Calcitonin inhibits invasion of breast cancer cells: Involvement of urokinase-type plasminogen activator (uPA) and uPA receptor

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Abstract. There is a growing body of evidence indicating that calcitonin (CT) and calcitonin receptor (CTR) are involved in the regulation of cell growth, differentiation, and survival and in tissue development. However, the precise functional role of CT/CTR in breast cancer is still unknown. It is well established that the urokinase plasminogen activator (uPA) system plays an important role in breast cancer invasion and metastasis. The goal of this study was to investigate the effects of CT on regulation of the uPA system and invasive capacity of breast cancer cells. In the highly invasive MDA-MB-231 cell line, 10^{-8} M CT decreased both uPA and uPAR mRNA and protein expression which was associated with inhibition of the extracellular signal-regulated kinase (ERK) 1/2 pathway. Furthermore, two weeks of CT administration to nude mice inhibited the expression of uPA mRNA in primary tumors by 25% ($P<0.05$), as compared to control, untreated animals. CT also inhibited the invasiveness of MDA-MB-231 cells by 37% (10^{-8} M CT, $P<0.05$), as determined by a Matrigel invasion assay. To the best of our knowledge, this is the first report describing a direct effect of CT on breast cancer cell invasion. Our data might suggest a close link between CT signaling, the uPA-mediated pathway, and breast cancer invasion.

Introduction

Calcitonin (CT) is a 32 amino-acid polypeptide hormone of thyroidal origin, whose main physiological role is to inhibit osteoclastic bone resorption (1). There is a growing body of evidence that CT and its receptor (CTR) are involved in the regulation of cell growth, differentiation, and survival and in tissue development (2-4). It has been demonstrated that cell proliferation is inhibited when human breast cancer cells T47D are treated with CT (5). There is also evidence that CT is involved in cell survival and apoptosis (6,7). However, a direct effect of CT on breast cancer invasion has not been reported.

Tumor invasion and metastasis are the leading causes of morbidity and mortality in patients with breast cancer (8,9). These events involve multiple processes requiring, as an intermediate step, destruction of the extracellular matrix (ECM) and basement membrane. One of the major proteolytic systems involved in this step is the urokinase-plasminogen activator (uPA) system, comprising uPA, its cell surface receptor uPAR, and its inhibitors plasminogen activator inhibitor types 1 and 2 (PAI-1 and PAI-2, respectively) (10-13). Upon binding to its receptor, uPA induces direct plasmin-mediated proteolysis or indirect activation of other proteinases, such as metalloproteases (MMPs) (10,13). In addition, uPA is associated with cell proliferation, chemotaxis and angiogenesis (10). PAI-1 inactivates uPA, but it also has an important role in cell adhesion and cell migration (10,12). The four major components of this system have been established as prognostic factors in primary breast cancer by various research groups (10-13). In particular, uPA and PAI-1 reached level-I evidence in terms of their prognostic impact (14,15), according to proposed guidelines (16). As a result of these compelling clinical data, the uPA system represents one of the most of interesting targets for breast cancer therapy. To date, experimental *in vitro* and *in vivo* studies have shown that inhibition of uPA activity or uPA-binding to uPAR inhibits tumor growth and reduces or abolishes metastasis (17-19).

In several human cancers, mitogenesis and expression of uPA and uPAR are frequently activated through common signaling complexes and pathways (20). Most mitogens and several oncogenes transmit signals through the proto-oncogene ras, which initiates a phosphorylation cascade resulting in the activation of a pair of M_r 42,000 and 44,000 mitogen-activated protein kinases, also known as extracellular signal-regulated kinase (ERK) 1/2 (21). It is well documented that the binding of CT to CTR leads to the activation of multiple signaling pathways, including the ERK 1/2 pathway (2). We have reported that CT suppressed constitutive phosphorylation of ERK 1/2 in DU145 prostate cancer cells (21). Our ongoing research has also shown that CT suppresses phosphorylation of ERK1/2

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in MDA-MB-231 breast cancer cells (unpublished data). In this regard, Ma *et al* have shown that uPA-uPAR system acts within a positive feedback loop with phosphorylated ERK 1/2 in MDA-MB-231 cells, which may promote cellular proliferation and cell invasion (22). Disruption of this loop, for example, via treatment with PD098059, a MEK-1 inhibitor, was reported to inhibit cell growth and invasiveness (22,23). Therefore, we hypothesized that CT might be also a potential modulator of the uPA system in MDA-MB-231 cells.

