Tamoxifen Induces Heparanase Expression in Estrogen Receptor – Positive Breast Cancer

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Abstract

Purpose: Mammalian heparanase degrades heparan sulfate, the main polysaccharide of the basement membrane. Heparanase is an important determinant in cancer progression, acting via the breakdown of extracellular barriers for invasion, as well as release of heparan sulfate – bound angiogenic and growth-promoting factors. The present study was undertaken to elucidate molecular mechanisms responsible for heparanase overexpression in breast cancer.

Experimental Design: To characterize heparanase regulation by estrogen and tamoxifen and its clinical relevance for breast tumorigenesis, we applied immunohistochemical analysis of tissue microarray combined with chromatin immunoprecipitation assay, reverse transcription-PCR, and Western blot analysis.

Results: A highly significant correlation ($P < 0.0001$) between estrogen receptor (ER) positivity and heparanase overexpression was found in breast cancer. Binding of ER to heparanase promoter accompanied estrogen-induced increase in heparanase expression by breast carcinoma cells. Surprisingly, heparanase transcription was also stimulated by tamoxifen, conferring a proliferation advantage to breast carcinoma cells grown on a naturally produced extracellular matrix. Heparanase overexpression was invariably detected in ER-positive second primary breast tumors, developed in patients receiving tamoxifen for the initial breast carcinoma. The molecular mechanism of the estrogenlike effect of tamoxifen on heparanase expression involves recruitment of transcription coactivator AIB1 to the heparanase promoter.

Conclusions: Heparanase induction by ligand-bound ER represents an important pathway in breast tumorigenesis and may be responsible, at least in part, for the failure of tamoxifen therapy in some patients. Our study provides new insights on breast cancer progression and endocrine therapy resistance, offering future strategies for delaying or reversing this process.

Heparanase is an endo-β-D-glucuronidase enzyme which cleaves heparan sulfate, the main polysaccharide component of the basement membrane and extracellular matrix (ECM; refs. 1 – 6). Heparan sulfate is a key element participating in the self-assembly, insolubility, and barrier properties of the basement membrane (7 – 9). In addition, heparan sulfate moieties in the ECM are responsible for specific binding of members of the heparin-binding family of growth factors [i.e., basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF)] and serve as the extracellular reservoir for these factors (7, 8, 10, 11). Thus, heparan sulfate – bound growth and angiogenic factors are protected, stabilized, and sequestered from their site of action, but upon enzymatic degradation of heparan sulfate, can be readily mobilized to induce growth factor – dependent processes (i.e., neovascularization and tumor growth). Therefore, cleavage of heparan sulfate in the ECM by heparanase is an important event in cancer progression, leading to the disassembly of extracellular barriers, enabling cell invasion, and releasing heparan sulfate – bound bioactive angiogenic and growth-promoting factors sequestered in the ECM (2, 4, 12, 13). Although clearly shown in a variety of cancer types (14 – 21), heparanase involvement in breast carcinoma progression is particularly well documented. Heparanase promoter activity, gene expression, and enzymatic activity correlate with the aggressiveness of human breast carcinoma cell lines (1, 2, 22). Elevated levels of heparanase were found in body fluids of patients with active breast cancer disease versus healthy donors (11). Immunohistochemical detection of heparanase in breast cancer biopsy specimens was associated with a larger primary tumor size and the presence of metastases (23). In addition, heparanase expression was more frequently found in invasive breast carcinoma than in non-/preinvasive

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ductal carcinoma *in situ* (24). Within the latter group, heparanase expression correlated with more aggressive subtypes (25). Mammary tumors produced in mice by breast cancer cells genetically engineered to overexpress heparanase grew significantly faster than tumors produced by control cells (26, 27). The enhanced primary tumor growth was accompanied by augmented vascularization and a higher degree of vessel maturation (27). Moreover, heparanase gene silencing or enzymatic inhibition in breast carcinoma cells significantly reduced their invasive (12) and metastatic (28) abilities. Although these experiments, together with corroborative evidences obtained from studies of clinical specimens of human breast cancer (11, 22–25), convincingly show involvement of heparanase in breast tumorigenesis, molecular mechanisms responsible for heparanase overexpression in breast cancer are only partially understood. Several transcription regulators (i.e., EGR1, ETS1 and 2, mutant p53) were shown to activate heparanase promoter *in vitro* (29–31); however, the relevance of these findings to breast tumor progression in animal models or in human patients remains to be shown. Recently, we reported that estrogen treatment induced heparanase gene transcription in estrogen receptor α (ERα)–positive breast cancer cells *in vitro*. Heparanase seems to serve as a novel downstream estrogen effector, promoting pathologic tumor-stromal interactions (i.e., ECM degradation, angiogenesis) and thus contributing to the development and progression of breast cancer (32).

In the present study, we characterized interactions between ER and the heparanase gene regulatory sequence. Following demonstration of a direct binding of estrogen-liganded ER to heparanase promoter, we validated the clinical relevance of this finding, applying a breast carcinoma tissue microarray, and showed a highly significant correlation between ER positivity and heparanase induction in breast cancer. We also revealed that tamoxifen, prescribed as the endocrine treatment of choice for ER-positive breast cancer, exerts estrogenlike stimulatory effect on heparanase expression in two ER-positive breast carcinoma cell lines. Tamoxifen-bound ER was found to recruit to the heparanase promoter ER coactivator AIB1 (amplified in breast cancer-1; ref. 33), providing an explanation for altering tamoxifen activity from an antagonist to agonist. The agonistic effect of tamoxifen on heparanase expression was further supported by immunohistologic findings in tissue specimens of a second ER-positive breast cancer that developed in patients receiving tamoxifen treatment for their initial breast tumor. In light of the well-documented role of heparanase in breast tumorigenesis (1, 2, 22–27), the estrogenlike effect of tamoxifen on heparanase expression may contribute, at least in part, to the lack of benefit observed in a non-negligible fraction of tamoxifen-treated breast carcinoma patients (34, 35). Detailed understanding of the mechanism through which estrogen and tamoxifen affect heparanase transcription is expected to provide new insights on breast cancer progression, as well as to suggest new strategies for delaying or reversing this process.

Materials and Methods

Reagents. Estrogen (17-β-estradiol) and an active cellular metabolite of tamoxifen, 4-hydroxy-tamoxifen, were obtained from Sigma and dissolved in absolute ethanol. Pure ER antagonist ICI 182,780 was purchased from Tocris Cookson and dissolved in DMSO.

Cell culture and treatment. MCF-7 human breast carcinoma cells were kindly provided by Dr. G. Neufeld (Technion, Israel). T47D and MDA-MB-231 human breast carcinoma cells were obtained from the American Type Culture Collection. Cells were routinely maintained in RPMI 1640 (MCF-7) or DMEM (T47D, MDA-MB-231), supplemented with 1 mmol/L glutamine, 50 μg/mL streptomycin, 50 units/mL penicillin, and 10% FCS (Biological Industries) at 37°C and 7.5% CO_2. Before estrogen and/or tamoxifen treatment, cells were maintained for 4 days in phenol red–free medium supplemented with charcoal-stripped FCS (Biological Industries). Then, the medium was changed to serum-free medium, and estrogen or tamoxifen was added at concentrations indicated in Results. When cells were treated with estrogen together with either tamoxifen or ICI 182,780, the inhibitors were added 2 h before estrogen. Control cultures were treated with the corresponding vehicle alone.

Cultures of bovine corneal endothelial cells were established from steer eyes and maintained in DMEM (1 g of glucose/L) supplemented with 5% newborn calf serum, 10% FCS (Life Technologies), and 1 ng/mL fibroblast growth factor-2 (FGF-2) as described previously (2, 36). Confluent cell cultures were dissociated with 0.05% trypsin and 0.02% EDTA in PBS and subcultured at a split ratio of 1:8.

