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ABSTRACT

Urokinase plasminogen activator (uPA) expression in breast cancer is associated with relapse and a reduction in disease-specific survival. Thus, efforts are under way to identify uPA inhibitors. By screening a chemical library of >1000 compounds, 17-allylamino geldanamycin (17AAG) was identified as a potent inhibitor of uPA by the National Cancer Institute and is now in Phase I clinical trials. At this time, it remains unclear how 17AAG blocks uPA; one possibility is through disruption of the insulin-like growth factor I receptor (IGF-IR) pathway. This would be consistent with studies from our laboratory showing that activation of IGF-IR results in the induction of uPA protein. In the study described herein, we observed that IGF-IR and uPA were highly expressed in 87 and 55% of breast cancer by screening tumor tissue microarrays representing 930 cases. A significant proportion (52.1% = 354 of 680 cases, Ρ < 0.0001) of the patients had tumors expressing both proteins. uPA alone (Ρ = 0.033) or in combination with IGF-IR (Ρ = 0.0104) was indicative of decreased disease-specific survival. Next, we demonstrated that treating MDA-MB-231 cells with increasing concentrations of 17AAG resulted in IGF-IR degradation (IC50 = 1.0 μM) and blocked signal transduction through the Akt and mitogen-activated protein kinase pathways. Finally, we found that 17AAG had a robust inhibitory effect on the production of uPA mRNA and protein in the presence of IGF-I. Thus, our study raises the possibility that 17AAG could prove to be an effective therapeutic agent for a large number of breast cancer patients by inhibiting the IGF-IR and ultimately uPA.

INTRODUCTION

The invasion protease urokinase plasminogen activator (uPA) is a promising therapeutic target in breast cancer based on several translational research initiatives. Basic research efforts reveal that uPA is integral to angiogenesis and breast cancer progression (1). This observation was carried through to a preclinical model where inhibition of uPA with a synthetic peptide in combination with Tamoxifen ultimately uPA. It remains unclear how 17AAG inhibits uPA and reduces tumor formation, but one possibility is by disrupting the insulin-like growth factor I receptor (IGF-IR) pathway. Our laboratory previously reported that uPA is induced by activation of IGF-IR (12) and subsequently determined this to be through the convergence of the phosphotyrosylinositol 3-kinaseAkt and mitogen-activated protein kinase (MAPK) pathways (13). We also discovered that Herbimycin A, which is structurally related to 17AAG, inhibits the induction of uPA by IGF-I through the inhibition of these pathways (13). Thus, a key objective of the study presented herein was to assess uPA and IGF-IR expression in clinical breast tumor specimens and determine whether their presence relates to survival. To achieve this, we screened tumor tissue microarrays (TMAs) representing 930 breast cancer patients. We also explored the possibility that 17AAG degrades IGF-IR as it does other tyrosine kinase receptors relevant to breast cancer such as Her-2 (14) and c-met (7). Finally, we sought to determine whether 17AAG inhibits uPA protein production in the presence of IGF-I.

MATERIALS AND METHODS

Tumor TMAs

TMAs were constructed from formalin-fixed, paraffin-embedded breast tissues sent to the Vancouver Hospital during the period 1976–1990. Biopsy
tissue was retrieved from 930 patients with stage I, II, or III breast cancer, who had participated in four British Columbia Cancer Agency clinical trials. Clinical data and outcome, including all breast cancer recurrences and deaths, were available for all patients. Mean and median follow-up time from original diagnosis until analysis, for patients still alive at the time of analysis, was 17.4 years (range, 9.8–28.1 years). Tumor grade was not included in the analysis because the standards for distinguishing this parameter changed during the recruiting period. To construct TMAs, a pathologist confirmed the presence and location of breast carcinoma in the archival tissue blocks and transferred 0.6-mm tissue cores (one from each patient) to recipient blocks using a Beecher Instruments tissue arrayer. In breast cancer tissue microarray series, single cores have been shown to be sufficiently representative to make accurate comparisons among biomarkers and correlations with clinical outcome data (15, 16). Three TMAs containing 333, 334, and 336 cores (including control tissues) from a total of 930 patients were constructed.