In the present study, the effects of CT on regulation of the uPA system and invasive capacity of MDA-MB-231 cells were investigated. We show that i) CT treatment causes down-regulation of uPA and uPAR in MDA-MB-231 cells, with the ERK1/2 pathway being involved in mediating this effect; ii) CT inhibits the invasiveness of MDA-MB-231 cells *in vitro*; and iii) CT treatment results in a decrease in uPA mRNA expression within MDA-MB-231-derived xenografts *in vivo*.

Materials and methods

Reagents and antibodies. Salmon CT was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Anti-human uPAR 3937 monoclonal antibody (MoAb) was obtained from American Diagnostics (Greenwich, CT). PD98059, a MEK1 inhibitor, was obtained from Calbiochem (La Jolla, CA).

Cell culture. Human breast cancer cell lines MDA-MB-231, MDA-MB-435, T47D and MCF-7 were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Invitrogen) with 10% FBS (HyClone, Logan, UT). LCC6 cells were a gift from Dr M.E. Lippman (Georgetown University, Washington, DC) and maintained in modified improved MEM (Gibco BRL, Invitrogen) containing 5% FBS. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C.

Cell growth experiments. DNA synthesis was assessed in MDA-MB-231, T47D and MCF-7 cells by measuring [^{3}H]-thymidine incorporation. Briefly, cells were seeded at a density of 1x10^6 cells/well in 8-well plates. After being cultured in 0.5% serum-containing DMEM for 18 h, cells were treated with different concentrations of CT, which was immediately followed by supplement of 1.0 μCi/ml [^{3}H]-thymidine. After incubation for 12 h at 37˚C, the media were aspirated, and cells were washed twice with ice-cold PBS, and then incubated with ice-cold 5% trichloroacetic acid (TCA) for 15 min. After aspiration of the TCA, the cells were dissolved in lysis buffer containing 0.4 N NaOH, and an aliquot was analyzed by liquid scintillation counting (LS6500, Beckman Coulter, Inc., Fullerton, CA). Eight replicate wells were prepared for each CT concentration. The results were expressed as the mean of determinations from three independent experiments.

RNA isolation and reverse transcription-PCR analysis. Total cellular RNA was extracted using the Ultraspec RNA Isolation system (Biotecx Labs. Inc., Houston, TX). Total RNA (2 μg) was transcribed to cDNA via use of the SuperScript RT-PCR kit (Life Technologies, Inc., Gaithersburg, MD). cDNAs of human uPA, uPAR, PAI-1 and CTR mRNA transcripts were amplified using the following primers: uPA forward 5'-AAGGACTACAGCGCTGACAC-3'; reverse 5'-AACTCCT GCAGGCTTCAGTC-3'. uPAR forward 5'-CGGTGCATGC AGTGTAAGAC-3'; reverse 5'-AGCAGGAGACATCAATG TGG-3'. PAI-1 forward 5'-TCATGGACAGACCCTTCCTC-3'; reverse 5'-GGACATTCACTCTGCCACCT-3'. CTR forward 5'-GACAAAGAGATCTTCAAAAATC-3'; reverse 5'-TTCT GATGGATCAAAATCCGG-3'.

PCR amplification was accomplished with an initial denaturation step at 95˚C for 5 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at either 62˚C (for uPA, uPAR and PAI-1) or 58˚C (for CTR) for 30 sec, and extension at 72˚C for 30 sec. RT-PCR analysis with ß-actin primers (forward 5'-AAGAGAGGCATCCTCACCCT-3' and reverse 5'-TACATGGCTGGGGTGTTGAA-3') was used as an internal RNA control. PCR conditions for ß-actin were the same as that used for analysis of the CTR gene. PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide.

Northern blot analysis. The cDNA probes synthesized via RT-PCR were a 789-bp fragment covering nucleotides 863-1652 of the *uPA* mRNA transcript (GeneBank accession no. NM_002658) and a 792-bp fragment covering nucleotides 496-1288 of the *uPAR* mRNA transcript (GeneBank accession no. NM_002659). Northern blot analysis was performed as described previously (24). Briefly, total RNA was extracted using the Ultraspec RNA Isolation System. Total RNA (20 μg) was electrophoresed on 1.2% agarose-formaldehyde gels, transferred to nylon membrane and fixed by Gs gene linker (Bio-Rad, Lab., Hercules, CA). Hybridization was performed using probes labeled with [α-^{32}P]-ATP via the Hotprime DNA labeling kit (GeneHunter, Nashville, TN). Quantitative analysis of the autoradiographs was performed using the NIH Image densitometry program, with the levels of 18s rRNA (as determined by staining with ethidium bromide) being used for normalization purposes. The data were expressed as the mean of determinations from two independent experiments.

