MCT-1 Oncogene Contributes to Increased In vivo Tumorigenicity of MCF7 Cells by Promotion of Angiogenesis and Inhibition of Apoptosis

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Abstract

Overexpression of a novel oncogene MCT-1 (multiple copies in a T cell malignancy) causes malignant transformation of murine fibroblasts. To establish its role in the pathogenesis of breast cancer in humans, we generated stable transfectants of MCF7 breast cancer cells negative for endogenous MCT-1 (MCF7-MCT-1). Overexpression of MCT-1 in these cells resulted in a slight elevation of estrogen receptor-α, and higher rates of DNA synthesis and growth in response to estradiol compared with the empty vector control (MCF7-EV). The pure antiestrogen fulvestrant inhibited the estradiol-stimulated proliferation of MCF7-MCT-1 cells. The MCF7-MCT-1 clones showed increased invasiveness in the presence of 50% serum compared with the MCF7-EV. In a tumor xenograft model, MCT-1–overexpressing cells showed higher take rates and formed significantly larger tumors than MCF7-EV controls. When we examined angiogenic phenotype and molecular mediators of angiogenesis in MCF7-MCT-1 tumors in vivo, we found greater microvascular density and lower apoptosis in the MCF7-MCT-1 tumors compared with MCF7-EV controls accompanied by a dramatic decline in the levels of angiogenesis inhibitor, thrombospondin-1 (TSP1). In vitro, blocking TSP1 in the medium conditioned by MCT-1–negative cells restored its angiogenic potential to that of the MCF7-MCT-1 cells. Conversely, despite an increase in mRNA encoding vascular endothelial growth factor upon MCT-1 overexpression, vascular endothelial growth factor protein levels have not been notably altered. Taken together, our results suggest that MCT-1 may contribute to the pathogenesis and progression of human breast cancer via at least two routes: promotion of angiogenesis through the decline of TSP1 and inhibition of apoptosis. (Cancer Res 2005; 65(23): 10651-6)

Introduction

The mechanism of breast cancer progression and metastases is extremely complex. Patients with estrogen receptor (ER)–positive breast cancers often respond to hormonal therapy and have a better prognosis than patients with ER-negative tumors. The malignant progression of breast tumors is often explained by the transition from the ER-positive to the ER-negative stage; however, the differences in gene expression profiles of breast tumors (1) point to additional factors involved in breast cancer evolution. One approach to understanding the role of such factors in breast cancer genesis and progression is expression of the potentially relevant factor(s) in the ER-positive breast cancer cell lines.

MCT-1 (multiple copies in a T cell malignancy) is a novel candidate oncogene on chromosome Xq22-24, amplified in a T cell lymphoma (2). MCT-1 causes transformation of NIH 3T3 mouse fibroblasts and MCF-10A mammary epithelial cells, shortens their doubling time and G1 phase of the cell cycle, and is associated with deregulation of the G1-S checkpoint and increased expression of cyclin D1 (2–4). MCT-1 stimulates PKB/Akt activation, thus, protecting cells against apoptosis by serum deprivation (5). MCT-1 overexpression has recently been shown in a number of B and T lymphoma cell lines and in a subset of primary diffuse large B cell lymphomas (2, 5).

Here we analyzed MCT-1 expression in breast cancer cells and found higher MCT-1 levels in ER-negative MDA-MB-231, MDA-MB-435, and SK-BR3, whereas ER-positive nonmetastatic MCF7, ZR-75-1, and T47D cells produced none or little MCT-1. This finding has raised the possibility that MCT-1 contributes to the more aggressive metastatic breast cancer phenotype. To verify this hypothesis, we generated stable clones of MCF7 breast cancer cells overexpressing MCT-1 (MCF7-MCT-1) and characterized them in vitro and in vivo. We found that MCT-1 prompts the transition to a more aggressive phase in breast cancer progression by (a) enhancing invasiveness and decreasing apoptosis, thereby promoting faster growth, and (b) increasing tumor angiogenesis via down-regulation of an endogenous angiogenesis inhibitor, thrombospondin-1 (TSP1). These novel functions of MCT-1, combined with the previously documented protective effect of MCT-1 against stress-induced apoptosis, support its potential role in promoting the progression of breast cancer.