uPA and IGF-IR Immunohistochemistry

Immunohistochemical staining for uPA was performed on TMAs. Endogenous peroxidases were blocked with 3% H2O2 for 30 min at room temperature, and nonspecific binding was inhibited with 10% normal horse serum plus 1% BSA for 30 min. Samples were then incubated at room temperature overnight with the uPA antibody (mouse monoclonal, MoAB 3689; American Diagnostica, Greenwich, CT), diluted 1:1000. The secondary antibody (biotinylated goat antirabbit, diluted at 1:600, incubated for 30 min. Visualisation was achieved using a streptavidin–biotin-horseradish peroxidase labelling system from Vectastain (Vector Laboratories, Burlingame, CA) followed by incubation with diaminobenzidine tetrahydrochloride for 6 min. Negative control slides were incubated with normal mouse serum in place of the primary antibody.

IGF-IR protein expression was identified using a polyclonal antibody to the β-subunit of IGF-IR (C20; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100. Optimal IGF-IR detection required antigen retrieval: 0.1 M citrate buffer (pH 6.0) was heated to boiling, and the slides were incubated 5 min. This step was repeated; slides were then rinsed in tap water and PBS. Slides were blocked as indicated above then incubated with the IGF-IR antibody for 1 h at room temperature. Negative control slides used normal rabbit serum in place of the primary antibody. The secondary antibody was biotinylated goat antirabbit, diluted at 1:600, incubated for 30 min, then visualized with diaminobenzidine tetrahydrochloride. Under these conditions, 12 of 12 colorectal adenocarcinomas in tissue microarray format gave moderate to positive IGF-IR immunostaining, consistent with results reported by others (17). When tested on multiple tissue microarrays (18), IGF-IR immunostaining was consistently negative in normal skin, brain, lung, esophagus, pancreas, spleen, ovary, bladder, and in a set of 17 soft tissue and brain tumors. TMA slides were analyzed, and the intensity of the immunostaining in tumor cells was scored based on the following system: 0, negative; 1, focal and/or weak staining only; 2, moderately positive; and 3, strongly positive in the majority of the tumor cells of the tissue core. Interobserver agreement between at least two of three investigators was considered as a final score. Cores scored as 0 or 1 was considered negative for the tested marker, 2 or 3 as positive. Cores that did not contain tumor cells or lacked tissue were excluded. For detection of the estrogen receptor (ER), the slides were pretreated with citrate buffer (pH 6.0) as an antigen retrieval step. The ER was then stained at a 1:50 dilution with the ID5 monoclonal antibody (Dako) using an automatic stainer.

Statistics

We performed Fisher’s exact test to evaluate the correlation between IGF-IR and uPA staining, and survival analyses, using SPSS 11.0 software (SPSS, Inc., Chicago, IL). Survival time was calculated from the date of breast cancer diagnosis until the date of death. For disease-specific survival (DSS), deaths due to causes other than breast cancer were censored. In univariate analyses, Kaplan-Meier curves and survival estimates were calculated for each outcome, and log-rank statistics were used to test for differences between groups. In multivariate analyses, a proportional hazards (Cox regression) model was fitted and Wald’s statistic used to assess each variable’s effect. Statistical significance was declared if the P from a two-tailed test was <0.05.

<table>
<thead>
<tr>
<th>Tumor size</th>
<th>n</th>
<th>% of total cases</th>
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<tbody>
<tr>
<td>&lt;2 cm</td>
<td>112</td>
<td>13</td>
</tr>
<tr>
<td>2.5–5 cm</td>
<td>620</td>
<td>71</td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>146</td>
<td>17</td>
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* a The percentage was calculated only for the total number of known cases.

b ER, estrogen receptor; IHC, immunohistochemistry; IGF-IR, insulin-like growth factor 1 receptor; uPA, urokinase plasminogen activator.