Preparation of dishes coated with basement membrane-like ECM. Bovine corneal endothelial cells were plated into 35-mm tissue culture dishes at an initial density of 2×10^5 cells/mL and cultured as described above, except that 4% dextran T-40 was included in the growth medium. On day 12, the subendothelial ECM was exposed by dissolving the cell layer with PBS containing 0.5% Triton X-100 and 20 mmol/L NH_4OH, followed by four washes in PBS (36). The ECM remained intact, free of cellular debris, and firmly attached to the entire area of the tissue culture dish (36).

Cell proliferation assay. MCF-7 and T47D cells were cultured in quadruplicates in 2-cm^2 dishes (0.5×10^5 cells per dish) in phenol red–free medium supplemented with charcoal-stripped FCS for 72 h. Then, estrogen, tamoxifen, and/or heparanase inhibitor ^{100}NA,RO-H (37) were added at concentrations indicated in Results. Forty-eight hours later, cells were washed with PBS and fixed to the plate with 4% formaldehyde in PBS (pH 7.4) for at least 2 h. After fixation, the plates were washed in 1% boric acid, and the cells were stained for 30 min with 1% methylene blue reagent (Sigma) in 1% boric acid. After extensive washing with tap water, the methylene blue stain was eluted by the addition of 500 μL of 1 mol/L HCl. The color intensity was measured at a wavelength of 620 nm.

In some experiments, the cells were seeded on ECM coated 35-mm culture dishes (5×10^4 cells per dish). Forty eight hours later, cells were harvested, diluted in isotone solution (Coulter), and counted in cell Coulter Z1 (CC Coulter Corporation, Scientific Instruments). Cell numbers were further confirmed by counting with a hemacytometer.

RNA isolation and reverse transcription-PCR. RNA was isolated with Tri-Reagent (Medical Research Council) according to the manufacturer's instructions. First, oligo(dT)-primed reverse transcription was done using 1 μg total RNA in a final volume of 20 μL, and the resulting cDNA was further diluted to 100 μL. The cDNA was amplified using Taq DNA polymerase (Promega). Comparative semiquantitative PCR was done as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was first amplified at low cycle number, applying GAPDH sense: 5′-CCACCCATGGCAAATTCCATGGCA-3′ and antisense: 5′-TCTAGACGG-CAGGTCAGGTCCACC-3′ primers. If needed, cDNAs were adjusted to obtain similar intensities for GAPDH signals with all the samples. The adjusted amounts of cDNA were subjected to PCR with the following primers: 355-sense: 5′-TTCGATCCCAAGGAATCAAC-3′ and 229-antisense: 5′-GTAGTGATGCCATGTAACTGAATC-3′, designed to amplify a 564-bp PCR product specific for human heparanase (2). The PCR conditions were an initial denaturation at 94°C for 2 min, denaturation at 94°C for 15 s, annealing for 45 s at 60°C, and extension for 1 min at 72°C (33 cycles). Aliquots (15 μL) of the amplified cDNA were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Intensity of each band was quantified using

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the Scion Image Program (Scion Corporation), and the results of three separate experiments are presented as band intensity relative to that of GAPDH. Only RNA samples that gave completely negative results in PCR without reverse transcriptase were used to rule out the presence of genomic DNA contamination.

Tissue microarray construction, immunostaining, and statistics. Formalin-fixed, paraffin-embedded breast carcinoma tissues from 214 nonselected invasive breast carcinoma patients (174 ductal, 21 lobular, 5 medullary, 8 mucinous, and 6 metaplastic) were available from the Department of Pathology, Hadassah Medical Center, Jerusalem. The use of these specimens and data in research were approved by the Ethics Committee of the Hadassah Medical Center. Sections (5 μm) stained with H&E were obtained to confirm the diagnosis and to identify representative areas of the specimen. From these defined areas, three tissue cores with a diameter of 0.6 mm were taken with a manual tissue arrayer MTA-1 (Beecher Instruments) as previously described (38). From each specimen, three tissue cores with a diameter of 0.6 mm were taken from the different regions of the tumor and arrayed in triplicate on a recipient paraffin block (39) and arrayed in triplicates on a recipient paraffin block. Sections of 5 μm of the recipient blocks were cut and placed on charged poly-lysine – coated slides. Immunodetection of heparanase was done as described (2) with minor modifications. Briefly, sections of the tissue array blocks were deparaffinized and rehydrated. Tissue was then incubated in 3% H_2O_2, denatured by boiling (3 min) in a microwave oven in citrate buffer (0.01 mol/L; pH 6.0), and blocked with 10% goat serum in PBS. Sections were incubated with polyclonal anti-heparanase antibody, raised against a peptide (^{105}DPKKESTFEERS^{116}) spanning the last five amino acids at the COOH terminus of the 8-kDa subunit and the first seven residues of the linking segment of the heparanase protein (40), kindly provided by Dr. Robert L. Heinrikson (Pfizer). We have also used polyclonal rabbit anti-heparanase antibody (733) directed against a synthetic peptide (^{158}KKFKNSTYRSSSVD^{171}) corresponding to the NH_2 terminus of the 50-kDa subunit of the heparanase enzyme (41). The antibody was diluted 1:100 in 10% goat serum in PBS. Control slides were incubated with 10% goat serum alone. Color was developed by using the Zymed AEC substrate kit (Zymed Laboratories) for 10 min, followed by counterstaining with Mayer's hematoxylin. For immunodetection of ERα and progesterone receptor (PR), monoclonal antibodies NCL-L-ER 6F11 and NCL-PGR-312, respectively, were used (Novocastra). For immunodetection of AIB1 H-270 antibody (Santa Cruz Biotechnology), slides were visualized with a Zeiss axioscope microscope and manually read by an expert pathologist (B.M.). Tumors were considered positive for ER/PR if nuclear staining was seen in at least 10% of the tumor cells. To define tumor as heparanase positive, a cutoff point of 25% immunostained tumor cells was chosen on the basis of an initial overview of the cases to improve signal-to-noise ratios. We have also tested additional, even stricter cutoffs (i.e., ≥50%, ≥75%) of tumor cells staining for heparanase. Cutoffs were chosen before any attempt at correlating ER status with heparanase expression.

χ^2 tests were done to study the relationship between heparanase and ER immunohistochemical results, using SPSS software (SPSS Inc.). Statistical analysis revealed significant association between ER positivity and heparanase expression by tumor cells at either 25% ($P < 0.0001$), 50% ($P < 0.001$), or 75% ($P < 0.013$) cutoff points.