Materials and Methods

Cell culture. The MCF7:WS8 (parental cells), T47D:A18, ZR-75-1, SK-BR3, MDA-MB-231, and MDA-MB-435 cells were maintained in RPMI or MEM with phenol red, 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 6 ng/mL bovine insulin, penicillin, and streptomycin. Antiestrogen-resistant MCF7:5C cells were cultured in phenol red–free medium with 5% charcoal-stripped calf serum. Prior to the hormone-sensitive experiments, cells were grown in estrogen-free medium for 3 days before treatment. Estradiol was purchased from Sigma (St. Louis, MO), fulvestrant (ICI 182,780) was a gift from AstraZeneca (Macclesfield, United Kingdom). All the cells were free of Mycoplasma.
**Plasmid constructions and transfections.** MCT-1 coding sequence was amplified from total RNA (Jurkat cells) with the forward 5'-CACCATGGTT-CAAGAAATTTGATGAA-3' and reverse primer 5'-TTTATATGCTTCCATATGG-CCACAGCC-3' and cloned in the TOPO sites of pcdNA3.1/V5-histidine tag vector (Invitrogen, Carlsbad, CA). To subclone into a retroviral vector, the pcdNA3.1-MCT-1-V5-histidine plasmid was again PCR amplified using the primers 5'-TGAATTCACCATGTTGAAATTGGTATG3' and 5'-AGTTACAGCGGGTTTAACCTCAAT-3' containing relevant restriction sites (underlined), digested with EcoRI and ligated into pLXSN (Clontech, Mountain View, CA). The pLXS-empty vector (EV) and pLXS-MCT-1-V5-histidine were transfected into PT67 packaging cells, the viral supernatants were collected and added to subconfluent MCF7. After 24 hours, the cells were subjected to selection with 400 μg/mL G418 for 2 weeks. Stable clones were evaluated by Western blot for V5-tagged MCT-1 expression.

Ex vivo cell culture was established by expansion of tumor cells from two EV-containing and three MCT-1 tumor bearing mice. Fresh tumors were minced with scalpel, smashed under coverslip in six-well tissue culture plate and maintained in the same media as original MCF7-MCT-1 cells.

**Growth assays.** The cells were seeded at 4 to 5 × 10⁴ cells/well. The following day, media containing the appropriate compound (estradiol or fulvestrant) were added. The medium was changed every other day for 8 days. The cells were then sonicated and DNA content was measured as described previously (8).

**Western blot analysis.** Western blot analysis was done as described previously (7, 8) using primary antibody for ER-α, TSP1 (NeoMarkers, Fremont, CA) and MCT-1 (Research Genetics, Huntsville, AL). TSP1 protein levels were examined in conditioned media collected from cell cultures after serum deprivation for 48 to 72 hours. Conditioned medium was concentrated using Amicon ultra filters (Millipore, Bedford, MA). The blots were reprobed for β-actin or stained with Ponceau S to confirm equal loading.

**Invasion assays.** Invasion assays were done using the Cell Invasion Assay kit (Chemicon International, Inc., CA). Briefly, cells (5,000/mL) were maintained in serum-free medium with or without 50% FBS as a chemoattractant at 37°C for 24 hours. Invaded cells were subsequently detached, lysed, and detected by CyQuant GR dye using fluorescence plate reader Mithras LB 940 (Berthold Technologies, Germany).

**Endothelial cell migration assay.** Endothelial cell migration assay was done as described previously (8). Briefly, the cells adhered to one side of the porous membranes were allowed 4 to 6 hours to migrate up the gradient of proangiogenic basic fibroblast growth factor or conditioned medium. The migrated cells were counted in 10 randomly selected fields (>100). All samples were tested in quadruplicate and each experiment repeated at least twice. ED₅₀ (50% effective dose) values were determined from the linear regression curves (SigmaPlot software).

**Tumorigenicity assay.** Ovariectomized BALB/c-nu/nu 5-week-old athymic mice (5-10 per group) were injected s.c. with 10⁶ MCF7-MCT-1 and MCF7-EV cells into axillary mammary fat pads. All animals were implanted with a 0.3 cm silastic estradiol capsules (replaced every 8 weeks). Tumors were measured weekly and tumor areas were calculated as 1/4 × length × width × π. The data is reported as mean tumor area per group. The tumors...
were excised and frozen, the lungs and livers were formalin-fixed and paraffin embedded for histologic examination.

