Tamoxifen Down-Regulates CD36 Messenger RNA Levels in Normal and Neoplastic Human Breast Tissues

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Abstract

Tamoxifen (TAM) exerts a long-term suppressive effect on human breast cancer cell proliferation. To determine whether the effects of TAM are mediated by specific gene activation or repression, normal and tumoral human breast tissues obtained before and during TAM treatment were analyzed by differential display technique. Total RNA for differential display analysis was obtained from breast tissues from two women with the diagnosis of estrogen receptor-positive stage II (T2N1M0) infiltrating ductal carcinoma performed after a 30-day treatment with TAM (20 mg/day). One 202-bp cDNA band, AP5.1, was present in normal and tumoral biopsy samples, but was absent in breast tissue obtained during TAM treatment, and was confirmed by Northern hybridization, which showed a 2.7-kb band in both patients. The differentially expressed cDNA fragment showed 99% homology to Homo sapiens CD36 gene, a glycoprotein that acts as a receptor for the extracellular matrix proteins thrombospondin-1, collagen types I and IV, and oxidized low-density lipoprotein. These results indicate that the down-regulation of CD36 induced by TAM might represent alternative or additional mechanisms of action of this drug affecting the functions of thrombospondin-1, which is involved in hematogenous tumor spread, invasion and angiogenesis, and oxidized low-density lipoprotein, playing a role in inhibition of arteriosclerosis. The multiple functions affected by the down-regulation of CD36 by TAM warrant the need for additional studies.

Introduction

TAM4 exerts a long-term suppressive effect on breast cancer cell proliferation. It has been used in the treatment of breast cancer patients for more than 20 years (1). TAM is a nonsteroidal triphenylethylene capable of adopting a structural conformation that resembles the steroid nucleus. It binds to the estrogen receptor in competition with this hormone, acting as both its antagonist and a partial agonist (1, 2). This orally effective, synthetic antiestrogen used first in women with metastatic disease has become an essential part of any therapeutic strategy for the control and prevention of breast cancer (3). Additional benefits reported with the use of TAM are a protective effect on the cardiovascular system as a result of its ability to decrease cholesterol levels and to reduce atheromatous plaque formation by blocking human low-density lipoprotein peroxidation (3).

The modulation of breast cancer cell proliferation by TAM has been reported to be mediated by several mechanisms, such as down-regulation of c-myc and c-erbB2 oncogenes (4), and by differentially influencing the cellular production of factors such as transforming growth factors α and β, cyclin A, cyclin D1, and p21 (5). TAM also decreases the proportion of cells in S phase and increases the number of cells in G2/G1, probably by arresting the cells in these early stages of the cell cycle (6).

Although many of the TAM actions are already established, very little is known about its influence on specific genes other than those involved in cell cycle control and proliferation (4—6). To determine whether other genes are up- or down-regulated by TAM therapy, we analyzed total RNA from both normal and neoplastic breast tissues obtained from breast cancer patients before and on the 30th day of TAM treatment using the DD method (7—9).

Materials and Methods

Specimens. Human breast cancer and adjacent normal breast tissue were obtained by one of us (I. D. C. G. S.) at the Mastology Group, Gynecology Department, Federal University of São Paulo, Escola Paulista de Medicina, São Paulo, Brazil, according to a protocol approved by the Human Investigations Committee. The patients were two women, who at the ages of 50 and 52 (patients 1 and 2, respectively) were diagnosed as having estrogen receptor-positive stage II (T2N1M0) infiltrating ductal carcinoma. The diagnosis was made by incisional biopsy; then, both women were treated with TAM (20 mg/day) for 4 weeks before the performance of a modified radical mastectomy. Neither patient had received radiotherapy, chemotherapy, or any hormonal treatment during the 6 months before the diagnosis of breast cancer. A fragment of both normal and malignant tissue was obtained at the time of performing the incisional biopsies and during mastectomy. All of the tissue samples were frozen in liquid nitrogen immediately upon removal. The remainder of the tissues was fixed in 10% neutral-buffered formalin and embedded in paraffin following standard histological procedures.