Western blot analysis. Western blotting was performed as previously described (21). Briefly, 70-80% confluent cells were cultured in 0.5% serum-containing medium for 18 h, and then treated with 10^{-8} M CT. At indicated time-points, cells were washed with ice-cold PBS once and extracted in ice-cold lysis buffer (21). Protein (20 μg) was directly electrophoresed on a 12% SDS-PAGE gel and then transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated with anti-uPAR MoAb (clone 3937, 1:1,000) overnight at 4˚C. The secondary antibody was horseradish peroxidase (HRP)-coupled anti-mouse IgG (Dako, Glostrup, Denmark) (1:1500), and the signals were detected with an enhanced chemiluminescence (ECL) kit (Amersham, Buckinghamshire, UK). To check the amounts of proteins transferred to the membrane, ß-actin was used as control, as detected by an anti-ß-actin MoAb (Ab-1, clone JLA20; Oncogene) at a 1:10,000 dilution. Three independent experiments were performed.

Measurement of uPA and PAI-1 protein in conditioned medium by ELISA. MDA-MB-231 cells were pre-cultured in 0.5% serum-containing medium for 18 h, plated at $5x10^4$ cells in 60-mm dishes, and treated with or without 10^{-8} M CT. After incubation for different time-periods, the medium was removed into tubes and centrifuged at 3,000 rpm for 15 min at 4˚C. uPA and PAI-1 ELISA kits (Imubind, American Diagnostica, Greenwich, CT) were used to measure the concentration of these proteins in cell culture supernatants. The cells were counted and the data corrected for cell number. The data were expressed as the mean of determinations from two independent experiments.

Matrigel invasion assay. Invasion capacities of MDA-MB-231 cells were analyzed using Biocoat Matrigel invasion chambers (Becton-Dickinson, Bedford, MA). Briefly, cells treated with or without CT for 24 h were trypsinized and resuspended in serum-free medium. Cell suspension (0.5 ml) ($2.5x10^4$ cells) was plated within the upper insert of the chamber in either the presence or absence of CT. The bottom chamber contained 10% FBS as a chemoattractant. After incubation for 22 h at 37˚C, cells that had invaded to the lower surface of the membranes were fixed and stained with Diff-Quick stain kit (International Reagents Co., Kobe, Japan). For quantification, cells were counted in five randomly selected microscopic fields (x200). Three independent experiments were performed.

Animals. Female, athymic BALB/c-nu/nu mice (5-6 weeks of age; CLEA Japan, Inc., Tokyo, Japan), were used for the studies. Animals were maintained in a specific pathogen-free environment under controlled conditions of light and humidity. All protocols for *in vivo* studies were approved by the Institutional Animal Care and Use Committee of the Wakayama Medical University.

Tumor cell inoculation and CT injection. MDA-MB-231 cells were injected into the subaxillary mammary fat pads of mice at both sites ($5x10^6$ cells/site). Animals were randomly divided into two groups immediately after tumor cell inoculation. Experimental group received s.c. injection of CT everyday (240 ng/day) in a volume of 70 μl. CT was dissolved in isotonic saline solution (0.9% NaCl) containing 1.2 M aminocaproic acid (pH 6.0) as peptidase inhibitor; the control group received comparable injection of isotonic saline solution. All mice were sacrificed 14 days after inoculation and primary tumors from experimental (n=6) and control group (n=5) were excised accordingly, with fresh tissues being stored at -80˚C immediately.

Real-time RT-PCR analysis. Total RNA was extracted from the primary tumors of mice to analyze uPA and uPAR mRNA levels *in vivo*. RNA (2 μg) was used to generate first-strand cDNA via reverse transcriptase (Superscript; Gibco, Grand Island, NY). The primer and probe sequences used for detection of GAPDH mRNA levels were as described previously (25). The primers and probes used for analysis of uPA and uPAR transcripts were purchased from Applied Biosystems Japan Ltd. (Applied Biosystems Japan Ltd, Tokyo, Japan). PCR amplification was performed with 5% of the resultant cDNA in a final volume of 50 μl containing 900 nM of each primer, 250 nM probe, and 0.5X PCR reagent mix (TaqMan Universal PCR Master Mix; Applied Biosystems). The amplification protocol comprised incubations at 50˚C for 2 min, and 95˚C for 10 min, followed by 40 cycles of incubation at 95˚C for 15 sec and 60˚C for 1 min. The reactions were run in triplicate, with mean values being determined by comparing threshold cycle (C_t) values. The method used to calculate C_t for statistical testing has been described previously (25).

