Cancer Letters 362 (2015) 106–115

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Inhibition of angiogenesis by selective estrogen receptor modulators through blockade of cholesterol trafficking rather than estrogen receptor antagonism

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ARTICLE INFO

Article history:
Received 29 December 2014
Received in revised form 16 March 2015
Accepted 17 March 2015

Keywords:
Angiogenesis
Selective estrogen receptor modulator
Tamoxifen
Cholesterol trafficking

ABSTRACT

Selective estrogen receptor modulators (SERM) including tamoxifen are known to inhibit angiogenesis. However, the underlying mechanism, which is independent of their action on the estrogen receptor (ER), has remained largely unknown. In the present study, we found that tamoxifen and other SERM inhibited cholesterol trafficking in endothelial cells, causing a hyper-accumulation of cholesterol in late endosomes/lysosomes. Inhibition of cholesterol trafficking by tamoxifen was accompanied by abnormal subcellular distribution of vascular endothelial growth factor receptor-2 (VEGFR2) and inhibition of the terminal glycosylation of the receptor. Tamoxifen also caused perinuclear positioning of lysosomes, which in turn trapped the mammalian target of rapamycin (mTOR) in the perinuclear region of endothelial cells. Abnormal distribution of VEGFR2 and mTOR and inhibition of VEGFR2 and mTOR activities by tamoxifen were significantly reversed by addition of cholesterol–cyclodextrin complex to the culture media of endothelial cells. Moreover, high concentrations of tamoxifen inhibited endothelial and breast cancer cell proliferation in a cholesterol-dependent, but ER-independent, manner. Together, these results unraveled a previously unrecognized mechanism of angiogenesis inhibition by tamoxifen and other SERM, implicating cholesterol trafficking as an attractive therapeutic target for cancer treatment.

Introduction

Tamoxifen and selective estrogen receptor modulators (SERM) have been used to treat hormone responsive, estrogen receptor (ER)-positive breast cancers since the 1980s. It has generally been accepted that the anticancer activity of tamoxifen is mainly attributable to its competitive antagonism to ER, thereby inhibiting the proliferation of ER-positive breast cancer cells [1]. However, whether this is the only mechanism of action underlying the anticancer activity of SERM has been questioned since tamoxifen and other SERM also showed anticancer activity in ER-negative breast cancers [2–4]. Since the 1990s, several groups have found that tamoxifen and SERM strongly inhibited angiogenesis by mechanisms independent of ER [5–7]. Based on these findings, tamoxifen and other SERM are now being actively investigated as anti-angiogenic agents in clinical trials for cancer treatment [8–10]. However, the underlying molecular mechanism by which tamoxifen inhibits angiogenesis has remained largely unknown.

Cholesterol is an essential component of cellular membranes and plays a key role in membrane permeability and fluidity. In addition to a structural role, it also functions in intracellular transport and cell signaling [11,12]. Serum cholesterol is delivered throughout the body in the form of low-density lipoprotein (LDL) and transported into cells through receptor-mediated endocytosis [13]. Endocytosed LDL is transported to the late endosomes and lysosomes (endolysosomes) where cholesteryl esters are hydrolyzed and free cholesterol is released from the endosomal system for delivery to other compartments, including the plasma membrane and endoplasmic reticulum [14]. One of the most important machineries of cholesterol trafficking in the endolysosomes is the Niemann–Pick type C (NPC) proteins (NPC1 and NPC2), which help acid lipase-mediated hydrolysis of cholesteryl esters and deliver free cholesterol out of the endolysosomes [15]. Inhibition of NPC1 or 2 causes accumulation of cholesterol and glycolipids in the endolysosomes, a phenotype called NPC after the genetic disease of the same name [16].

We have previously reported that a newly identified anti-angiogenic drug itraconazole inhibited cholesterol trafficking and induced NPC-like phenotype in endothelial cells [17]. Inhibition of cholesterol trafficking by itraconazole is accompanied by inhibition of mTOR signaling and VEGFR2 glycosylation, both of which are essential signaling components for endothelial cell proliferation

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http://dx.doi.org/10.1016/j.canlet.2015.03.022
0304-3835/

[17,18]. Recently, Fang et al. showed that upon over-expression, apoA-I binding protein (AIBP), which is responsible for cholesterol efflux from endothelial cells, inhibited angiogenesis by depleting cholesterol from the plasma membrane, thereby inhibiting the VEGFR2 signaling pathway in endothelial cells and animal models [19]. Similar to AIBP over-expression, cells with NPC phenotype induced by small molecules showed accumulation of cholesterol in the endolysosomes leading to cholesterol depletion in plasma membrane and inhibition of the VEGFR2 signaling pathway [17,18]. These results strongly suggest that cholesterol trafficking in endothelial cells is critical for proper angiogenesis.