Immunoprecipitation and Western Blots

The MDA-MB-231 cells were serum starved for 24 h, treated with varying concentrations of 17AAG (0, 0.01, 1.0, and 10 μM) for 24 h and pulsed with IGF-I (100 ng/ml; GroPep, Adelaide, Australia) for 30 min to initiate IGF-IR-mediated signal transduction. IGF-IR was immunoprecipitated with α-I3 antibody (Calbiochem, Cambridge, MA) and detected with the C20 antibody (Santa Cruz Biotechnology), as described previously (19). AKT, phosphorylated AKT, extracellular signal–regulated kinase (ERK)1/2, phosphorylated ERK1/2 (Cell Signaling, Beverly, MA), and phosphorylated glycogen synthase kinase-3 (Upstate Biotechnology, Inc.) were detected by immunoblotting with antibodies against the respective proteins. The antibodies were rabbit polyclonal except Grb2 (Transduction Laboratories, Lexington, KY).

MDA-MB-231 cells were serum starved for 24 h and treated with increasing doses of 17AAG (0, 0.01, 1.0, 10 μM) for 24 h and evaluated for ubiquitination of the IGF-IR complex. 17AAG was a generous gift from Dr. Len Neckers, National Cancer Institute/National Institute of Health (Bethesda, MD). The IGF-IR complex was immunoprecipitated and analyzed by Western blot analysis with an ubiquitin antibody (Santa Cruz Biotechnology). This series of Western blot analyses was done on prepoured 4–12% gradient gels (Invitrogen-Life Technologies, Inc.) to achieve maximal separation Mf >80,000.

uPA mRNA and Protein Detection

uPA mRNA Detection. The MDA-MB-231 cells were plated in a 100-mm dish at a density of 1.4 × 106 and allowed to adhere for 24 h before serum starvation. The cells were subsequently treated with increasing amounts of 17AAG for 24 h, then IGF-I was added for 30 min, and RNA was isolated using the RNA easy kit (Qiagen, Valencia, CA). RNA (1 μg) was reverse transcribed using Superscript II reverse transcriptase and oligodeoxythymidylic acid primers (Invitrogen) in a 20-μl final volume. PCR was performed on 2 μl of the cDNA in a total volume of 50 μl, using TaqDNA polymerase (Invitrogen) and uPA primers (forward primer, CTGTGAACGTCTAATG-GAGG; reverse primer, GACGATGTAGTCCTCCTTCTT; a generous gift from Dr. Peter Leung, University of British Columbia). Monoclonal, Imperial Cancer Research Fund gene no. 2 was amplified as an endogenous control using the following primers (forward primer, ATGACTTTGACTTAG-
GAGATGC; reverse primer, CGACAGCCCCCACCACAATCCCG). Amplification was as follows: 5 min at 94°C, 27 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C, then 10 min at 72°C (20). Monoclonal, Imperial Cancer Research Fund gene no. 2 is routinely analyzed in our Molecular Pathology Laboratory (Children’s Hospital, Vancouver, British Columbia, Canada) because after testing several housekeeping genes, we determined that it was the most reliable.

**uPA Protein Detection.** MDA-MB-231 cells plated 5000/96-well and allowed to attach overnight. The media was removed the next day and replaced without or with 17AAG (0.01, 0.1, 1.0, and 10μM) for 24 h. The next day, the media was replaced with fresh 17AAG (same concentrations) in DMEM: Ham’s F-12 along with IGF-I (100 ng/ml Long R; Gro-Pep), and the cells were incubated for another 24 h to allow for uPA protein production and secretion. Cells that received IGF-I only were also treated with DMSO as a vehicle control for 17AAG. Conditioned media was removed and stored at −30°C until tested. The uPA protein was quantified by ELISA (American Diagnostica). The statistical significance of each treatment was assessed using the Student t test. To ensure that 17AAG was not having a cytotoxic effect, we evaluated the viability of the cells using the Celltiter 96 Aqueous cell proliferation/survival assay (modified MTS assay, Promega). We measured cell survival in the presence of increasing concentrations of the 17AAG (1 or 10 μM) or DMSO for 24 h. Each sample was replicated six times.