**Immunohistochemistry.** To visualize tumor vasculature and TSP1, 5-μm-thick sections were simultaneously incubated with TSP1 and CD31

![Diagram](Image)

**Figure 2.** A and B, MCF7-MCT-1 cells retained functional ERα. A, ERα protein levels in MCF7-MCT-1 cells were determined by Western blotting. Proteins were resolved on 7.5% SDS-PAGE (20 μg cell extract/lane), MCF7:WS8 and MDA-MB-231 cells were used as positive and negative controls, respectively. B, cells treated with ethanol, estradiol (10⁻³ mol/L), and fulvestrant (10⁻⁶ mol/L) for 24 hours were analyzed as in (A). Western blots were repeated at least thrice with similar results, representative blots are shown. C and D, dose- and time-estradiol response proliferation of MCF7-MCT1 cells assessed by DNA content. Points, mean of triplicate wells; bars, SE. C, a representative growth curve of N1 clone with varying estradiol concentrations. The cells were maintained in estradiol-free medium and treated with increasing concentrations of estradiol or vehicle (ethanol) for 6 days. D, the inhibition of estradiol-stimulated proliferation of MCF7-MCT-1 by pure antiestrogen fulvestrant (F). The cells were treated for 8 days with ethanol, 10⁻⁵ mol/L estradiol, 10⁻⁶ mol/L fulvestrant, or a combination of estradiol and fulvestrant. Each treatment was done in triplicate, the experiment was repeated five times.

**Results**

**Aggressive estrogen receptor-negative breast cancer cells express MCT-1 at higher levels.** To investigate the role of MCT-1 in breast cancer, we analyzed MCT-1 levels in a panel of human breast cancer cells that differ in their aggressiveness and metastatic ability (Fig. 1A). MCF7:WS8, MCF7:5C, and T47D:A18 are all nonaggressive ERα-positive cell lines (9, 10). In contrast, SK-BR3, MDA-MB-231, and MDA-MB-435 cells are ER-negative and highly aggressive and metastatic. As seen in Fig. 1A, MCT-1 was expressed at high levels in the extracts from malignant, ER-negative cells, and at much lower levels in the ER-positive cells.

**Construction of MCT-1 stable transfectants.** We stably transfected MCF7:WS8 cells with pLXSN-EV and pLXSN-MCT1-V5-histidine tag vector and selected three clones with high steady-state levels of MCT-1 (N1, N4, and N7). Western blot analysis showed the bands of expected molecular weight (2) in the cell extracts from MCF7-MCT-1 clones but not from vector controls (Fig. 1B).

**MCF7-MCT-1 cells display increased invasiveness in vitro.** As shown in Fig. 1C, in the presence of chemotactant (50% FBS), MCF7-MCT-1 cells showed significantly increased invasiveness almost reaching the level of MDA-MB-231 cells, whereas the invasiveness of MCF7-EV remained low. The invasiveness varied between the three MCF7-MCT-1 clones in the presence of 50% FBS, however, even the least aggressive N1 clone was considerably more invasive than MCF7-EV control (Fig. 1D).

**MCF7-MCT-1 cells maintain functional estrogen receptor-α and respond to estradiol.** We analyzed ERα protein levels by Western blot (Fig. 2A) and found that MCF7-MCT-1 express ERα at slightly higher levels than the control cells. To further characterize the biological activity of ERα in these cells, we determined the effect of estradiol on ERα protein expression. Consistent with published data (11), we found an estradiol-dependent decrease of ERα protein levels in MCF7-MCT-1 cells and a substantial ERα degradation by fulvestrant (Fig. 2B).

Next, we examined the effect of estradiol and fulvestrant alone and in combination on the growth of MCT-1–overexpressing cells in vitro. The ER-positive MCF7-MCT-1 clones showed dose-dependent growth stimulation by estradiol (Fig. 2C). Time-dependent growth analysis with fixed concentrations of estradiol (10⁻³ mol/L), fulvestrant (10⁻⁶ mol/L), or the combination of these two compounds showed strong proliferation in response to estradiol, no effect by fulvestrant alone, and the inhibition of the estradiol-stimulated growth by fulvestrant, further substantiating the functional role of ERα in estradiol-stimulated growth (Fig. 2D).

Although MCF7-MCT-1 cells showed a clear trend towards more robust mitogenesis in response to estradiol compared with the MCF7-EV and parental MCF7 cells, the differences were not statistically significant (data not shown).
MCT-1 overexpression resulted in increased tumorigenicity. To test the effect of MCT-1 overexpression on tumorigenicity, two MCT-1-overexpressing MCF7 clones (N1 and N7) as well as MCF7-EV cells, were bilaterally injected into the mammary fat pads of ovariectomized athymic mice in the presence of s.c. estradiol-release pellets. Both MCT-1 transfectants showed higher tumor take at week 7 (37.5% for N1 and 65% for N7) compared with MCF7-EV (20%). At week 14, tumors formed by MCF7-MCT-1 cells were clearly larger than in the control group (Fig. 3A). However, due to the high variability within MCF7-MCT-1 groups and the unexplained mortality, the statistical significance was marginal (P < 0.06 for N1 and P < 0.07 for N7). Nevertheless, at week 17, the surface area of the MCF-MCT-1 tumors was 3.5 to 3.8 times higher than of MCF7-EV tumors (Fig. 3A, right).