In the present study, we found that tamoxifen and other SERM inhibited cholesterol trafficking in endothelial cells. Blockade of cholesterol trafficking by SERM led to an abnormal subcellular distribution of mTOR and VEGFR2 and caused inhibition of their signaling pathways in a cholesterol-dependent manner. These data suggest that tamoxifen and other SERM inhibit angiogenesis by interfering with cholesterol trafficking in endothelial cells and that cholesterol trafficking is a novel target for anti-angiogenesis therapy.

Materials and methods

Cells and reagents

Pooled human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Allendale, NJ) and were grown in endothelial cell growth medium-2 (EGM-2) using the EGM-2 bullet kit (Lonza). MCF-7 (ER-positive) breast cancer cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY) and 1% antibiotics (penicillin and streptomycin) solution (Life Technologies). MDA-MB-231 (triple negative) breast cancer cells were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS (Life Technologies) and 1% antibiotics. All the cells were maintained in a humidified incubator at 37 °C adjusted to 5% CO_2. Methyl-β-cyclodextrin, cholesterol and filipin were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human VEGF-165 was purchased from R&D Systems (Minneapolis, MN).

Filipin staining

Filipin staining was performed as described with slight modifications [17]. HUVEC were cultured in a Nunc Lab-Tek II 8-Chamber Slide (Thermo Scientific, Rockford, IL) at 1×10^4 cells/well. Cells were treated with SERM with or without cholesterol and cyclodextrin complex for 24 h. Cells were then fixed with 4% paraformaldehyde for 20 min at room temperature and stained with filipin at a final concentration of 50 μg/ml in the dark for 1 h at room temperature. Cells were washed with PBS, mounted with Immu-mount (Thermo Scientific), and observed under a Zeiss 510 Meta multiphoton confocal microscope (Carl Zeiss, Thornwood, NY).

Immunofluorescence imaging

For co-staining of proteins and cholesterol, HUVEC (1×10^4 cells/well) grown in a Nunc Lab-Tek II 8-Chamber Slide were treated with compounds for 24 h, fixed with 4% paraformaldehyde for 20 min at room temperature and stained with filipin (50 μg/ml) for 1 h at room temperature. Cells were then permeabilized with 0.2% saponin supplemented with 50 μg/ml filipin and 5% bovine serum albumin (BSA) in PBS for 30 min. Cells were incubated with primary antibodies in PBS together with 50 μg/ml filipin, 0.05% saponin and 5% BSA overnight at 4 °C. Cells were then incubated with secondary antibodies in PBS with 50 μg/ml filipin, 0.05% saponin and 5% BSA at room temperature for 1 h. Cells were washed with PBS, mounted with Immumount, and observed under a Zeiss 510 Meta multiphoton confocal microscope. For general immunofluorescence, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 10 min and washed with PBS prior to blocking in 1% BSA in PBS containing 0.1% Tween 20 (PBST) for 1 h. Cells were then incubated with primary antibodies including anti-VEGFR2 (Cell Signaling Technology, Danvers, MA), anti-LAMP1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mTOR (Cell Signaling Technology) and anti-GM130 (BD Biosciences, San Jose, CA) in the blocking solution overnight at 4 °C, and then incubated with secondary antibodies conjugated with Alexa-Fluo488 or Alexa-Fluo594 for 1 h. The cellular nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and actin cytoskeleton was stained with rhodamine-phalloidin (Life Technologies). The immunofluorescence images were obtained using the Zeiss 510 Meta multiphoton confocal microscope.