Chromatin immunoprecipitation. Following overnight treatment of cells with estrogen/tamoxifen or vehicle alone, cross-linking between DNA and protein was preformed by adding formaldehyde (Merck) directly into the culture medium to a final concentration of 1%. Fixation proceeded at room temperature for 10 min and was stopped by the addition of glycine to a final concentration of 0.125 mol/L. Plates were rinsed twice with PBS, the cells were removed by scraping and collected by centrifugation. Pellets were incubated with lysis buffer 1 [50 mmol/L HEPES-KOH (pH 7.5), 140 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton X-100, and a mixture of protease inhibitors], rocked at 4°C for 10 min, and centrifuged. The pellets were then resuspended in lysis buffer 2 [200 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L Tris-HCl (pH 8.0)], rotated for 10 min at room temperature, and collected by centrifugation. Pellets were resuspended in lysis buffer 3 [1 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L Tris-HCl (pH 8.0), 0.1% deoxycholic acid], and sonicated into chromatin fragments of an average length of <500 bp, as determined by agarose gel electrophoresis of fragmented chromatin samples. ChIP was done with anti – ERα D-12 or anti – AIB1 H-270 antibodies (Santa Cruz Biotechnology) preincubated with magnetic bead-conjugated mouse immunoglobulin G (IgG) (Dynabeads M-280 sheep anti-mouse IgG, Dynal Biotech ASA) at 4°C, overnight with rotation. Immunoprecipitates were washed eight times with wash buffer [50 mmol/L HEPES (pH 7.6), 1 mmol/L EDTA, 0.7% deoxycholic acid, 1% NP40, 0.5 mol/L LiCl, and protease inhibitors mixture]. Elution of immune complexes was carried out by the addition of 50 μL of elution buffer [50 mmol/L Tris-HCl (pH 8), 10 mmol/L EDTA, 1% SDS] at 65°C for 15 min with brief vortexing every 2 min. Reverse cross-linking was carried out by incubating at 65°C for overnight. RNA and unbound proteins were removed by the addition of 0.2 mg/mL of RNase A for 1 h at 37°C, followed by the addition of 0.2 mg/mL proteinase K for 2 h at 55°C. DNA was extracted by PCR Purification Kit (Genomed). Recovered chromatin was suspended in 50 μL TE, and PCR analysis was done using 5 μL of immunoprecipitated chromatin or input chromatin using Titanium Taq PCR kit (BD Biosciences Clontech). Amplifications (38 cycles) were done using specific primer sets flanking the putative estrogen-response elements (ERE) in the heparanase promoter. EREp-1: sense 5′-TTGCCAAATTTCTCCTCTGC-3′, antisense 5′-TTCACATCCC-GATTCTGACA-3′, PCR product size = 184 bp; EREp-2: sense 5′-CAT-GATGAAGCCCCATCTCTA-3′, antisense 5′-GAGAGGGTCTCAC-TTTGTCACC-3′, PCR product size = 278 bp; EREp-3: sense 5′-CTACT-TCCTTGCTCGCTTTCC-3′, antisense 5′-GAGGAAGGGATGAATACT-CCA-3′, PCR product size = 301 bp. Primers for the promoter region of the PS2 gene: sense 5′-GGCCATCTCTCACTATGAATCACTTCTGCA-3′ and antisense 5′-GGCAGGCTCTGTTTGCTTAAAGAGCGTTAGATA-3′ (42).

SDS-PAGE and Western blot analysis. Equal number of cells, harvested at each treatment condition, were lysed and fractionated as described (43). Briefly, cells were scraped off the plate with a rubber policeman in ice-cold 3 mmol/L imidazole buffer (pH 7.4) containing 0.25 mol/L sucrose, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L EDTA. The cell suspension was incubated on ice for 15 min before being homogenized with 20 strokes of a Dounce homogenizer using pestle A. Unbroken cells and nuclei were pelleted by centrifugation at 16,000 × *g* for 2 min (Eppendorf Centrifuge 5417R). The supernatant was collected and loaded on a sucrose gradient generated from 0.7 mol/L (5 mL) and 1.6 mol/L (5 mL) sucrose in 20 mmol/L imidazole (pH 7.4) and centrifuged (Beckman TST-41 I Ultracentrifuge) at 28,000 rpm for 3 h at 4°C. Ten fractions (1 mL each) were collected, and protein concentration was determined with Coomassie Plus Protein Assay Reagent (Pierce). A total of 50 μg of protein from the heparanase-containing fraction (fraction 3) were diluted into the SDS-PAGE sample buffer, boiled for 5 min, and loaded on SDS-PAGE (10% acrylamide) under reducing conditions. Proteins were transferred to a polyvinylidene diflouride membrane (Pierce), and Western blot analysis was done using anti-heparanase rabbit polyclonal antibody 1453, raised against the full-length 65-kDa heparanase (41), followed by horseradish peroxidase – conjugated secondary antibody and a chemiluminescent substrate (Pierce). The membrane was stripped and incubated with anti-β-actin antibody (Sigma, clone AC-15) to ensure equal protein load.

Results

Interaction between ER and heparanase promoter* in vitro *and demonstration of its functional significance in clinical samples of breast carcinoma. The presence of several regions homologous to consensus estrogen response element (ERE) in the regulatory

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sequences of the heparanase gene (32) prompted us to apply ChIP analysis to test whether ER directly binds to the heparanase promoter. MCF-7 cells were treated with estrogen or vehicle alone, and chromatin was isolated following chemical cross-linking, as described in Materials and Methods. The cross-linked chromatin was sonicated to ≤500-bp fragments and immunoprecipitated with antibody against ERα. DNA obtained from the immunoprecipitated chromatin was amplified using three sets of heparanase promoter–specific primers (EREp1-3), flanking all the putative EREs (Fig. 1A), as well as set of primers specific to the unrelated GAPDH gene sequence. Enrichment of heparanase promoter sequence in estrogen-treated but not in untreated MCF-7 cells was reproducibly detected when primer set EREp-3 was used to amplify the chromatin DNA immunoprecipitated with anti-ERα antibody (Fig. 1A). No enrichment was observed when the antibody against an irrelevant protein (Flt-1 tyrosine kinase receptor) was used for ChIP (data not shown). These results show direct binding of estrogen-liganded ER to heparanase gene regulatory sequence and attest regions of homology to the consensus ERE, located most proximally to the transcription initiation site (bp -147 to -362), as functional ERE responsible for the previously shown estrogen induced heparanase expression (32). We next assessed whether the *in vivo* observed ER interaction with heparanase promoter may account for the repeatedly reported up-regulation of heparanase expression in clinical samples of breast tumors (1, 2, 22, 24, 25). For this purpose, tissue microarray comprised of tumor tissue specimens from 214 nonselected invasive breast carcinoma patients (each tumor represented by three core samples taken from different areas of the original paraffin block) was immunostained for heparanase (Fig. 1B) and estrogen/progesterone receptor (data not shown). A summary of clinical and pathologic characteristics of the study population is shown in Table 1. The frequency of ER positivity was 64% (136 tumors), and PR was expressed in 36 (18%) tumors. Heparanase expression was revealed in 33.6% of all the tumors. In agreement with previously reported data (1, 2), no heparanase expression was detected in any of the five control specimens of nonmalignant breast tissue included in the array (data not shown).

χ^2 analysis was then used to assess the relationship between heparanase up-regulation and the hormone receptor status. Strong and highly significant association was observed between ER positivity and heparanase expression by tumor cells. As shown in Fig. 1C, heparanase expression was detected in 43.5% of ER-positive tumors, whereas among ER-negative tumors, only 15.8% expressed detectable levels of heparanase (χ^2 test, $P < 0.0001$; Fig. 1C). Moreover, majority of all heparanase-overexpressing cancers found on the tissue microarray (58 out of 72; 80%) were ER positive.

It should be noted that the number of PR-positive tumors in the study population was relatively low; however, as expected, PR positivity was strongly related to ER positivity (χ^2 test $P < 0.003$).

