tumours by a factor of 2.5–5 (Figure 7). These nude mouse 'selected' AS-2 cells were capable of producing large tumors within the first 2 weeks of growth on CAMs (Table V). Therefore, it appears that *in vivo* selection resulting in a phenotype with a full complement of uPAR and uPA was a prerequisite for the commencement of exponential growth *in vivo* (Figure 5).

These results show that it is possible to target specific genes for inactivation of expression by antisense RNA and that interference with one of the many steps in the proteolytic cascade results in a dramatic down-modulation of the malignant potential of tumor cells. Further experiments using cells transfected with constructs with inducible promoters will be required to identify the mechanism by which uPAR-bound uPA modulates specific stages of tumor growth.

Materials and methods

Preparation of constructs

Total RNA was isolated (Chomczynski and Sacchi, 1987) from uPAR-rich WISH cells, and 1 μg of RNA was reverse transcribed (Chen *et al.*, 1992) using a synthetic oligonucleotide (30 mere; American Synthesis, Pleasanton, CA) corresponding to the 3′ end (nucleotides 1123–1154) of the uPAR cDNA (Roldan *et al.*, 1990). A 306 bp (−46 to 246) cDNA fragment was PCR-amplified using synthetic primers (ATCTCGAGAGAGAAGACGTGCAGGGACCC, ATAAGCTTGCCAGTCCGATAGCTCAGGGTCC; non-specific *Xho*I and *Hin*dIII restriction enzyme recognition sequences are in bold) that included *Xho*I and *Hin*dIII recognition sites. The conditions used were as described previously (Chen *et al.*, 1992). The amplified cDNA fragment was subcloned into a *Xho*I- and *Hin*dIII-digested pCDM8 vector (Seed, 1987) or pCDNAI/NEO obtained from Invitrogen Co. (San Diego, CA). These antisense constructs are referred to as AS-pCDM8 and AS-pCDNA/NEO. An oligonucleotide located between the *Pst*I and *Hin*dIII sites of the uPAR insert (nucleotides 211–250; Roldan *et al.*, 1990) was used as a probe. A cDNA containing the full coding sequence of human uPAR was also prepared and cloned in sense and antisense orientations into pCDM8 and pCDNAI/NEO. COS cells transfected with the pCDM8 sense or the pCDNAI/NEO sense constructs expressed surface uPAR as determined by their ability to bind 2.7- or 1.6-fold more, respectively, radioiodinated pro-uPA than mock-transfected COS cells (results not shown). Sequence analysis of 300 bp of internal sequence showed 100% homology with the published sequence for uPAR cDNA (Roldan *et al.*, 1990).

Transfection and selection

Human squamous cell carcinoma (HEp3) cells (Toolan, 1954), isolated from a tumor serially transplanted on chick embryo (COFAL-negative from SPAFAS, Norwich, CT) CAMs and passaged *in vitro* for 2 months, were plated at 1.4×10^6 cells per 100 mm dish in DME with 10% FBS; when 70% confluent, they were transfected with either pSVneo DNA alone (10 μg/dish) or pCDM8 (8 μg/dish) and pSVneo (3 μg/dish), or AS-pCDM8 (8 μg/dish) pSVneo (3 μg/dish) in medium without serum containing 7 μl/ml Transfectam (Promega) for 3 h at 37°C. All cultures were kept in Dulbecco's medium with 10% serum for 24 h and then 400 μg/ml of G418 was added. After 8 days, when most cells died and detached, the medium was changed to RPMI with 15% serum and 300 μg/ml of G418. Eighty pSVneo-resistant colonies were isolated (40 transfected with pSVneo alone, 20 with pSVneo and pCDM8 and 20 with AS-pCDM8) from a single transfection experiment, their receptor numbers determined and five clones (two controls and three antisense) were selected for further study. In a separate experiment, HEp3 cells were transfected by electroporation using 5×10^5 cells and 10 μg plasmid DNA in a Cell-Porator (BRL Life Technologies Inc., Grand Island, NY) with a single pulse. The cells were grown in medium with 10% FBS for 48 h at which time 200 μg/ml of G418 was added. Twenty clones (10 transfected with pDCNAI/NEO-uPAR in sense orientation and designated S-pCDNA/NEO, and 10 with the same vector but with the uPAR cDNA 5′ fragment described above in the antisense orientation, designated AS-pCDNAI/NEO) were selected and nine characterized further.

Determination of uPA receptor numbers

Recombinant pro-uPA (Abbot Laboratories, Abbot Park, IL) was iodinated to a specific activity of $1–4\times10^7$ c.p.m./μg protein using Iodo-Gen. Cells (1×10^5 cells per well in a 24-well tray) were acid stripped of endogenous uPA (Stoppelli *et al.*, 1986) and radioiodinated pro-uPA binding measured as described (Ossowski *et al.*, 1991). Briefly, the cells were incubated for 60 min at 4°C and after three washes in DME-HEPES-BSA the radioactivity was solubilized in 0.5% Triton X-100 for 20 min at 37°C and counted in a gamma counter. Each of the pSVneo-

resistant clones was tested for pro-uPA binding by incubation with a single near saturating concentration of ^{125}I-labeled pro-uPA, with and without a 50-fold excess of unlabeled pro-uPA. Clones which showed reduced binding were characterized with respect to their total uPAR content and the k_D of uPA binding.