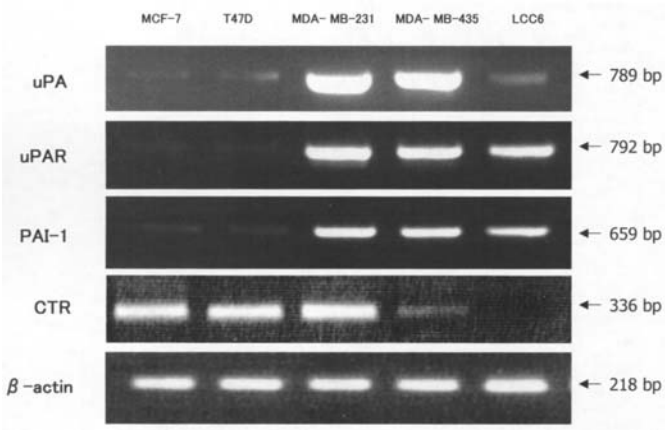

Figure 1. Characterization of human breast cancer cell lines for uPA system and CTR gene expression. Total RNA was extracted from various breast cancer cell lines and analyzed for uPA, uPAR, PAI-1 and CTR gene expression via RT-PCR analysis, as described in Materials and methods. Level of ß-actin was used as an internal control.

Statistical analysis. All results were expressed as mean ± SE and statistical comparisons were based on a One-way ANOVA or Student's t-test using StatView software 5.0 (SAS Institute, Cary, NC, USA). Significance was considered as $P<0.05$.

Results

Characterization of human breast cancer cell lines for uPA, uPAR, PAI-1 and CTR gene expression. The expression levels of uPA, uPAR, PAI-1 and CTR mRNA in various breast cancer cell lines are shown in Fig. 1. In agreement with studies by Guo *et al* (26) and Holst-Hansen *et al* (27), uPA, uPAR, PAI-1 transcripts were found to be barely detectable in MCF-7 and T47D cells, both of which are ER-positive, of low invasive potential, and representative of an early stage in terms of breast cancer progression. In contrast, the ER-negative, highly invasive MDA-MB-231, MDA-MB-435, and LCC6 cells, which are representative of later stages in respect to breast cancer, exhibited high levels of uPA system gene expression. CTR mRNA is expressed in all breast cancer cell lines, apart from LCC6 cells. As MDA-MB-231 cells exhibit the highest level of uPA and uPAR mRNA expression in our series and it is more invasive *in vitro* than either MDA-MB-435 or LCC6 cells (28), we selected it as a cell model for our study.

Effect of CT on DNA synthesis in MDA-MB-231, MCF-7, and T47D cells. To explore whether CT has direct anti-proliferative

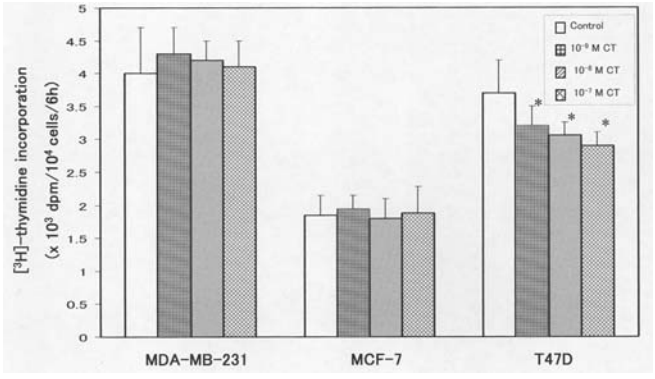

Figure 2. Effect of CT on DNA synthesis in breast cancer cells. Breast cancer cells were cultured with or without CT (10^{-9}, 10^{-8}, 10^{-7} M), which was immediately followed by supplement of [^{3}H]-thymidine. After incubation for 12 h, incorporated thymidine was measured by liquid scintillation counting, as described in Materials and methods. Columns, mean of eight wells; bars, SE; *P<0.05. Similar results were obtained from three separate experiments.

properties or not, we examined its effect on DNA synthesis in breast cancer cells by quantifying [^{3}H]-thymidine incorporation. As shown in Fig. 2, CT treatment reduced the extent of DNA synthesis in T47D cells in a concentration-dependent fashion. For example, CT treatment at 10^{-7} M decreased [^{3}H]-thymidine incorporation in T47D cells by 22% as compared to the control (P<0.05). However, CT treatment had little effect on DNA synthesis in MDA-MB-231 and MCF-7 cells.