We established a new generation of MCT-1 tumors by implanting 1 mm³ pieces of the MCF7-MCT-1 (N1) and MCF7-EV tumors from the previous experiment, into the mammary fat pads of a new group of mice. The transplanted MCT-1 tumors showed rapid growth and better tumor take than the tumors developed from cell injection. Visible tumors emerged almost immediately in the MCF7-MCT-1 group whereas tumors remained latent for 6 weeks in the MCF7-EV group (Fig. 3B). By week 11, mean tumor size was significantly greater in the MCF7-MCT-1 (N1) group compared with the MCF7-EV (0.91 and 0.38 cm², respectively; P < 0.001; Fig. 3B, right), these differences remained significant (P < 0.01) by week 14 despite the diminished group size due to mortality.

Gross and microscopic examination of H&E-stained lung and liver tissues from all tumor-bearing mice revealed no signs of micrometastases (data not shown), suggesting that MCT-1 overexpression alone is insufficient to promote spontaneous dissemination of MCF7 cells.

MCF7-MCT-1 tumors were more vascularized and less apoptotic than control MCF7-EV tumors. Histologic analysis revealed low cellular density and sizeable necrotic areas for MCF7-EV and a “healthy” appearance for MCF7-MCT-1 tumors (Fig. 4A, H&E). Moreover, MCF7-MCT-1 tumors showed significant, 2.6-fold reduction of apoptosis (Fig. 4A and B, TUNEL; P < 0.00000000001) compared with the control. Such central necrotic areas and high apoptotic rates are likely to emerge from poor vascularization and resulting hypoxia. We, therefore, compared the microvascular densities of MCF7-MCT-1 and MCF7-EV tumors at week 14.

Figure 3. A, increased tumorigenicity of MCT-1 overexpressing MCF7 cells. MCF7-MCT-1 (N1 and N7) and MCF7-EV cells were inoculated into mammary fat pads of ovariectomized athymic mice implanted with estradiol pellets. Left, tumor growth curves; (▲) N1, (●) N7, and (▼) MCF7-EV. Right, tumor-bearing mice representative of each group at 17 weeks, arrows point at tumors. B, tumor progression in mice bearing implanted fragments from MCF7-EV and MCT-1 (N1) tumors from experiment shown in (A). Left, tumor growth curves. Note larger tumor sizes and significant differences between groups starting at week 8 (P < 0.001). Right, representative mice from each group at week 14 after implantation.
Indeed, MCT-1 overexpression resulted in a dramatic ~3.5-fold increase in the median tumor microvascular density (Fig. 4A and B, CD31; P < 0.00000015).

**MCT-1 overexpression caused a decrease in the angiogenesis inhibitor, thrombospondin-1.** Seeking a mechanism behind the proangiogenic effect of MCT-1, we examined two angiogenic factors critical in breast cancer, proangiogenic VEGF and antiangiogenic TSP1 (12). Immunostaining revealed relatively high VEGF levels typical for breast cancer, with no significant differences between MCF7-MCT-1 and MCF7-EV tumors (data not shown). In contrast, TSP1 levels were dramatically lower in MCF7-MCT-1 compared with vector controls. Interestingly, VEGF mRNA levels were increased. Conditioned medium was collected from cells maintained in hypoxia chamber (1.5% O2; Coy Instruments, Grass Lake, MI) for 48 hours and subjected to Western blotting with anti-VEGF and anti-TSP1 antibodies. To determine the functional consequences of the MCT-1-driven decline of TSP1, we measured endothelial cell chemotaxis in the presence of TSP1 peptide (100 nmol/L) and migration stimulated by basic fibroblast growth factor (bFGF, 10 ng/mL). Points, means; bars, SE.