Effect of tamoxifen on heparanase expression. Tamoxifen is the most frequently prescribed drug in ER-positive breast cancer endocrine therapy. Having shown that a majority of breast tumors with elevated heparanase levels were ER positive, we next examined the effect of tamoxifen on heparanase expression. Two ER-positive breast cancer cell lines, MCF-7 and T47D, were treated with tamoxifen at concentrations ranging from 3×10^{-6} to 3×10^{-7} mol/L, in the presence or absence of estrogen, and heparanase mRNA levels were assessed by semiquantitative reverse transcription-PCR (RT-PCR). The ER status of the cells

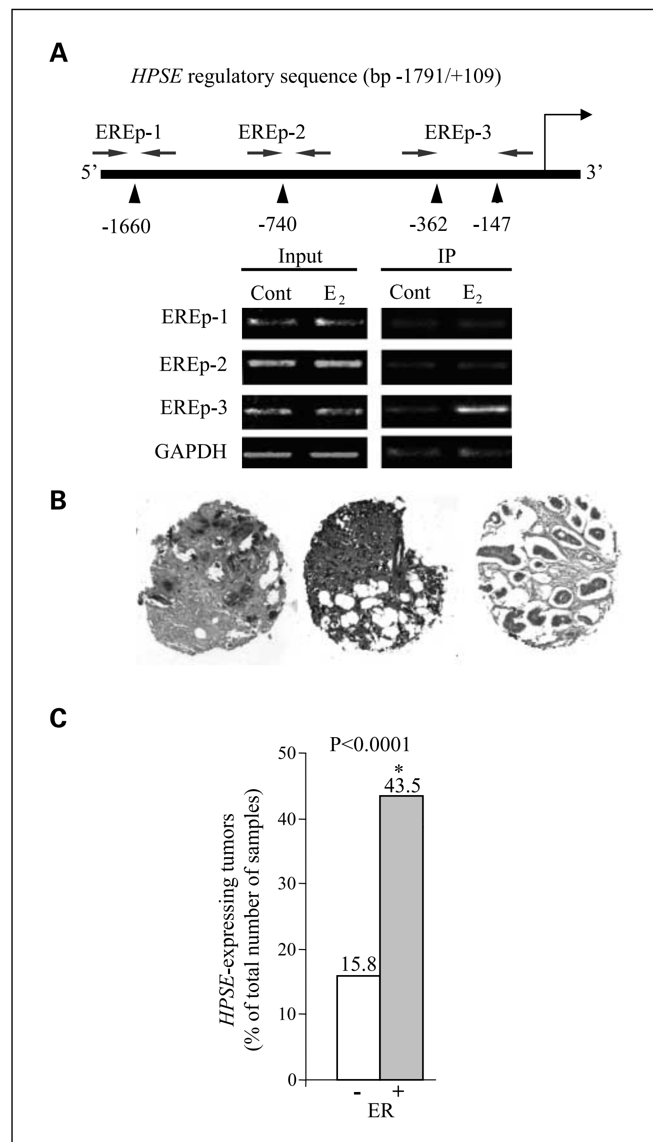

Fig. 1. *A,* top, schematic representation of regions along the heparanase promoter with ≥70% homology to the consensus ERE (*black arrowheads*). The numbers below show the location of putative EREs relative to the transcription initiation site (*bent arrow*). The sequences of putative EREs (with 70-80% homology to the consensus ERE sequence GGTCAnnnTGACC) in heparanase promoter region are GGTCACTGcaACC at -1,660 bp; GaaaAGTCTGACC at -740 bp; aGTgAACGTGACC at -362 bp, and GGTCAGAGgGAta at -147 bp. Arrow pairs, primer sets flanking putative EREs, which were used in ChIP analysis. Bottom, occupancy of heparanase promoter by ER. ChIP assay was done with MCF-7 cells treated with vehicle (*Cont*) or estrogen (E_2) to examine ER binding to the putative ERE sites. Following cross-linking of proteins to DNA, chromatin was sonicated into fragments of average length ≤500 bp; the ER protein was immunoprecipitated with the specific antibody, and PCR analysis was done on the immunoprecipitated DNA samples (*IP*) using primer sets EREp1-3, as described in Materials and Methods. Set of primers specific to unrelated GAPDH gene sequence was used as a control. Input, DNA that was PCR amplified from chromatin preparations before immunoprecipitation. Gels are representative of three independent experiments. *B,* representative images of positive heparanase immunostaining of breast carcinoma tissue specimens from tissue microarray (*left,* invasive ductal carcinoma; *right,* invasive lobular carcinoma), as well as negative staining (*right,* invasive ductal carcinoma). Magnification, ×100. *C,* tissue microarray analysis of heparanase expression relationship to ER status. χ^2 statistical analysis revealed a highly significant correlation ($P < 0.0001$) between heparanase expression and the presence of ER.

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used in these experiments was confirmed by RT-PCR with ERα-specific primers and Western blot analysis with anti-ERα antibody (data not shown), as well as by their estrogen responsiveness in proliferation assay (Fig. 2C). In agreement with the previously reported data (32), treatment with estrogen alone resulted in a marked increase in the levels of heparanase mRNA in both cell lines (Fig. 2A). This effect of estrogen was completely abolished in the presence of 1×10^{-7} mol/L of the pure ER antagonist fulvestrant (ICI 182,780; ref. 44; data not shown). As shown in Fig. 2, at concentrations similar to those observed in the serum of drug-treated patients (3×10^{-7} mol/L; ref. 45), tamoxifen was unable to halt estrogen-induced increase in heparanase mRNA expressed by MCF-7 and T47D cells. Moreover, even in the absence of estrogen, tamoxifen at 3×10^{-7} mol/L (and, to a lesser extent, at 10^{-6} mol/L) exerted an estrogenlike stimulatory effect on heparanase mRNA levels expressed by MCF-7 and T47D cells (Fig. 2A). In ER-negative MDA-231 breast carcinoma cells, treatment with tamoxifen did not result in any change in heparanase mRNA levels (data not shown). In agreement with the RT-PCR results, increased levels of heparanase protein were detected in lysates of the cells treated with 10^{-9} mol/L estrogen or 3×10^{-7} mol/L tamoxifen, as compared with untreated cells (Fig. 2B), further corroborating the estrogenlike effect of tamoxifen on heparanase expression. In fact, tamoxifen was able to exert a notable antagonistic effect on estrogen-induced heparanase expression only at a concentration as high as 3×10^{-6} mol/L (Fig. 2A), which is 10 times higher than that observed in the serum of tamoxifen-treated breast cancer patients (45) or that required to inhibit proliferative effects of estrogen on breast carcinoma cells growing on plastic culture dishes (Fig. 2C). Interestingly, when the same cells were grown on dishes coated with naturally produced basement membranelike ECM (36), their proliferation was not inhibited by tamoxifen (Fig. 2D). The ECM better mimics the microenvironment found in epithelial tissues/tumors and provides a natural substrate for heparanase enzymatic activity, which efficiently releases various growth factors sequestered by heparan sulfate in the ECM (10, 13, 46). To verify that the lack of tamoxifen-inhibitory effect on MCF-7 cell maintained on ECM is due (at least in part) to the tamoxifen-induced increase in heparanase expression, we compared the proliferation of tamoxifen-treated cells on the ECM in the presence or absence of a specific inhibitor of heparanase enzymatic activity ^{100}NA RO-H (100% N-acetylated, 25% glycol-split heparin, ref. 37). A statistically significant decrease in proliferation of tamoxifen-treated MCF-7 cells growing on ECM was obtained in the presence of 1 μg/mL ^{100}NA,RO-H ($P < 0.0001$; Fig. 2D). At the same concentration ^{100}NA,RO-H exerted no effect on proliferation of the cells growing on plastic (data not shown).