CT decreased mRNA expression and secretion of uPA in MDA-MB-231 cells. Using Northern blot analysis, we showed that CT treatment at 10^{-8} M suppressed uPA mRNA (2.4 kb transcript size) expression in MDA-MB-231 cells, reaching maximal inhibition after 6 h (4-fold) (Fig. 3A). Accordingly, we measured the effect of CT on the secretion of uPA from MDA-MB-231 cells. Our results showed that 10^{-8} M CT

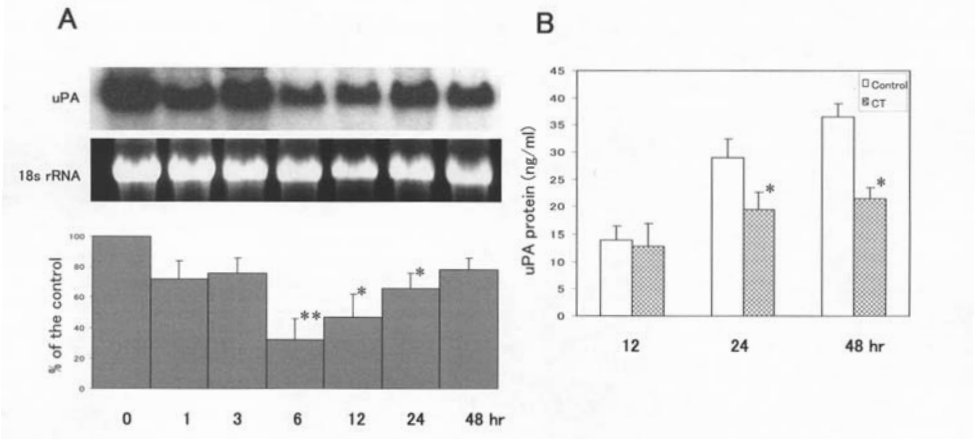

Figure 3. The effect of CT on the mRNA level and secretion of uPA in MDA-MB-231 cells. Cells at 80% confluence were cultured in 0.5% serum-containing medium for 18 h, and incubated in the presence or absence of CT (10^{-8} M) for the indicated time. (A), Total RNA was isolated, and levels of uPA mRNA was detected by Northern blot analysis. To control for equal loading of the gels, the expression of uPA was normalized to level of 18s rRNA (as determined from ethidium bromide-stained gel). To compare expression levels among samples, the expression levels of the uPA gene in the control cells were set to 100%. Quantitation of uPA mRNA is shown in the bottom panel. (B), Conditioned media were assayed for uPA protein level using an ELISA kit. Data are presented as the mean of results from two experiments, each performed in triplicate. Bars ± SE; *P<0.05; **P<0.01.

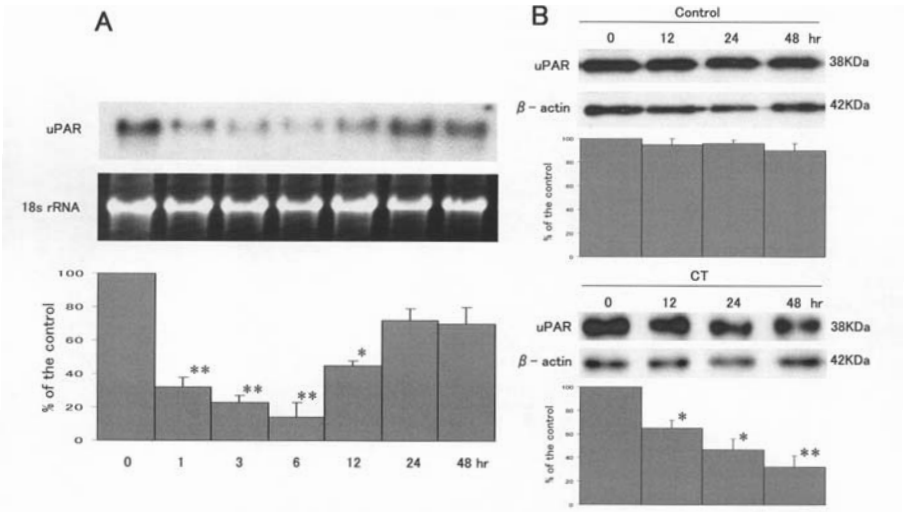

Figure 4. The effect of CT on the expression of uPAR mRNA and protein in MDA-MB-231 cells. Cells were cultured in 0.5% serum-containing medium for 18 h before treatment with or without CT (10^{-8} M) for indicated periods of time. (A), uPAR mRNA expression levels as detected by Northern blot analysis. The expression of uPAR was normalized to the level of 18s rRNA, with the expression level of uPAR mRNA in the control cells being set to 100%. Quantitation of uPAR mRNA is shown in the bottom panel. (B), Total cell lysates were prepared and subjected to Western blot analysis for assessment of uPAR protein expression. The same blots were stripped and reprobed with an anti-ß-actin antibody. Data are presented as the mean of results from two experiments for Northern blot analysis and three experiments for Western blot analysis. Bars ± SE; *P<0.05; **P<0.01.