**RESULTS**

uPA Is Commonly Overexpressed with IGF-IR in Primary Breast Cancer. TMAs were evaluated for both uPA and IGF-IR proteins to assess whether they were coordinately expressed in human breast cancer specimens. The clinical characteristics of the patients and protein expression results are displayed in Table 1. Patient median age at the time of surgery was 46.2 years (range, 22–86 years). We were able to interpret IGF-IR immunostaining for 707 cases (92 negative, 615 positive). IGF-IR was highly expressed in 87% of the cases (615 of 707, Table 1). Similarly, uPA was evaluated in 743 cases and was highly expressed in 55% (335 negative, 408 positive; Table 1). We then went on to examine the number that expressed both uPA and IGF-IR. Our analyses revealed a significant association between uPA and IGF-IR expression (P < 0.0001; Table 2), which were coordinately expressed in 354 of 680 cases (52.1%). Examples of coordinate IGF-IR and uPA expression are illustrated in Fig. 1. In the first case, both IGF-IR and uPA were highly expressed in serial sections taken from the same patient (Fig. 1, A and B, respectively). Likewise, serial sections of tissues from another patient revealed correspondingly low IGF-IR and uPA expression (Fig. 1, C and D, respectively).

We then addressed whether patients who expressed both uPA and IGF-IR were less likely to survive long term. To achieve this, we retrospectively tracked the patient cohort over a 20-year period. The potential relationship between DSS and the tumor markers alone or in combination with each other was assessed. In a univariate analysis, uPA was associated with poor DSS (P = 0.033, Fig. 2A). In a multivariate analysis, the significance of uPA persisted after DSS was
adjusted for tumor size, nodal, and ER status ($P = 0.0343$). In contrast, IGF-IR was not significantly associated with DSS in either univariate ($P = 0.068$) or multivariate ($P = 0.2411$) analyses (data not shown). Next, we considered the impact of both proteins on DSS. Interestingly, DSS was significantly reduced for patients who were uPA positive and expressed IGF-IR ($P = 0.0104$, Fig. 2B). In contrast, if IGF-IR was not expressed, uPA was not associated with survival ($P = 0.9306$, Fig. 2C). In a multivariate analysis, the interaction of uPA and IGF-IR was not significant ($P = 0.3827$), although this might be because of the limited number of tumors that were uPA positive and IGF-IR negative. On the basis of the profound difference in the survival curves for patients with positive and negative IGF-IR tumors, we conclude that the TMAs provide supportive evidence for a link between IGF-IR and uPA in primary breast cancer, with adverse prognostic implications. Our data are strengthened by the fact that we screened patients over an extended period of time. These observations suggest that inhibitors that target IGF-IR and uPA might have therapeutic potential for the treatment of a large proportion of human breast cancers.

17AAG Inhibits IGF-IR/AKT/MAPK-Mediated Induction of uPA. In vitro experiments were performed using MDA-MB-231 breast cancer cells because they are both highly malignant and produce elevated levels of uPA in response to IGF-I. Initially, the effect of 17AAG was evaluated by assessing IGF-IR and downstream signaling intermediates in the phosphotyrosidinositol 3-kinase/Akt pathway. We observed that 17AAG treatment resulted in the degradation of IGF-IR protein in a dose-dependent manner (Fig. 3A). Because half of the amount of IGF-IR was degraded by 1.0 µM 17AAG, we estimated this to be the IC$_{50}$. Next, we investigated the effect of 17AAG on IGF-IR initiated signal transduction through the phosphotyrosidinositol 3-kinase/AKT pathway. We found that total AKT

![Fig. 2. Disease-specific survival (DSS) for breast cancer patients with tumors that expressed urokinase plasminogen activator (uPA) and/or insulin-like growth factor I receptor (IGF-IR). A, in a univariate analysis, DSS was lower for patients with tumors overexpressing (relative to normal) uPA ($P = 0.0331$). B, when IGF-IR is expressed, uPA positive patients show a decreased DSS ($P = 0.0104$). C, when IGF-IR was not expressed, uPA was not related to DSS ($P = 0.9306$). There was no significant difference in mean patient age between the groups.](image1)