Tamoxifen recruits ER coactivator AIB1 to heparanase promoter. ER coactivator AIB1 (SRC-3) was shown upon binding to tamoxifen-ER complex to alter tamoxifen activity from antagonist to agonist (47). We applied the ChIP assay to elucidate whether AIB1 is a part of the transcription complex assembled by tamoxifen-bound ER on the heparanase promoter and, therefore, is responsible for the agonist activity of tamoxifen in our system (Fig. 3A). MCF-7 nuclear extracts incubated in the absence or presence of either 3×10^{-7} mol/L tamoxifen or 10^{-9} mol/L estrogen were immunoprecipitated with antibodies against ERα or AIB1, followed by PCR using the heparanase promoter-specific primer set EREp-3. As expected, either estrogen or tamoxifen treatment induced occupancy of the heparanase promoter by ER (Fig. 3A). Interestingly, the occupancy of the heparanase promoter by AIB1 was also induced by tamoxifen, even more effectively than by estrogen itself, as indicated by the enhanced enrichment of the heparanase promoter sequence in tamoxifen-treated versus untreated or estrogen-treated cells (Fig. 3A). When PCR primers specific to pS2 gene (well-characterized estrogen target gene) were used in the same ChIP assay, estrogen induced occupancy of the pS2 promoter by both ER and AIB1, whereas tamoxifen, as expected (42), recruited ER, but not AIB1, to the pS2 promoter (Fig. 3A). No recruitment of either AIB1 or ER was observed when primers specific to the unrelated GAPDH gene sequence were used as a negative control (data not shown). In addition, no enrichment of any promoter sequence was observed when the antibody against an irrelevant protein (i.e., Flt-1 tyrosine kinase receptor) was used for ChIP. These results show the specific recruitment of AIB1 to the heparanase promoter by tamoxifen-bound ER and may explain the agonistic action of tamoxifen on heparanase expression. To examine the relevance of this finding to a clinical situation in which ER-positive breast carcinoma cells are exposed to tamoxifen, we examined heparanase and AIB1 expression in the second primary ER-positive breast carcinomas, developed in patients under tamoxifen treatment for their first tumor. We examined eight tissue specimens of the second ER-positive breast cancer from tamoxifen-receiving patients (four were contralateral metachronous tumors and the other four represented disease recurrence within the conserved breast): in all tested tumors, heparanase overexpression was detected by

Table 1. Clinical and pathologic characteristics of the study population (N = 214 subjects)

Criteria	Median (range)
Age (y)	49 (20-82)
Tumor size (cm)	2.0 (0.2-13)
	Number of cases (%)
Tumor type	
Ductal	174 (81.3)
Lobular	21 (9.8)
Mucinous	8 (3.7)
Metaplastic	6 (2.8)
Medullary	5 (2.3)
Lymph node status	
LN positive	66 (48)
LN negative	71 (52)
Unknown	77
Grade	
1	33 (15.4)
2	79 (36.9)
3	102 (47.7)
ER	
Negative	78 (36)
Positive	136 (64)
PR	
Negative	176 (88)
Positive	38 (18)
AIB1	
Negative	139 (80)
Positive	35 (20)
Unknown	40

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immunostaining. Moreover, five out of eight tumors (60%) were also positive for AIB1. In these tumors, the pattern of AIB1 protein staining was similar to that of heparanase expression in defined areas of the tumor (Fig. 3B), further supporting the notion that recruitment of AIB1 to tamoxifen-liganded ER contributes to heparanase up-regulation. AIB1 expression was also assessed in the primary breast tumor tissues presented on the microarray previously analyzed for heparanase and ER status. AIB1 was revealed in 20% of all the tested primary tumors (Table 1), which is thrice less than the incidence of AIB1 expression in tumors developed under tamoxifen treatment (60%). Within the group of primary AIB1-positive tumors presented on the microarray, the incidence of heparanase overexpression was almost twice as high in ER-positive (42%) than in ER-negative (25%) tumors, in agreement with the notion that recruitment of AIB1 by ER contributes to heparanase up-regulation.

Discussion

About 70% of breast cancers are ER positive, and ER is one of the few tumor markers recommended for routine clinical use by the American Society of Clinical Oncology (48). Estrogen-liganded ER, complexed with several coregulatory molecules, binds to ERE sequences in the promoter region of target genes and activates their transcription. Numerous cell growth-regulatory proteins have been identified as ER-target genes (49, 50), thus implicating ER signaling in breast tumorigenesis, primarily via stimulation of cell proliferation. More recently, several lines of evidence indicated that ER signaling, independently of its proliferative effects, contributes to breast tumorigenesis by enhancing abnormal tumor-stromal interactions (i.e., neovascularization, metastatic spread; refs. 51 – 53). Estrogen-mediated regulation of heparanase gene transcription may be an integral part of this phenomenon. Overexpression of heparanase in breast tumors and the associated potential contributions to cancer progression (i.e., enhanced primary tumor growth, invasiveness, angiogenesis, metastasis) are well documented (1 – 4, 12, 23 – 27) and further supported by a significantly higher frequency of heparanase positivity in metastatic versus the nonmetastatic ER-positive breast tumors (this study). Our previous report, describing estrogen-driven heparanase expression (32) was recently supported by findings reported by Xu et al. (54). However, the underlying molecular mechanism remained poorly understood. Physical interaction between estrogen-bound ER and a specific region of the heparanase promoter containing ERE sequences shown in the present study clearly implicates classic ER signaling in