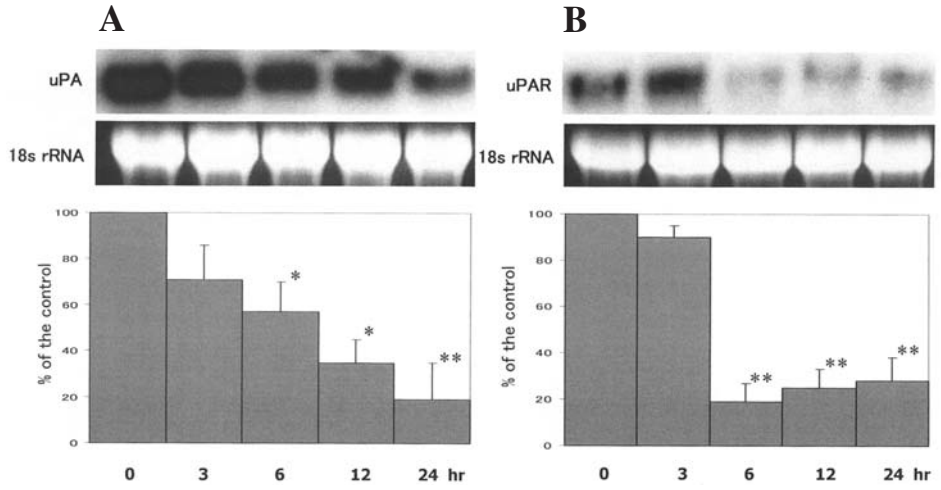

Figure 5. PD98509 blocks uPA and uPAR mRNA expression in MDA-MB-231 cells. Cells were treated with PD98059 (50 μM) or with vehicle alone for indicated time in 0.5% serum-containing medium. Total RNA isolates were analyzed for uPA (A) and uPAR (B) mRNA expression by Northern blot analysis. The level of 18s rRNA in each sample was used for normalization. Quantitation of uPA and uPAR mRNA is shown in the bottom panel. Bars ± SE; *P<0.05; **P<0.01.

significantly inhibited the secretion of uPA (by 31% after treatment for 24 h and by 41% after 48 h), as compared with that exhibited by untreated cells (P<0.05) (Fig. 3B).

Suppression by CT of uPAR mRNA and protein expression in MDA-MB-231 cells. We next evaluated the potential regulation of uPAR mRNA expression by CT in MDA-MB-231 cells. CT rapidly inhibited the expression of uPAR mRNA (by 3-fold after 1 h), with this inhibitory effect lasting 12 h (Fig. 4A). To determine whether the observed changes in the level of uPAR mRNA expression were associated with a corresponding decrease in the expression of uPAR protein, Western blot analysis was performed. From this, the expression of the uPAR protein in MDA-MB-231 cells was observed to be significantly decreased after 24 and 48 h of CT treatment, as compared with those in control groups (P<0.05) (Fig. 4B).

Suppression of uPA and uPAR expressions by CT is associated with inhibition of ERK1/2 pathway. Ongoing research in our laboratory has demonstrated that CT inhibited phosphorylation of ERK1/2 in MDA-MB-231 cells (unpublished data). To confirm that the ERK1/2 pathway in response to CT is critical for its effect on the regulation of uPA and uPAR in MDA-MB231 cells, we treated the cells with PD98095, a specific MEK1/2 inhibitor. Consistent with the findings of Ma *et al* (22), decreased uPA and uPAR mRNA expression was observed by Northern blot analysis (Fig. 5). Thus, it seems very likely, that CT inhibits uPA and uPAR expression via regulation of the ERK1/2 pathway.

CT inhibited invasion of MDA-MB-231 cells. The ability of CT to mediate down-regulation of uPA and uPAR expression led us to explore the effect of CT on the invasive capacity of MDA-MB-231 cells. Cells were pre-treated with CT for 24 h, followed by transfer to invasion chambers. The doses of CT used were selected on the basis of our previous reports and those used in other investigations (21,24,29). As shown in Fig. 6, CT suppressed invasiveness of MDA-MB-231 cells in a dose-dependent manner. CT treatment at 10^{-7} M and 10^{-8} M inhibited cell invasion by 41 and 37%, respectively, when compared to control cells (P<0.05, n=3). We next evaluated if pretreatment of the cells was necessary for this anti-invasive effect. For this purpose, untreated cells were added to invasion chambers in the presence of increasing concentrations of CT, but no significant inhibitory effect was found (data not shown). These results suggest that CT may slowly induce these changes in the phenotype of MDA-MB-231 cells, which counteract the ability of these cells to invade.

