Coordinate Up-Regulation of Sp1 DNA-binding Activity and Urokinase Receptor Expression in Breast Carcinoma

Antonella Zannetti, Silvana Del Vecchio, Maria V. Carriero, Rosa Fonti, Paola Franco, Gerardo Botti, Giuseppe D’Aiuto, M. Patrizia Stoppelli, and Marco Salvatore


Abstract

The regulatory mechanisms underlying the overexpression of urokinase-type plasminogen activator (uPA) and its receptor (uPAR) in highly invasive breast carcinomas remain poorly understood. In this study, we have simultaneously determined the level of uPAR and the activity of the transcription factor Sp1 in 14 breast carcinomas and 5 benign lesions. We found that uPAR levels and Sp1-binding activity are coordinately elevated in malignant tumors (r = 0.94; P < 0.001). On the contrary, undetectable or only barely detectable levels of uPAR and Sp1 activity were found in benign breast lesions. Finally, the engagement of uPAR by catalytically inactive uPA in the MDA-MB-231 breast carcinoma cell line results in a rapid up-regulation of Sp1-binding activity followed by an increase of uPAR protein. These results, taken together, suggest the existence of a uPA-dependent positive regulatory loop that may progressively enhance malignant breast cell invasiveness.

Introduction

Proteolytic degradation of the extracellular matrix constituents and modification of cell adhesion properties are required for tumor invasion and metastasis. Recent evidence shows that selective inhibitors of proteolytic enzymes prevent tumor growth and dissemination in animal models (1). In particular, uPA and its cognate receptor (uPAR) play a key role in tumor invasion and progression because this receptor focuses the uPA proteolytic activity at the cell surface and mediates, in a ligand-dependent manner, a variety of cell responses such as migration, adhesion, and transcription of specific genes (2).

The uPAR is a highly glycosylated Mr 55,000–60,000 glycosylphosphatidylinositol-anchored protein that comprises three structural domains including the NH2-terminal uPA-binding site (3). Many malignant cultured cell lines and human neoplasms have been characterized by their increased uPA and uPAR expression (4). Accordingly, levels of uPA and uPAR in malignant tumors are reported to be significantly associated to a poor prognosis, which suggests that the inhibition of uPAR activity could be a promising strategy to prevent cancer invasion and metastasis (5).

In a previous study (6), we reported a coordinate overexpression of uPA and uPAR in breast carcinomas leading to a net enhancement of cell invasiveness. This finding raises an interesting question concerning the mechanisms responsible for the modulation of uPAR expression, which is also controlled by a variety of hormones and growth factors (4).

Soravia et al. (7) recently identified a minimal promoter region required for the basal transcription of the human uPAR gene. This region lacks TATA and CAAT boxes and contains GC-rich proximal sequences that are specifically bound by the transcription factor Sp1. The uPAR gene is transcriptionally regulated by Src activity via an upstream sequence (−152/−135) bound with Sp1 (8). The transcription factor Sp1 is a member of the C2-H2 zinc finger family, which acts in the absence of TATA boxes and regulates the transcription of a wide variety of different genes including those involved in general cellular metabolism such as the housekeeping genes (9) and a number of growth factor and growth factor receptor genes (10, 11). Traditionally considered as a constitutive transcription factor, it is becoming increasingly clear that Sp1 activity may be induced by many oncoproteins such as v-Rel, v-Ras, v-Src, c-Fes, and the human retinoblastoma gene product (12–14). Furthermore, Sp1-binding activity is decreased by phosphorylation (15), whereas it is increased by dephosphorylation mediated by protein phosphatase 1 (16).

Despite the proven ability of Sp1 to regulate the transcriptional activity of a variety of genes involved in cell differentiation and proliferation (17, 18), little is known about its role in tumor growth and progression. High levels of Sp1 have been reported in human gastric carcinomas (19), and a coordinate overexpression of Sp1 and laminin-y1 was found in human hepatocellular carcinomas (20).