[^{3}H]-thymidine DNA incorporation assay

HUVEC, MCF-7 or MDA-MB-231 were seeded at 3×10^3 cells/well in 96-well plates and allowed to adhere at 37 °C for 24 h. Cells were then treated with compounds for 24 h prior to being pulsed with 0.5 μCi [^{3}H]-thymidine (PerkinElmer, Waltham, MA) for 16 h and then trypsinized. The cells were harvested onto FilterMat A glass fiber filters (Wallac, Turku, Finland) using a Harvester 96 cell harvester (Tomtec, Hamden, CT), and the radioactivity of [^{3}H]-thymidine incorporated into DNA was counted using a MicroBeta liquid scintillation plate reader (PerkinElmer). The IC_{50} values and 95% confidence intervals were calculated using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

AlamarBlue cell viability assay

HUVEC, MCF-7 or MDA-MB-231 were seeded at 3×10^3 cells/well in 96-well plates and allowed to adhere at 37 °C for 24 h. The cells were treated with compounds in the presence or absence of cholesterol or cyclodextrin, or both for 24 h. AlamarBlue reagent (Life Technologies) was added to the media at a final concentration of 10% and the incubation was continued for an additional 2 h. The fluorescence signal was read with an excitation wavelength of 570 nm and an emission wavelength of 590 nm using a SpectraMax M5 fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

Western blot analysis

HUVEC (2×10^5 cells/well) were seeded in 6-well plates and allowed to adhere overnight. Following drug treatment for 24 h, cells were lysed by adding 2× Laemmli buffer containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris–HCl, pH 6.8 and the lysates were boiled for 10 min and vortexed. After SDS-PAGE, the proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked with 5% non-fat dried milk at room temperature for 1 h, incubated with the primary antibodies including anti-VEGFR2 (Cell Signaling Technology), anti-phospho-VEGFR2 (Tyr1175, Cell Signaling Technology), anti-FGFR1 (Cell Signaling Technology), anti-mTOR (Cell Signaling Technology), anti-phospho-mTOR (Ser2448, Cell Signaling Technology), anti-S6K (Cell Signaling Technology), anti-phospho-S6K (Thr389, Cell Signaling Technology), anti-PDGFRβ (Santa Cruz Biotechnologies), anti-actin (Santa Cruz Biotechnologies) or anti-α-tubulin (Santa Cruz Biotechnologies) antibodies overnight at 4 °C and then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. The immune-complexes were detected using enhanced chemiluminescence (ECL) detection reagent (GE Healthcare, Pittsburgh, PA).

Endothelial cell tube formation assay

A 96-well plate was coated with 50 μl Matrigel (BD Biosciences) and was incubated at 37 °C for 1 h to allow for polymerization. HUVEC were mixed with appropriate compounds and seeded (2×10^4 cells/well) on the Matrigel-coated wells, followed by incubation in a CO_2 incubator for 16 h. Cells were washed carefully with PBS once and Calcein-AM (2 μM in PBS, Life Technologies) solution was added to the cells. After incubation at 37 °C for 30 minutes, the cells were washed gently with PBS and the fluorescence-labeled tubular structures were observed under a Nikon Eclipse TS100 fluorescence microscope with an excitation wavelength of 485 nm and an emission wavelength of 520 nm at magnification ×100. The total tube lengths, sizes and number of junctions from the fluorescence images were quantified using the AngioQuant v1.33 software (The MathWorks, Natick, MA) and plotted using the GraphPad Prism 5.0 software.

Statistical analysis

Statistical significance of the data between control and test groups was determined by two-sided Student's *t*-test using the GraphPad Prism 5.0 software. The *P* values less than 0.05 were considered significant.

Results

Tamoxifen and SERM induce NPC-like phenotype in HUVEC

We tested four FDA-approved SERM including tamoxifen, toremifene, clomifene and raloxifene (Supplementary Fig. S1) for effects on cholesterol trafficking in HUVEC. Intracellular cholesterol was visualized by staining fixed cells with filipin [20]. All four SERM induced accumulation of cholesterol in the perinuclear region of HUVEC, a phenotype similar to NPC and that induced by itraconazole (Fig. 1A and Supplementary Fig. S2A). The cell morphology was also changed from large flat morphology into a partially shrunken form. These effects, however, were reversed upon addition of exogenous cholesterol and its carrier methyl-β-cyclodextrin (CD) (Fig. 1B and Supplementary Fig. S2B), suggesting that they are mediated largely through inhibition of cholesterol trafficking.