Semiquantitative RT-PCR showed lower TSP1 mRNA levels in MCF7-MCT-1 tumors compared with the MCF7-EV tumors (data not shown; Supplementary data; Fig. 3). Interestingly, VEGF mRNA was higher in MCT-1 tumors compared with vector controls. Western blot analysis of ex vivo cultures showed higher levels of TSP1 secreted by MCF7-MCT-1 conditioned medium compared with MCF7-EV (Fig. 4C, top). When conditioned medium was collected under hypoxia (1.5% O2), to mimic the in vivo tumor environment, VEGF levels became similar in both EV- and MCT-1-expressing cells. In contrast, TSP1 levels were dramatically lower in MCF7-MCT-1 conditioned medium compared with MCF7-EV (Fig. 4C, bottom). To determine the functional consequences of the MCT-1-driven decline of TSP1, we measured endothelial cell chemotaxis in the presence of conditioned medium from the cells collected in hypoxia. The secretions of the MCT-1 expressing cells were more angiogenic as was reflected by their lower ED50 values (0.3 versus 2.9 μg/mL for EV). Adding TSP1-neutralizing antibodies restored the migratory capability of MCF7-EV cells (ED50 ≈ 0.7 μg/mL; Fig. 4D, left),...
whereas isotype control IgA had no effect (data not shown). In contrast, TSP1 active antiangiogenic peptide ABT-510 (13) completely blocked the ability of conditioned medium from MCF7-MCT-1 cells to stimulate endothelial cell chemotaxis (Fig. 4D, right).

Discussion

The fact that MCT-1 was expressed at high levels in the aggressive breast cancer cells prompted us to examine its potential role in breast cancer progression. Like parental cells, MCF7-MCT-1 retained functional ER-α and estradiol-responsiveness, however, they gained enhanced ability to invade in vitro, further supporting the role of MCT-1 in increasing metastatic potential (2).

Although MCF7-MCT-1 and MCF7-EV grew at a similar rate in culture, in nude mice, MCF7-MCT-1 formed estradiol-dependent tumors much faster, especially when previously established tumors were transplanted to a new group of mice. Thus, we have shown for the first time that MCT-1 overexpression in MCF7 breast cancer cells results in a higher rate of tumor progression. Despite enhanced invasiveness of MCF7-MCT-1 cells, MCT-1 was insufficient to cause MCF7-MCT-1 tumors to metastasize, suggesting the requirement for additional factors.

The lack of differences between the growth rates in vitro combined with considerably increased tumorigenicity in vivo suggested a possible role for MCT-1 in the regulation of tumor angiogenesis. This hypothesis was corroborated by a dramatic 2.7-fold increase in the vascularity of the MCT-1–positive tumors. The MCT-1–driven increase in angiogenesis coincided with an extensive decrease of tumor cell apoptosis, which explains, at least in part, the accelerated growth of MCF7-MCT-1 tumors. Moreover, central necrotic areas characteristic of hypoxia due to insufficient vascularization (14) were present in all MCT-1–negative tumors and were absent in the MCF7-MCT-1 tumors.

Tumor angiogenesis is determined by the balance between extracellular mediators of angiogenesis, increased expression of the proangiogenic molecules (inducers), or decrease in the secreted inhibitor(s) (12), which in turn might be controlled by the loss of tumor suppressors and the activation of oncogenes (15). Here, we established that the MCT-1 oncogene participates in angiogenic switch in MCF7 breast cancer cells in vitro and in vivo.

Several mediators of angiogenesis have been linked to breast cancer progression. Of the inducers, VEGF is the most prominent; its overexpression promotes local tumor growth and brain metastases in the mouse model of breast cancer (16). Conversely, the angiogenic switch may occur due to the loss of potent inhibitor, TSP1, which is expressed at high levels in normal breast tissue. Higher TSP1 levels in the stroma around ductal carcinoma in situ indicate favorable prognoses (17), moreover, forced TSP1 expression reverses the angiogenic switch and reduces tumorigenicity (18).

In our estrogen-dependent breast cancer model, MCT-1 overexpression severely decreased the levels of TSP1 mRNA and protein, suggesting that MCT-1 causes angiogenic switch predominantly by suppressing inhibitory TSP1. Indeed, by neutralizing TSP1, we were able to restore the angiogenic activity of the MCF7-EV cells to the levels close to those of MCF7-MCT-1, whereas exogenous TSP1 blocked angiogenesis by MCF7-MCT1. Interestingly, the changes in TSP1 expression could only be registered under hypoxic conditions, suggesting that MCT-1 may suppress TSP1 via one of the hypoxia-inducible transcription factors (15). Conversely, MCT-1 overexpression resulted in constitutively high VEGF levels, even in high oxygen content, whereas hypoxia caused increased VEGF secretion by MCF7-EV, thus, muffling the differences in VEGF as was registered by immunostaining.

In summary, this is the first study to show the role of MCT-1 in breast cancer. Our observations suggest a new function for MCT-1, the control of angiogenic phenotype via down-regulation of the antiangiogenic protein, TSP1. In addition, MCT-1 overexpression inhibited tumor apoptosis. Taken together with documented proliferative and antiapoptotic roles of MCT-1 (2, 3, 5), our observations support a role for MCT-1 in the progression of breast cancer towards aggressive, highly vascularized tumors.

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References

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