In an effort to identify the transcriptional activators involved in the regulation of uPAR, we investigated whether there is a relationship between uPAR expression and Sp1 activity in invasive breast carcinomas. Therefore, we determined the levels of uPAR and the DNA-binding activity of Sp1 in 4 malignant tumor cell lines, 14 breast carcinomas, and 5 benign breast lesions. In addition, the ability of uPA or its catalytically inactive ATF to regulate both uPAR levels and Sp1 activity was tested in MDA-MB-231 and MCF-7 mammary carcinoma cell lines.

Materials and Methods

Cell Lines and Culture Conditions. MDA-MB-231 and MCF-7 human breast carcinoma, HeLa carcinoma, and HT1080 human fibrosarcoma cell lines were grown in DMEM containing 10% fetal bovine serum, 100 IU/ml penicillin, and 50 µg/ml streptomycin. To determine Sp1 activity and uPAR expression, MDA-MB-231 and MCF-7 human breast cancer cell lines were starved in serum-free medium for 18 h and then treated with 10 nM uPA, recombinant single-chain pro-uPA, or ATF for the indicated times at 37°C. In a set of experiments, cells were preincubated for 18 h with 100 nM mithramycin (Sigma Chemical Co, St. Louis, MO), a competitive inhibitor of Sp1 DNA-binding activity (21).

Patients and Tissue Samples. Fourteen patients with breast carcinoma (mean age, 68 ± 13 years) and 5 with benign breast lesions (mean age, 43 ± 16 years) were studied. Malignant tumors included nine invasive ductal carcinomas, four invasive lobular carcinomas, and one ductallobular carcinoma.

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3 The abbreviations used are: uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; ATF, NH2-terminal fragment of urokinase.
with a diameter ranging between 1.7 and 5 cm. The benign lesions included three fibroadenomas and two fibrocystic diseases. Tumor biopsy specimens were immediately frozen in liquid nitrogen after surgical removal and stored at −80°C.

**Nuclear Extracts from Cells and Tissues.** Nuclear extracts were prepared according to the method of Dignam et al. (22). Briefly, cells were washed in cold PBS and collected by centrifugation. The cell pellet was resuspended in 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.1 mM EGTA (homogenization buffer); homogenized by passage through a 26-gauge needle; and centrifuged for 5 min at 1,500 rpm. The pellet representing nuclei was resuspended in 10 mM HEPES (pH 7.9), 5% glycerol, 1.5 mM MgCl₂, 0.5 mM DTT, 0.4 M NaCl, and 0.1 mM EGTA (extraction buffer). The protease inhibitors leupeptin (5 mM), aprotinin (1.5 mM), phenylmethylsulfonyl fluoride (2 mM), and benzamidine (1 mM) were used throughout the extraction procedure. The suspension was rocked at 4°C for 30 min, cleared by centrifugation for 30 min at 12,000 rpm, and stored at −80°C.

Frozen samples of benign and malignant human breast tissues (500–1000 mg) were pulverized in a Mikro-Dismembrator (Braun, Melsungen, Germany). The resulting powder was suspended at 1:10 (w/v) in the homogenization buffer supplemented with 0.25 M sucrose (buffer A), homogenized, and centrifuged for 15 min at 2,300 rpm. The pellet was resuspended in buffer A, mixed with two volumes of homogenization buffer containing 2.5 M sucrose (buffer B), layered on top of 3:1 (v/v) buffer B, and finally centrifuged at 27,000 rpm for 1 h at 4°C. Nuclear proteins were then extracted as described above for cells. Protein concentration was determined by the method of Bradford (Bio-Rad Laboratories, Richmond CA).

**Electrophoretic Mobility Shift Assay.** The double-stranded DNA oligonucleotide containing Sp1 consensus sequence (5'-ATT CGATCGGGGGCGGG-GGAGGC-3') was from Promega, Madison, WI. The oligonucleotide was end-labeled with T4 polynucleotide kinase and [γ-32P]ATP and purified by gel electrophoresis. Ten and 40 μg of nuclear extracts from cells and tissues, respectively, were preincubated in a binding buffer containing 20 mM HEPES (pH 7.5), 40 mM KC1, 5% glycerol, 2 μg of poly(dI:dC) for 10 min at 22°C. The radiolabeled DNA probe (100,000 cpn/0.1 ng) was added in the presence or absence of a large molar excess (300-fold) of unlabeled Sp1 oligonucleotide, and the incubation was continued for an additional 20 min at 22°C. An excess of unlabeled AP1 oligonucleotide (Promega) was also used as competitor when indicated. Free and bound probes were separated on a 8% nondenaturating polyacrylamide gel followed by autoradiography.