![Fig. 3. A, 17-allylaminoelliamycin (17AAG) degrades insulin-like growth factor I receptor (IGF-IR) in a dose-dependent manner. MDA-MB-231 breast cancer cells were pretreated with varying concentrations of 17AAG (Lane 1: DMSO carrier only; Lanes 2–5: 0.01, 0.1, 1, or 10 µM 17AAG) for 24 h before the cells were pulsed with IGF-I (100 ng/ml for 30 min). Lysates were immunoprecipitated for IGF-IR protein or evaluated by immunoblotting for AKT, phosphorylated AKT (P-AKT) or phosphorylated glycogen synthase kinase-3 (P-GSK). B, 17AAG induces ubiquitination of IGF-IR receptor complex in a dose-dependent manner. MDA-MB-231 cells were treated with increasing concentrations of 17AAG (Lane 1: DMSO carrier only; Lanes 2–5: 0.01, 0.1, 1, or 10 µM 17AAG) for 24 h. Lysates were immunoprecipitated for IGF-IR receptor and evaluated for IGF-IR (top panel) and ubiquitin (bottom panel).](image2)
was degraded in a dose dependent manner (Fig. 3A). Likewise, levels of phosphorylated AKT and phosphorylated glycogen synthase kinase-3 were also reduced in a dose-dependent manner after 17AAG treatment (Fig. 3A).

Because the MAPK pathway is also stimulated by IGF-I and is involved in uPA production (13), we decided to examine the effect of 17AAG on ERK1/2. Although total Erk1/2 was not degraded by 17AAG, the drug did suppress phosphorylated Erk1/2 in response to IGF-I (Fig. 3A). This suggests that 17AAG can block signal transduction in the absence of proteolysis and is specific for individual signaling intermediates.

We went on to determine how 17AAG degrades IGF-IR by looking for changes in ubiquitination. The cells were treated with increasing concentration of 17AAG as described above, then the IGF-IR was immunoprecipitated and probed for ubiquitin. IGF-IR was ubiquitin-tagged in response to 17AAG as compared with the DMSO control (Fig. 3A). A statistically significant reduction in uPA was observed after 48 h of exposure to 17AAG as indicated by the asterisks. Significance was determined using the Student’s t test; *, P < 0.05.

DISCUSSION

In this study, we found a link between IGF-IR and uPA in primary breast cancers. We also identified 17AAG as a drug that degrades IGF-IR and its signaling intermediate Akt. When cells were exposed to increasing concentrations of 17AAG, the loss of signaling through Akt and MAPK blocked the induction of uPA by IGF-I. Furthermore, we show that IGF-IR degradation may be mediated through the ubiquitin-proteasome pathway as we find increased ubiquitination of the receptor complex after 17AAG treatment. Our data are consistent with the observations that the parent compound of 17AAG, geldanamycin (21), and a related ansamycin antibiotic, Herbinymicin A (22), also result in the degradation of IGF-IR. To this effect, the exposure of MCF-7 cells to 1 μM geldanamycin (21) and 5 μg/ml Herbinymicin A (22) significantly degrades IGF-IR after exposure for 24 h. It was also shown that the mechanism whereby Herbinymicin A induced IGF-IR degradation is via the 20S proteasome pathway (22). It therefore appears that degradation of IGF-IR by ansamycin antibiotics is generally through ubiquitination and subsequent proteolysis.

The results from our study demonstrate that TMAs can be used to reliably screen uPA protein in formalin fixed tissues. This is in contrast to the traditional method of measuring uPA protein from tumor extracts by ELISA (23). The TMA platform provides a rapid means of screening large numbers of patients for uPA expression in a fraction of the time it would take to analyze the protein by ELISA. This approach also provides a new avenue to pursue possible regulators of uPA as we have done by evaluating IGF-IR expression. TMAs can also be used to determine what proportion of breast cancers that might benefit from uPA inhibitors such as 17AAG. For example, we find that IGF-IR is expressed in 87% of the breast cancers evaluated, and it positively regulates uPA (12) but is blocked by 17AAG.
REFERENCES


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