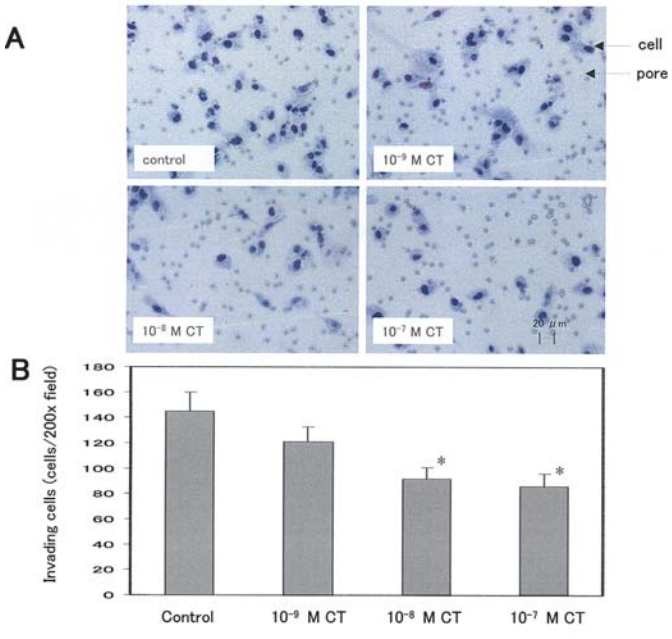

Figure 6. Inhibition of invasion by CT in MDA-MB-231 cells. MDA-MB-231 cells were pretreated for 24 h with CT (10^{-9}, 10^{-8}, 10^{-7} M), and cell invasion was determined after an additional 22 h of incubation with CT. Shown above (A) is a section of the visual field of one representative experiment (magnification x200). The data shown are the means ± SE of duplicate determinations. Similar results were obtained in two additional experiments. Bars ± SE; *P<0.05; scale bar, 20 μm.

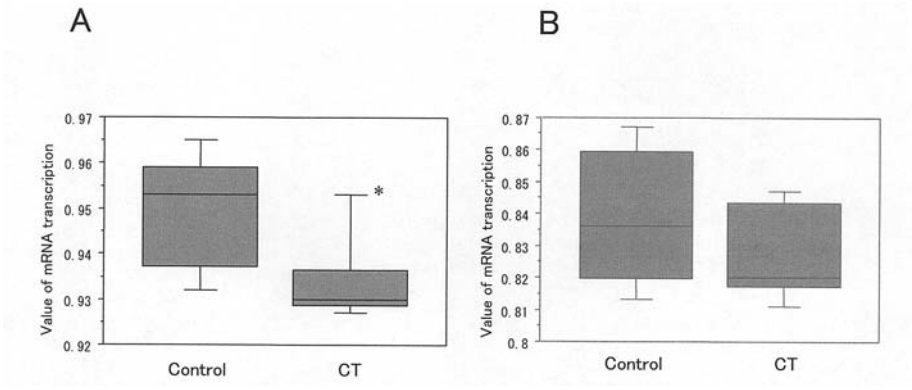

Figure 7. Real-time RT-PCR analysis of uPA and uPAR mRNA levels *in vivo*. Values of uPA (A) and uPAR (B) mRNA transcriptions were determined from real-time RT-PCR analysis, as described in Materials and methods. Data were expressed a percentage of the mean control value ± SE; *P<0.05 (Student's t-test, two-tailed).

CT decreased uPA mRNA levels in vivo. Using relative quantitation by real-time RT-PCR analysis, uPA mRNA levels from tumors of mice receiving CT treatment were shown to be decreased by 25% (P<0.05), as compared with those of the control group. Alteration of uPAR mRNA levels showed the same tendency, but this did not reach statistical significance (Fig. 7).