Sp1-binding activity was quantitated by morphodensitometric scanning of autoradiograms using an image analysis system (Sistemi Avanzati, Milan, Italy) including a high resolution charge coupled device camera (High Technology Holland) and the Micro Computer Imaging Device (Imaging Research, Inc, Ontario, Canada). Each gel included a nuclear extract sample from HeLa cells as a control. For each sample, the product of absorbance and the area of the band was calculated and normalized against the Sp1 signal and protein content of HeLa cells, which were taken as 100%.

**Cross-linking of uPAR.** Human recombinant ATF of urokinase (amino acids 1–135), obtained from Dr. J. Wang (Abbot laboratories, Abbot Park, IL), was labeled with Na125I using Iodo-Gen method as described previously (6). Membrane fractions from cultured cell lines and benign and malignant human breast tissues were prepared and treated with an acidic buffer (pH 3) to remove bound uPA. After washing with PBS they were incubated with 125I-ATF (7 nM) and cross-linked using disuccinimidyl suberate as reported elsewhere (6). uPAR expression was determined by morphodensitometric scanning of autoradiograms as described above using HT1080 cells as an internal control for each gel.

**Western Blotting and Immunoperoxidase Staining.** Western blot analysis of proteins from nuclear extracts and whole cell lysates was carried out using a standard procedure (23). Sp1 was detected using 1 μg/ml anti-Sp1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and uPAR was detected with 5 μg/ml anti-uPAR R4 monoclonal antibody, generously provided by Dr. G. Hoyer-Hansen (the Finsen Laboratory, Righospitalet, Copenhagen, Denmark). The immunoreaction was revealed by the enhanced chemiluminescence detection system (ECL) according to the manufacturer’s recommendations.

To determine the rate of proliferation, immunoperoxidase staining was performed as described previously (6) using the antibody Ki67 directed against a nuclear antigen expressed in G1, S, G2, or M phases of the cell cycle.

**Statistics.** The uPAR levels, Sp1 activity, and rate of proliferation were compared using simple regression analysis and Pearson’s coefficient of correlation. A probability value (P) lower than 0.05 was considered significant.

**Results**

**Sp1-binding Activity and uPAR Levels in Tissue Specimens.** Equal amounts of nuclear extracts from a total of 14 breast carcinomas and 5 benign breast lesions were incubated with a radiolabeled oligonucleotide probe containing the consensus sequence of Sp1 in the presence or absence of a large molar excess of unlabeled oligonucleotide. The resulting complexes were separated by SDS-PAGE and revealed by autoradiography. Table 1 summarizes the clinical data and pathological findings of the patients with breast cancer and benign breast lesions that we have analyzed.