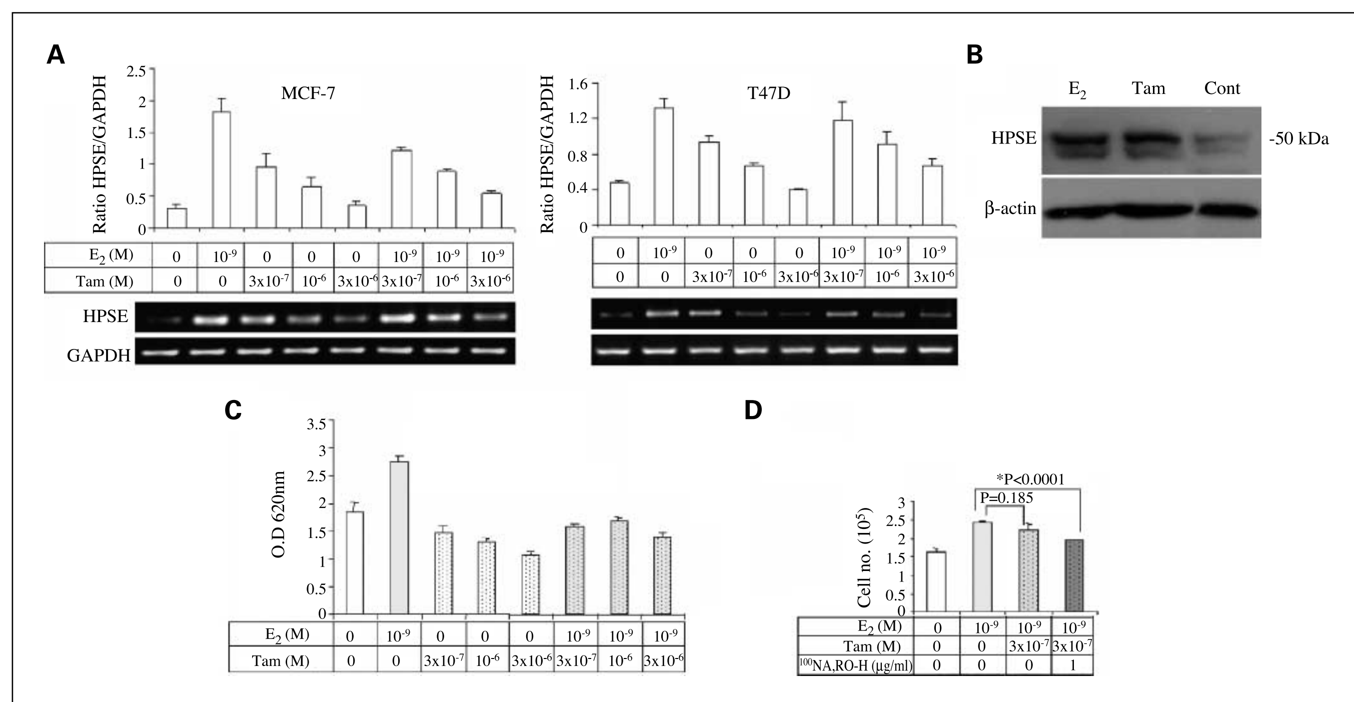

Fig. 2. *A,* effect of estrogen and tamoxifen on heparanase mRNA expression in ER-positive breast carcinoma cells. Before estrogen treatment, MCF-7 (*left*) and T47D (*right*) cells were maintained for 96 h in phenol red – free medium supplemented with charcoal-stripped fetal bovine serum. Then, the medium was changed to serum-free medium with or without 1×10^{-9} mol/L estrogen (E_2) in the presence or absence of tamoxifen at concentrations of 3×10^{-7}, 10^{-6}, or 3×10^{-6} mol/L. Sixteen hours later, RNA was isolated from the cells, and comparative semiquantitative RT-PCR was done as described in Materials and Methods. Aliquots of 10 μL of the amplification products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining (*bottom*). Intensity of each band was quantitated using Scion Image Software, and the results of three separate experiments (mean ± SD) are expressed as band intensity relative to that of GAPDH (*top*). Note the marked increase in heparanase mRNA levels obtained upon tamoxifen (3×10^{-7} mol/L) treatment, as compared with untreated cells. *B,* heparanase protein content in lysates of T47D cells untreated or treated with either 10^{-9} mol/L estrogen or 3×10^{-7} mol/L tamoxifen. Equal protein aliquots of cell lysates (50 μg of total protein per lane) were separated on SDS-PAGE and analyzed by Western blot with rabbit polyclonal antibody using anti-heparanase rabbit polyclonal antibody 1453 (ref. 41; *top*) or anti – β-actin antibody (*bottom*). *C,* MCF-7 cell proliferation in the presence of estrogen and tamoxifen. The cells were seeded in quadruplicates into 2-cm^2 dishes (0.5×10^5 cells per dish) and treated with the indicated concentrations of estrogen and/or tamoxifen. Forty eight hours later, cell growth on plastic was assessed by staining with methylene blue reagent and by color spectrometry at a wavelength of 620 nm as described in Materials and Methods. *D,* MCF-7 cell proliferation on ECM. The cells were cultured in quadruplicates on ECM-coated plates and treated with estrogen, tamoxifen, and/or ^{100}NA,RO-H, as described in *C*. Heparanase inhibitor ^{100}NA RO-H (1 μg/mL) was added daily. The cells grown on ECM were dissociated with trypsin/EDTA and counted. Bars, mean of quadruplicate dishes; bars, SD.

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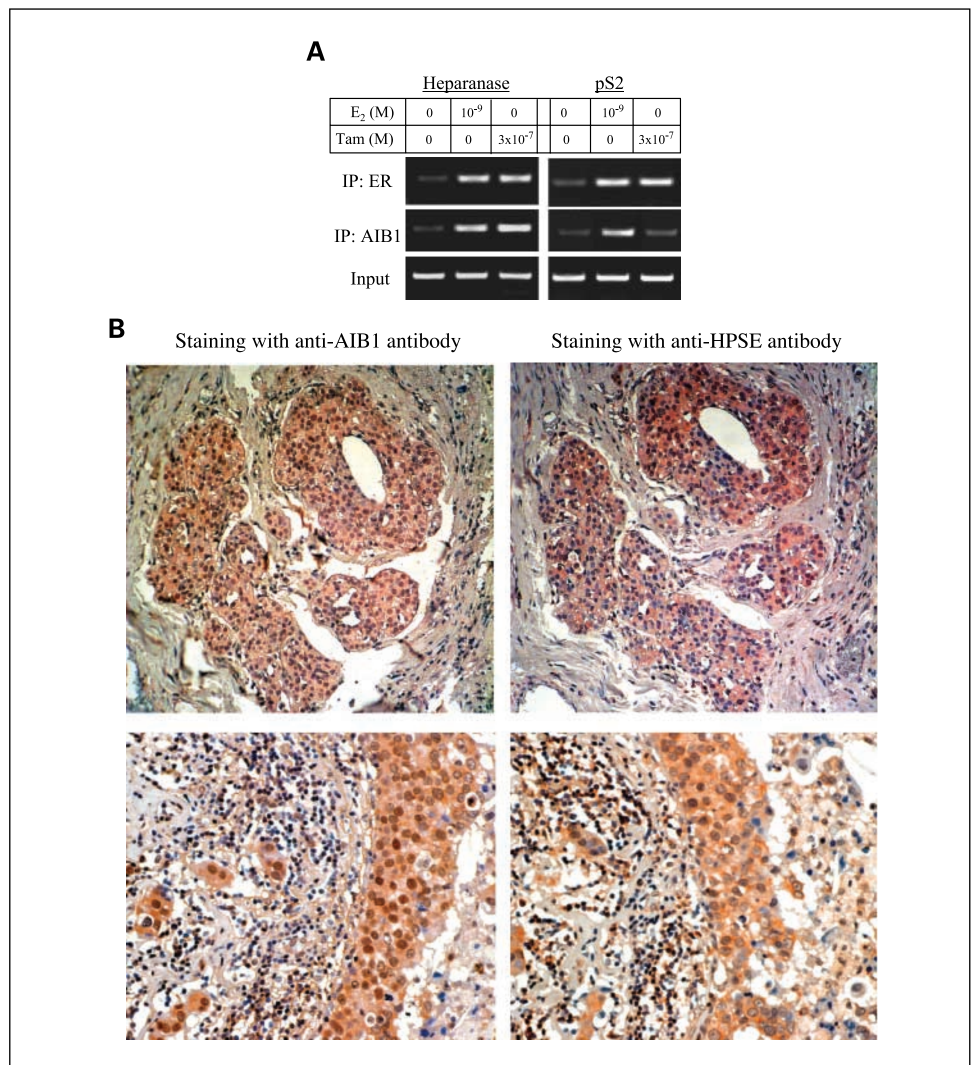

Fig. 3. *A,* recruitment of ER and AIB1 to the heparanase and pS2 promoters in the presence of estrogen and tamoxifen. Estrogen-depleted MCF-7 cells were treated with 10^{-9} mol/L estrogen, 3×10^{-7} mol/L tamoxifen, or with the vehicle alone; chromatin was prepared as described in Materials and Methods and immunoprecipitated (*IP*) with antibodies against ER or against AIB1. The final DNA extractions were amplified using EREp3 primer set that covers functional EREs in heparanase promoter (*left*) or primer set specific to pS2 promoter sequence (*right*). Primers specific to unrelated GAPDH gene sequence were used as a control. Input, DNA that was PCR amplified from chromatin preparations before immunoprecipitation. The gels shown are representative of three independent experiments. *B,* coexpression of AIB1 and heparanase in histologic specimens of metachronous ER-positive breast tumor developed in a patient receiving tamoxifen treatment for her initial breast cancer. Immunostaining with the antibodies against AIB1 (*left*) and heparanase (*right*) was done as described in Materials and Methods. Note the similarity in the spatial pattern of staining between AIB1 (*left*) and heparanase (*right*), as well as the prominent nuclear staining of AIB1, consistent with its involvement in promoter regulation. Magnification: top, ×200; bottom, ×400.

heparanase regulation and attests heparanase as a novel downstream estrogen effector involved in breast tumor progression. Importantly, we were able to extend the mechanistic studies of the heparanase-ER relationship to human breast cancers. Tissue microarray analysis revealed that ER status is a determinant of heparanase expression in breast tumors, corroborating the physiologic relevance of the ChIP findings. A highly statistically significant ($P < 0.0001$) 3-fold increase in the frequency of heparanase overexpression in ER-positive versus ER-negative primary breast cancers shows that the ER-dependent mechanism of heparanase regulation is operative *in vivo*. Nevertheless, the 15% incidence of heparanase expression in ER-negative tumors (Fig. 1C) suggests that alternative molecular mechanisms (55) may be responsible for heparanase up-regulation in a subset of ER-negative breast carcinomas.