Discussion

Breast cancer is a hormone-dependent malignancy that generally initiates as a less aggressive, hormone therapy-responsive type and gradually progresses to a highly invasive and hormone therapy-resistant phenotype (11). Finding an appropriate strategy for treating the latter stage of this disease is a challenging problem in the clinical setting. Receptor-bound uPA is now believed to play an important role in several cancers, with it being associated with late stage cancers (10). From the series of breast cancer cell lines analyzed in our study, MDA-MB-231 cells are hormone-insensitive, highly invasive and representative of late stage breast cancer. Animals inoculated with MDA-MB-231 develop large tumors that can metastasize *in vivo* (18). In this cell line, high levels of uPA and uPAR expression correlate with invasive capacity and provide a link between uPA expression and tumor stage (26).

Recently, much interest has arisen in terms of the relationship between the CT/CTR system and breast cancer. Firstly, CT is able to inhibit growth of the breast cancer cell line T47D, as well as abolish the growth stimulation provided by either epidermal growth factor or insulin in this cell model (5,30). Secondly, CT is suggested to be involved in the regulation of metastasis-related genes in breast cancer, such as tissue inhibitors of metalloproteases (TIMP) and parathyroid hormone-related protein (PTHrP), a potential local mediator of skeletal metastasis (29,31). Thirdly, we and other investigators have shown that CTRs are commonly expressed in breast cancer cell lines and primary breast cancers (32,33). Using a laser capture microdissection technique, we also reported that decreased CTR mRNA expression was much more frequently seen in cases with lymph node metastasis and lymphatic invasion (33). However, a precise functional role for CT/CTR in breast cancer is still largely unknown.

We have previously reported that CT induced significant up-regulation of uPA in the LLC-PK1 cell line (24). In the present study, for the first time, we could show that CT was also a potent regulator of the uPA system in the metastatic human breast cancer cell line MDA-MB-231. CT treatment down-regulated both uPA and uPAR mRNA and protein expression, leading to a decrease in uPA secretion. The inhibitory effect of CT treatment on uPA mRNA expression was further verified *in vivo*. CT treatment did not exhibit any significant effect on PAI-1 expression in MDA-MB-231 cells (data not shown). We highlighted these findings because potent paracrine uPA-uPAR signaling may be required for breast cancer metastasis (10). It is recognized that the uPA-uPAR system acts within a positive feedback loop with phosphorylated ERK 1/2 in MDA-MB-231 cells. The MEK inhibitor PD98059, which targets a kinase upstream of ERK1/2, has been shown to substantially decrease uPA and uPAR mRNA expression (22); this finding was confirmed in our present study. Strategies that block this loop, in some conditions, may have therapeutic efficacy. On the other hand, CT has been used for treatment of osteoporosis (3). Therefore, our new data provides additional information to revive a long forgotten area of CT and cancer biology.

Another important finding in this study is that CT inhibits the invasiveness of MDA-MB-231 cells. To the best of our knowledge, this is the first report describing a direct effect of CT on breast cancer cell invasion. This observation is of great interest, since invasiveness of tumor cells represents one of several important factors necessary for metastasis. Our result has clearly shown that CT inhibits tumor cell invasion in a concentration-dependent manner. However, this effect was only seen in cells which had been cultured in the presence of CT for a prolonged period of 24 h. Assuming that inhibition of invasion requires changes in gene transcription and protein modulation, this observation is not surprising. In MDA-MB-231 cells, Holst-Hansen *et al* (27) and Guo *et al* (26) have suggested that *in vitro* invasiveness is dependent on uPA activity. The MMP system is another family of invasion-associated proteases in breast cancer (34); however, CT had no apparent effect on MMP-9 expression in this cell line (data not shown). Therefore, it is most likely that CT-mediated inhibition of

MDA-MB-231 cell invasion *in vitro* depends on the down-regulation of uPA and uPAR.

Our present data support the hypothesis that CT, under certain conditions, may protect against breast cancer invasion, which would help to explain why decreased CTR mRNA expression has been much more frequently seen in cases with advanced breast cancer. However, in contrast to our findings, Sabbisetti *et al* (35) and Chigurupati *et al* (36) have shown that CT increased the invasiveness of prostate cancer cells, as well as stimulating intratumoral angiogenesis by directly acting on endothelial cells. In our opinion, these conflicting data might be partially explained by the fact that CTRs exist in several isoforms in different tissues and are functionally heterogeneous (2). The CT-activated signaling pathway is not only tissue- and cell type-specific, but sometimes even cell cycle-specific (37).

In conclusion, our findings that CT regulates the level of expression of uPA and uPAR and the invasion of breast cancer cells opens a new avenue for investigations examining more closely the link between CT-signaling, the urokinase pathway, and breast cancer invasion. We propose that the MDA-MB-231 cell line is a useful model of advanced breast cancer and can be used to develop novel therapeutic agents involving the CT/CTR system.

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