We first tested whether breast carcinomas exhibit increased levels of Sp1 activity as compared with benign breast lesions. As shown in Fig. 1A, a major specific complex was observed in two invasive breast carcinoma samples (T2 and T8), whereas only a barely detectable Sp1 signal resulted from one of the fibroadenoma specimens (B15). The remaining two benign fibroadenomas (samples 18 and 19) and two fibrocystic diseases (samples 16 and 17) did not exhibit any Sp1 activity (not shown). Nuclear extracts from control HeLa cells sub-
Fig. 1. Sp1 DNA-binding activity and uPAR levels in breast carcinomas and benign breast lesions. A, 40 μg of nuclear extracts from two invasive breast carcinomas (T2 and T8) and one benign fibroadenoma (B15) or diluents (None) were incubated with a 32P-labeled consensus oligonucleotide for Sp1, in the presence (+) or absence (−) of a large molar excess (300-fold) of unlabeled Sp1 consensus oligonucleotide. The resulting complexes were analyzed by 8% gel electrophoresis and revealed by autoradiography. Ten μg of nuclear extracts from control HeLa cells were subjected to the same procedure. B, nuclear extracts from breast carcinomas or diluents (None) were subjected to gel-shift assay as described for A. Five representative samples exhibiting low (T7), intermediate (T4, T5), and high (T2, T3) levels of Sp1-binding activity are shown. Nuclear extracts from HeLa cells were included for relative quantitation. C and D, 50 μg of acid-treated membrane fractions from breast carcinomas (T2, T3, T4, T7, and T8) and a benign fibroadenoma (B15), 25 μg of HT1080 membranes or diluents (None) were incubated with 125I-ATF in the presence (+) or absence (−) of an excess unlabeled urokinase. Cross-linking was performed with 1 μM disuccinimidyl suberate for 20 min at 4°C, and the resulting products were analyzed by 10% SDS-PAGE under reducing conditions followed by autoradiography. Arrow, the position of the M, 70,000 125I-ATF-uPAR complex.

Table 2 summarizes the results of the cross-linking experiments and electrophoretic mobility shift assays, as obtained by morphodensitometric analysis of autoradiograms. The levels of uPAR and Sp1 activity were expressed as a percentage of the HT1080 and HeLa cells content, respectively. This quantitative analysis could not be applied to a few cases because of the low intensity of the signal (samples 13 and 14) or an unsuitable internal standard (samples 1 and 6). A positive and significant correlation was found between uPAR levels and Sp1 activity in breast carcinomas (Pearson’s coefficient of correlation, r, 0.94; P < 0.001).

No significant correlation was found between Sp1 activity and uPAR levels with any of the known prognostic factors of breast cancer, including tumor size, nuclear grade, and lymph-node and hormone-receptor status, reported in Table 1. Furthermore, although high levels of Sp1 activity were observed in highly proliferative breast carcinomas, the correlation between Sp1 activity and the rate of proliferation was not statistically significant (r, 0.71; P = 0.07). On the contrary, a direct and significant correlation was found between uPAR levels and the rate of proliferation (r, 0.82; P < 0.01).

Coordinate Up-Regulation of Sp1 Activity and uPAR Expression by uPA. To test the possibility that uPA may regulate the levels of uPAR and Sp1 activity in a receptor-dependent and coordinate manner, four malignant tumor cell lines were analyzed. First, nuclear extracts from HeLa cells, HT1080 fibrosarcoma cells, and MDA-MB-231 and MCF-7 breast carcinoma cell lines were subjected to a gel-shift assay for the detection of Sp1 activity. In all of the cases, this procedure resulted in the formation of a major complex that was

We have previously shown (6, 24) that malignant breast lesions overexpress uPARs and that the relative abundance of uPAR can be estimated by quantitative cross-linking using 125I-ATF. In agreement with previous findings (25), in all of the tissue samples, a single and specific band at Mr 70,000 with varying intensity was detected (Fig. 1, C and D). The electrophoretic mobility of the 125I-ATF-uPAR complex was similar to that exhibited by HT1080 cells, and 125I-ATF binding was competed for by an excess of unlabeled urokinase in both cell and tissue samples (Fig. 1, C and D). A detectable, although low, amount of 125I-ATF-uPAR complex was found in the B15 fibroadenoma specimen (Fig. 1C) and a fibrocystic disease sample (sample 16; not shown).
competed for by an excess of unlabeled Sp1 consensus oligonucleotide. As shown in Fig. 2A, different levels of Sp1 activity were found in the four cell lines tested, as follows: HT1080 > HeLa > MDA-MB-231 > MCF-7. These binding data reflect Sp1 protein relative levels as assessed by Western blot analysis of the same samples (data not shown). The relative abundance of uPA receptors was assessed by cross-linking 125I-ATF to acid-pretreated membrane fractions from the same cell lines. According to previous findings (6, 26), a single and specific band at Mr 70,000 was detected in all of the cell lines tested, although the amount of the complex varied among samples, i.e., HT1080 > HeLa > MDA-MB-231 > MCF-7 (not shown).