Plasminogen activator determinations

These were performed essentially as described (Mira-y-Lopez and Ossowski, 1987). Briefly, 10 µl of sample (obtained by incubation of semi-confluent cultures with DME for 24 h or by extracting cells with 0.5% Triton X-100 in 0.1 M Tris, pH 8.1) were mixed with 105 µl of Tris–HCl buffer (0.1 M, pH 8.1 with 0.1% Triton X-100) and 10 µl of 0.2 mg/ml of purified human plasminogen and incubated for 2 h at 37°C. Chromogenic substrate (Spectrozyme PL, American Diagnostica, Greenwich, CT) for plasmin was added and A_{410} was measured in a microplate reader (Dynatech Laboratories Inc., Chantilly, VA). A human uPA standard curve was included in every assay.

Zymographic assay of collagenase

Collagenases were identified by zymography (Ossowski, 1992). Briefly, conditioned media without serum were electrophoresed on polyacrylamide gels copolymerized with 1 mg/ml of gelatin (Sigma), washed in 2.5% Triton for 30 min, incubated in calcium-containing buffer for 40 h, stained, destained and examined for areas of lysis.

Southern blot analysis

High molecular weight DNA was extracted from confluent cultures of the transfected clones according to standard procedures (Ausubel *et al.*, 1987), digested with *Hin*dIII overnight at 37°C and size fractionated (10 µg per lane) on a 0.85% agarose gel. The DNAs were transferred onto N-Hybond nylon membrane (Amersham) and probed with a 0.6 kb uPA cDNA obtained by *Eco*RI digestion of plasmid pUK0321 (a gift from Dr W.-D.Schleuning, Institute of Biochemistry, Schering A, Berlin, Germany) or with the full-length uPAR cDNA (prepared by PCR, see above); probes were labeled by random priming with [^{32}P]dCTP to a specific activity of $3–7\times10^8$ c.p.m./µg DNA (Ausubel *et al.*, 1987). For construct integration analysis, a 592 bp fragment of the CMV promoter was amplified by PCR using specific oligonucleotides as primers and the genomic DNA of each clone as a template. The identity of the amplified fragments was determined by Southern blotting using either ^{32}P-labeled pCDM8 DNA or synthetic oligonucleotide as probes.

Northern blot analysis

Total RNA was extracted from confluent cultures by the one-step acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987), electrophoresed on 1.5% agarose gels (20 µg/lane), transferred to nylon membranes and hybridized with ^{32}P-labeled uPAR or uPA cDNA (as above). After stripping the membranes were rehybridized with murine GAPDH cDNA. In some experiments a labeled uPAR sense oligonucleotide (221–257 nucleotides) was used in hybridization. Hybridization and washing conditions were as before (Zelent *et al.*, 1991).

Quantitation of tumor cell invasion of chorioallantoic membrane

The method used was essentially as described previously (Ossowski, 1988b). Experiments described in Table III were carried out in embryonated eggs, and all the rest were carried out in COFAL-negative embryonated eggs obtained from SPAFAS (Norwich, CT). Tumor cells were labeled in culture with [^{125}I]UdR (0.2 µCi/ml DME with 5% FBS) for 20 h, to a specific activity of 0.07–0.20 c.p.m./cell, washed extensively, detached, resuspended in PBS at 4×10^6/ml and inoculated on CAMs of 10 day-old chick embryos (50 µl/CAM). The number of invading cells was determined after 24 h. The results are expressed as the percentage of total recovered radioactivity.

Growth of tumor cells on the CAMs

Tumor cells were detached with trypsin–EDTA, resuspended and counted in DME with 10% FBS, centrifuged, resuspended at 10×10^6 cells/ml PBS, inoculated (50 µl) on CAMs of 10 day-old chick embryos and incubated for 7 days. Even when there was no tumor growth, the site of inoculation was always evident as a small nodule. The nodules were measured (Table III) or removed, minced and reinoculated on a fresh CAM (Figure 4 and Table V).

Characterization of transfectants in nude mice

HEp3 parental cells, Neo-1 and AS-2 cells were inoculated subcutaneously at 2.0×10^6 cells per nude mouse (Nu/nu in Balb/C background, 6 weeks or older females, Life Sciences, FL). The mice were inspected and their tumors measured twice weekly. Tumors (calculated weight of ~1 g) were excised surgically and the mice observed for the appearance of dyspnea. If no symptoms appeared the mice were sacrificed 13 weeks following inoculation. Tumors, lungs and lymph nodes, known from previous work to be the preferred sites of metastasis (Ossowski *et al.*, 1987), were examined histologically. Primary tumors were re-established in culture and tested for G418 resistance, uPA binding and uPA and uPAR mRNA levels. Control HEp3 and Neo-1 cells produced identical results in nude mice; therefore only Neo-1 results are shown.

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