In recent years, aromatase inhibitors (agents that block the synthesis of estrogen) were reported to be superior to tamoxifen in postmenopausal breast cancer patients. However, tamoxifen is the drug of choice for premenopausal women and is an essential part of hormonal therapy both in the adjuvant setting and the treatment of metastatic disease in postmenopausal women. Thus, the fact that 83% of all heparanase-overexpressing cases of breast cancer found on the tissue microarray were ER positive implies that majority of patients whose tumors are characterized by elevated levels of heparanase are treated with tamoxifen. However, 40% to 50% of breast cancers fail to respond to the drug despite the presence of ER (34, 35). Tamoxifen therapy failure was often attributed to the tumor ability (either intrinsic or acquired) to be stimulated rather than inhibited by the drug (35). Along with proliferation-related genes, reportedly induced by tamoxifen in some scenarios of endocrine therapy resistance, tamoxifen-triggered heparanase expression, described in this study, may promote tumor progression, affecting pathologic tumor-stromal interactions (i.e., breakdown of extracellular barriers; release of heparan sulfate-bound angiogenic and growth-promoting factors).

Tamoxifen has long been known to exert both ER agonist and antagonist effects, depending on the species or tissue. Tamoxifen is predominantly an agonist in bone and endometrium, but an antagonist in breast, at least on genes important for cell proliferation (56). In our experiments, the physiologically relevant concentration of tamoxifen stimulated heparanase expression and, at the same experimental setting, exerted an inhibitory effect on estrogen-dependent proliferation of MCF-7 and T47D breast carcinoma cells growing on tissue culture

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plastic (Fig. 2) However, tamoxifen-induced increase in heparanase expression conferred a marked proliferation advantage to the cells grown on a naturally produced basement membranelike ECM. It was previously shown that heparanase enzymatic activity efficiently releases from the ECM various growth factors and bioactive molecules capable of supporting cell growth (10, 46). The growth-promoting activity of these factors may be of particular significance under tamoxifen treatment, when some of the genes important for cell proliferation are inhibited (56).

Our findings indicate that the molecular basis of the estrogenlike activity of tamoxifen on heparanase expression may involve the recruitment of transcription coactivator AIB1 to the tamoxifen-ER complex bound to the heparanase promoter. AIB1, a member of the SRC family of nuclear receptor coactivators, is known to enhance ER-dependent transcription (33). Increased levels of AIB1 were previously shown to alter tamoxifen activity from antagonist to agonist on a wide range of genes involved in cell proliferation (42). This effect, however, required both high AIB1 levels and growth factor receptor cross-talk, which brought about the phosphorylation of both ER and AIB1 (42). Our results suggest that even when antagonistic action of tamoxifen on the proliferation of cells grown on culture plastic (Fig. 2C) or on the recruitment of AIB1 to pS2 promoter (Fig. 3A) is preserved, the drug is capable of exerting a gene-specific agonistic effect on heparanase expression.

It should be noted that the inverse effect of higher doses of tamoxifen on heparanase expression is in agreement with the previous reports, describing biphasic effects of estrogen/tamoxifen in various systems (57, 58).

Identification of coactivator molecule(s) responsible for the estrogenlike effect of tamoxifen on heparanase expression may help to define the target patient population, in which the combination of tamoxifen therapy with anti-heparanase inhibitors (which are currently under development; refs. 59, 60) could be beneficial. It also might be speculated that the observed superiority of aromatase inhibitors over tamoxifen may be attributed, at least in part, to the lack of a stimulatory effect on the heparanase gene. Future retrospective clinical studies, comparing benefits of aromatase inhibitors versus tamoxifen therapy of heparanase-positive and heparanase-negative breast tumors will clarify whether aromatase inhibitors might be suggested to replace tamoxifen in patients with heparanase-expressing cancer.

Taken together, the emerging link between ligand-bound ER activity and heparanase overexpression may represent an important pathway underlying breast cancer progression to a more aggressive phenotype, as well as the failure of tamoxifen therapy in some patients. A detailed understanding of the molecular events occurring along this pathway will provide a more comprehensive insight into the biology of estrogen-driven breast tumorigenesis and may have important implications for recommendations on treatment and risk-reduction strategies.

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References

1. Zcharia E, Metzger S, Chajek-Shaul T, et al. Molecular properties and involvement of heparanase in cancer progression and mammary gland morphogenesis. J Mammary Gland Biol Neoplasia 2001;6:311–22.

2. Vlodavsky I, Friedmann Y, Elkin M, et al. Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. Nat Med 1999;5:793–802.

3. Vlodavsky I, Friedmann Y. Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. J Clin Invest 2001;108:341–7.

4. Parish CR, Freeman C, Hulett MD. Heparanase: a key enzyme involved in cell invasion. Biochim Biophys Acta 2001;1471:M99–108.

5. Hulett MD, Freeman C, Hamdorf BJ, Baker RT, Harris MJ, Parish CR. Cloning of mammalian heparanase, an important enzyme in tumor invasion and metastasis. Nat Med 1999;5:803–9.

6. Dempsey LA, Brunn GJ, Platt JL. Heparanase, a potential regulator of cell-matrix interactions. Trends Biochem Sci 2000;25:349–51.

7. Timpl R. Macromolecular organization of basement membranes. Curr Opin Cell Biol 1996;8:618–24.

8. Iozzo RV. Heparan sulfate proteoglycans: intricate molecules with intriguing functions. J Clin Invest 2001;108:165–7.

9. Kjellen L, Lindahl U. Proteoglycans: structures and interactions. Annu Rev Biochem 1991;60:443–75.

10. Vlodavsky I, Bar-Shavit R, Ishai-Michaeli R, Bashkin P, Fuks Z. Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? Trends Biochem Sci 1991;16:268–71.

11. Vlodavsky I, Miao H-Q, Benezra M, et al. Involvement of the extracellular matrix, heparan sulphate proteoglycans, and heparan sulphate degrading enzymes in angiogenesis and metastasis. In: Lewis CE, Bicknell R, Ferrara N, editors. Tumour angiogenesis. Oxford: Oxford University Press; 1997. p. 125–40.

12. Edovitsky E, Elkin M, Zcharia E, Peretz T, Vlodavsky I. Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. J Natl Cancer Inst 2004;96:1219–30.

13. Elkin M, Ilan N, Ishai-Michaeli R, et al. Heparanase as mediator of angiogenesis: mode of action. FASEB J 2001;15:1661–3.

14. Bitan M, Polliack A, Zecchina G, et al. Heparanase expression in human leukemias is restricted to acute myeloid leukemias. Exp Hematol 2002;30:34–41.

15. Friedmann Y, Vlodavsky I, Aingorn H, et al. Expression of heparanase in normal, dysplastic, and neoplastic human colonic mucosa and stroma. Evidence for its role in colonic tumorigenesis. Am J Pathol 2000;157:1167–75.

16. Gohji K, Hirano H, Okamoto M, et al. Expression of three extracellular matrix degradative enzymes in bladder cancer. Int J Cancer 2001;95:295–301.