The relative levels of uPAR and Sp1 activity in tumors and in cell lines suggested the possibility of a coordinate regulation. To gain insights into this regulatory mechanism, we tested whether uPA may activate Sp1, thereby stimulating uPAR promoter. Therefore, MDA-MB-231 and MCF-7 human breast cancer cells were incubated with 10 nM recombinant single-chain pro-uPA for different times, and the relative nuclear extracts were subjected to the gel-shift assay. Treatment with pro-uPA caused a marked increase of Sp1-binding activity in both MDA-MB-231 (Fig. 2B) and MCF-7 (not shown). In both of the cell lines, the elevation of Sp1 activity reached a maximum at 30 min and returned to near baseline level by 6 h. The same results were obtained after cell exposure to ATF, which indicated the independence of such an effect from the uPA catalytic activity (not shown).

To test whether the enhancement of Sp1 activity indeed corresponded to

Table 2. uPAR levels, Sp1 binding activity and rate of proliferation of 14 breast carcinomas and 5 benign breast lesions

<table>
<thead>
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<th>Patient no.</th>
<th>uPAR (%)</th>
<th>Sp1 (%)</th>
<th>Rate of proliferation (%)</th>
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<td>14</td>
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<td>Pos</td>
<td>10</td>
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<td>Benign breast lesions</td>
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<td>11</td>
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<td>19</td>
<td>Neg</td>
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* Pos, although a specific signal was detected, the morphodensitometric analysis could not be performed; ND, not determined; Neg, no specific signal was detected by cross-linking or gel-shift assay.

**Fig. 2. Coordinate up-regulation of Sp1 activity and uPAR expression by uPA.** A, 10 μg of nuclear extracts obtained from HeLa cells, HT1080, MDA-MB-231, MCF-7 cell lines or diluents (None) were subjected to gel-shift assay using the 32P-labeled oligonucleotide for Sp1. In this case, a large molar excess of unlabeled oligonucleotide for either Sp1 or AP1 consensus sequences were used as competitors. B, MDA-MB-231 cells were incubated with 10 nM recombinant single-chain pro-uPA (uPA) for different times. Nuclear proteins were then extracted and subjected to gel-shift assay with 32P-labeled oligonucleotide for Sp1, in the presence (+) or in the absence (−) of excess unlabeled competitor. C, the same nuclear extracts from MDA-MB-231 and HeLa cells analyzed in B were subjected to Western blot analysis with an anti-Sp1 polyclonal antibody. As expected, two species at Mr 97,000 and 105,000 resulted in all of the samples (arrow). However, a transient increase of the band at Mr 70,000 was observed at 30 min. D, MDA-MB-231 cells were incubated with 100 nM mitramycin (+) or diluents (−) for 18 h and then treated with 10 nM uPA for 0, 5, and 10 h. Whole lysates were analyzed by 10% SDSPAGE, and uPAR was detected by immunoblotting with the anti-uPAR monoclonal antibody R4.
an increase in the nuclear levels of Sp1 protein, the same nuclear extracts were analyzed by Western blotting. Two species at M, 97,000 and 105,000, corresponding to the described dephosphorylated and phosphorylated forms of Sp1 (15, 16), respectively, were detected in all of the samples (Fig. 2C). In agreement with the previous findings, a transient increase of the dephosphorylated form of Sp1 was observed at 30 min.

Next, whole cell lysates obtained from MDA-MB-231 cell lines exposed to uPA were analyzed for their uPAR content by Western blotting. A marked increase of uPAR expression was observed at 5 h after the addition of uPA (Fig. 2D). Treatment with catalytically inactive diisopropyl-fluorophosphate-inactivated uPA resulted in a similar uPAR induction that was prevented by the addition of cycloheximide, which indicated that uPAR up-regulation is due to neotranslation (not shown). Pretreatment with mithramycin, a competitive inhibitor of Sp1 DNA-binding activity, strongly reduces the extent of basal and induced uPAR (Fig. 2D).