17. Gohji K, Okamoto M, Kitazawa S, et al. Heparanase protein and gene expression in bladder cancer. J Urol 2001;166:1286–90.

18. Kelly T, Miao HQ, Yang Y, et al. High heparanase activity in multiple myeloma is associated with elevated microvessel density. Cancer Res 2003;63:8749–56.

19. Koliopanos A, Friess H, Kleeff J, et al. Heparanase expression in primary and metastatic pancreatic cancer. Cancer Res 2001;61:4655–9.

20. Rohloff J, Zinke J, Schoppmeyer K, et al. Heparanase expression is a prognostic indicator for postoperative survival in pancreatic adenocarcinoma. Br J Cancer 2002;86:1270–5.

21. Tang W, Nakamura Y, Tsujimoto M, et al. Heparanase: a key enzyme in invasion and metastasis of gastric carcinoma. Mod Pathol 2002;15:593–8.

22. Breidenbach M, Rein DT, Schondorf T, et al. A new targeting approach for breast cancer gene therapy using the heparanase promoter. Cancer Lett 2005;240:114–22.

23. Imada T, Matsuoka J, Nobuhisa T, et al. COX-2 induction by heparanase in the progression of breast cancer. Int J Mol Med 2006;17:221–8.

24. Maxhimer JB, Quiros RM, Stewart R, et al. Heparanase-1 expression is associated with the metastatic potential of breast cancer. Surgery 2002;132:326–33.

25. Maxhimer JB, Pesce CE, Stewart RA, Gattuso P, Prinz RA, Xu X. Ductal carcinoma *in situ* of the breast and heparanase-1 expression: a molecular explanation for more aggressive subtypes. J Am Coll Surg 2005;200:328–35.

26. Kelly T, Suva LJ, Huang Y, et al. Expression of heparanase by primary breast tumors promotes bone resorption in the absence of detectable bone metastases. Cancer Res 2005;65:5778–84.

27. Cohen I, Pappo O, Elkin M, et al. Heparanase promotes growth, angiogenesis and survival of primary breast tumors. Int J Cancer 2006;118:1609–17.

28. Miao HQ, Elkin M, Aingorn E, Ishai-Michaeli R, Stein CA, Vlodavsky I. Inhibition of heparanase activity and tumor metastasis by laminarin sulfate and synthetic phosphorothioate oligodeoxynucleotides. Int J Cancer 1999;83:424–31.

29. de Mestre AM, Khachigian LM, Santiago FS, Staykova MA, Hulett MD. Regulation of inducible

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heparanase gene transcription in activated T cells by early growth response 1. J Biol Chem 2003;278: 50377–85.
30. Lu WC, Liu YN, Kang BB, Chen JH. Trans-activation of heparanase promoter by ETS transcription factors. Oncogene 2003;22:919–23.
31. Baraz L, Haupt Y, Elkin M, Peretz T, Vlodavsky I. Tumor suppressor p53 regulates heparanase gene expression. Oncogene 2006;25:3939–47.
32. Elkin M, Cohen I, Zcharia E, et al. Regulation of heparanase gene expression by estrogen in breast cancer. Cancer Res 2003;63:8821–6.
33. Anzick SL, Kononen J, Walker RL, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 1997;277:965–8.
34. Hayes DF. Tamoxifen: Dr. Jekyll and Mr. Hyde? J Natl Cancer Inst 2004;96:895–7.
35. Schiff R, Massarweh S, Shou J, Osborne CK. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. Clin Cancer Res 2003;9:447–54S.
36. Vlodavsky I, Fuks Z, Bar-Ner M, Ariav Y, Schirrmacher V. Lymphoma cell-mediated degradation of sulfated proteoglycans in the subendothelial extracellular matrix: relationship to tumor cell metastasis. Cancer Res 1983;43:2704–11.
37. Naggi A, Casu B, Perez M, et al. Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol splitting. J Biol Chem 2005;280:12103–13.
38. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844–7.
39. Hoos A, Urist MJ, Stojadinovic A, et al. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. Am J Pathol 2001;158:1245–51.
40. Fairbanks MB, Mildner AM, Leone JW, et al. Processing of the human heparanase precursor and evidence that the active enzyme is a heterodimer. J Biol Chem 1999;274:29587–90.
41. Zetser A, Levy-Adam F, Kaplan V, et al. Processing and activation of latent heparanase occurs in lysosomes. J Cell Sci 2004;117:2249–58.
42. Shou J, Massarweh S, Osborne CK, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 2004;96:926–35.
43. Diment S, Leech MS, Stahl PD. Cathepsin D is membrane-associated in macrophage endosomes. J Biol Chem 1988;263:6901–7.
44. Wakeling AE. Similarities and distinctions in the mode of action of different classes of antioestrogens. Endocr Relat Cancer 2000;7:17–28.
45. Decensi A, Gandini S, Guerrieri-Gonzaga A, et al. Effect of blood tamoxifen concentrations on surrogate biomarkers in a trial of dose reduction in healthy women. J Clin Oncol 1999;17:2633–8.
46. Ishai-Michaeli R, Eldor A, Vlodavsky I. Heparanase activity expressed by platelets, neutrophils, and lymphoma cells releases active fibroblast growth factor from extracellular matrix. Cell Regul 1990;1:833–42.
47. Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 2003;95:353–61.
48. Bast RC, Jr., Ravdin P, Hayes DF, et al. 2000 update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. J Clin Oncol 2001;19:1865–78.
49. Charpentier AH, Bednarek AK, Daniel RL, et al. Effects of estrogen on global gene expression: identification of novel targets of estrogen action. Cancer Res 2000;60:5977–83.
50. Katzenellenbogen JA, O'Malley BW, Katzenellenbogen BS. Tripartite steroid hormone receptor pharmacology: interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. Mol Endocrinol 1996;10:119–31.
51. Banka CL, Lund CV, Nguyen MT, Pakchoian AJ, Mueller BM, Eliceiri BP. Estrogen induces lung metastasis through a host compartment-specific response. Cancer Res 2006;66:3667–72.
52. Losordo DW, Isner JM. Estrogen and angiogenesis: a review. Arterioscler Thromb Vasc Biol 2001;21:6–12.
53. Ruohola JK, Valve EM, Karkkainen MJ, Joukov V, Alitalo K, Harkonen PL. Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. Mol Cell Endocrinol 1999;149:29–40.
54. Xu X, Ding J, Rao G, et al. Estradiol induces heparanase-1 expression and heparan sulphate proteoglycan degradation in human endometrium. Hum Reprod 2007;22:927–37.
55. Gotte M, Yip GW. Heparanase, hyaluronan, and CD44 in cancers: a breast carcinoma perspective. Cancer Res 2006;66:10233–7.
56. Osborne CK, Schiff R. Growth factor receptor cross-talk with estrogen receptor as a mechanism for tamoxifen resistance in breast cancer. Breast 2003;12: 362–7.
57. Calabrese EJ. Estrogen and related compounds: biphasic dose responses. Crit Rev Toxicol 2001;31: 503–15.
58. Glaeser M, Niederacher D, Djahansouzi S, et al. Effects of the antiestrogens tamoxifen and raloxifene on the estrogen receptor transactivation machinery. Anticancer Res 2006;26:735–44.
59. Ishida K, Hirai G, Murakami K, et al. Structure-based design of a selective heparanase inhibitor as an antimetastatic agent. Mol Cancer Ther 2004;3:1069–77.
60. Karoli T, Liu L, Fairweather JK, et al. Synthesis, biological activity, and preliminary pharmacokinetic evaluation of analogues of a phosphosulfomannan angiogenesis inhibitor (PI-88). J Med Chem 2005; 48:8229–36.

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