Discussion

The present study shows that Sp1-binding activity and uPAR expression are coordinately up-regulated in breast cancer. We also report that urokinase binding to its cognate receptor enhances both Sp1 activity and uPAR protein levels in human breast carcinoma MDA-MB-231 and MCF-7 cell lines.

Alteration of transcription factor function has now been established as a frequent cause of neoplastic transformation. However, little is known about how dysfunction of transcriptional activators and consequent alteration of specific transcription factors lead to specific tumor phenotypes. In the present study, we report that uPAR ligation with uPA first enhances Sp1 activity and, subsequently, uPAR protein. This observation suggests that Sp1 may play an important role in driving induced uPAR mRNA transcription either directly or indirectly via other transcriptional activators. In any event, this finding highlights a regulatory circuitry triggered by uPA that may substantially contribute to the enhancement of cell invasiveness during breast tumor progression. The relevance of this ligand-receptor system to the malignant phenotype is shown by the finding that elevated levels of uPA and uPAR in several types of cancer are associated with a higher relapse rate and with a poor prognosis (27, 28). In agreement with these observations, we have previously reported that uPA and uPAR are overexpressed in breast carcinomas with respect to benign lesions (6) and that engaged uPARs are present on the surface of malignant epithelial cells (26).

The data presented here do not exclude the contribution of other growth factor-dependent pathways to the Sp1-dependent regulation of uPAR gene, because there is great evidence for transcriptional and posttranscriptional regulation of uPAR by a variety of cytokines and tumor promoters (4, 29). To date, the signaling mechanisms mediating such a response have not been completely elucidated and include activation of protein kinase C, increased mRNA stability, and increased levels or activity of transcription factors (30–33). Previous studies have shown that uPAR, despite the lack of a cytoplasmic domain, is able to trigger intracellular signals by modulating the activity of some Src family kinases, such as p56/crk and p55crk in myeloid cells (34, 35). Activation of p56crk has been also reported in HT1080 fibrosarcoma cells on binding of catalytically inactive uPA to uPAR (36). The strict relationship between uPAR, Src kinases, and Sp1 activity is further supported by the finding that a constitutively active Src kinase up-regulates uPAR gene expression and enhances Sp1-binding activity in the SW480 colon cancer cell line (8). In the emerging picture, uPA may be the trigger of a signaling pathway, involving Src kinases and Sp1, that impinges on the uPAR gene and results in an amplification of the uPA/uPAR axis. The possibility to interfere at any level with this uPA-dependent autogenerating loop is of relevance for designing new antimetastatic drugs.

The use of GC-specific DNA-binding drugs that inhibit the Sp1-binding to regulatory elements (such as mithramycin) is at hand (21, 37). Mithramycin has been used in the treatment of acute hypercalcemia associated with malignancy (38) as an inhibitor of osteoclastic activity, especially in patients with osteolytic bone lesions of breast carcinoma and myeloma. However, the wide variety of genes that contain an Sp1 regulatory element in their promoter and the consequent limited specificity of mithramycin for the relevant target sequences have narrowed the therapeutic range of this drug and limited its clinical use. The availability of Sp1 competitors with a greater sequence specificity than mithramycin would hopefully result in a more effective and less toxic interference with the mechanisms of cancer invasion and metastasis.

Interestingly, a role for receptor-bound uPA in angiogenesis is emerging because uPA and plasmin activities are required for the outgrowth of capillary-like structures in a fibrin gel (39). The dependence of blood-vessel formation and tumor growth on the extracellular matrix degradation and cell migration is a target for the development of novel anticancer strategies, as shown by the metalloprotease inhibitors in late-stage Phase III trials (40). Sp1 is also involved in tumoral angiogenesis because it mediates the induction of vascular endothelial growth factor (VEGF) and its receptor-2 by tumor necrosis factor α (41, 42). It is likely that future cancer therapies will use a combination of drugs to interfere with proteolysis, adhesion, angiogenesis, and other mechanisms that are proven to be essential for the establishment and the progression of tumors in vivo.

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References


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