RNA Preparation and Northern Blot Hybridization. Total RNA was isolated from tissues using the technique described by Chomczynski and Sacchi (10) and then treated with DNAase I using the Message Clean kit (GenHunter, Nashville, TN) to remove genomic DNA. Total RNA was separated on 1% agarose/formaldehyde gel and transferred to a Duralon membrane (Stratagene, La Jolla, CA). After UV cross-linking, membranes were hybridized overnight to probes labeled with 32PdCTP using a random-primer DNA system (Life Technologies, Gaithersburg, MD) at 42°C.

Differential Display. One one-base anchored oligodeoxy thymidylate thymidylic acid primer HT11G (5'-AAGCTTTTTTTTTTTT-3') was used to reverse transcribe total RNA from tissues into first-strand cDNA, which was amplified subsequently by PCR using the arbitrary upstream primers H-AP3 (5'-AAGCTTTGTCAAGC-3'), H-AP5 (5'-AAGCTTAGTAGGC-3'), and H-AP8 (5'-AAGCTTTATACCCG-3'); RNA Image kit, GenHunter). PCR conditions followed essentially the manufacturer’s instructions. PCR products were analyzed on a 6% DNA sequencing gel using 0.25 μl of α-32PdATP (2000 Ci/mmol). The bands that were unique before or during TAM treatment in the normal and/or breast cancer tissues were cut out from the gel, eluted, and amplified by PCR.

Cloning and Sequencing. The reamplified cDNA bands were cloned using the TA cloning kit (Invitrogen, San Diego, CA). The isolated fragment was sequenced using an automated DNA sequencer (Perkin Elmer ABI Prism model 377, version 2.1.1).

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4 The abbreviations used are: TAM, tamoxifen; DD, differential display; ox-LDL, oxidized low-density lipoprotein.

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Fig. 1. DD in duplicate (a, b) using total RNA of normal breast tissue from patient 1 showing the AP5-1 band (arrow). A, normal tissue before ($N_a$ and $N_b$) and on the 30th day of TAM treatment ($N_a + TAM$ and $N_b + TAM$). B, DD of invasive ductal carcinoma from the same patient before ($T_a$ and $T_b$) and on the 30th day of TAM treatment ($T_a + TAM$ and $T_b + TAM$).

Fig. 2. Northern blot hybridization using labeled AP5-1 cDNA as a probe. A, patient 1; B, patient 2. The AP5-1 RNA transcript (2.7-kb band) present in normal (N) and tumoral (T) tissues at the time of biopsy was down-regulated by TAM treatment in both normal ($N + TAM$) and tumor ($T + TAM$) total RNA samples from both patients. The 2.0-kb band was interpreted to be due to alternative splicing. β-actin mRNA detection was used as a control for verifying the loading of the RNA samples.

Fig. 3. Nucleotide sequence of AP5-1 cDNA. The underlined sequences are those used as primers for the DD.

Discussion

In the present study, we found that a 30-day TAM treatment down-regulates the expression of CD36 in both normal breast and invasive ductal carcinomas. CD36 is a cell-surface glycoprotein expressed in mammary epithelial cells, endothelial cells, monocytes, erythrocytes, and platelets (11–15). It is composed of a single polypeptide chain whose size ranges, depending on the cell type, from $M_r$ 78,000–88,000 ($M_r$ 50,000 deglycosylated; Ref. 16). CD36 is predicted to possess two transmembrane domains spanning residues 7–34 and 440–466, two short cytoplasmic tails at both the NH2- and COOH-terminal ends (residues 1–6 and 467–472, respectively), and a large, highly glycosylated extracellular domain comprising residues 35–439 (Fig. 4). The human CD36 gene consists of 15 exons on chromosome 7q11.2. The exon 14 encodes for the 54 terminal amino acids (149–472) included in one of the two transmembrane domains, one of the two cytoplasmic tails, and part of the extracellular domain. An additional exon, located 1.9 kb downstream from exon 14, presents a consensus acceptor splicing site at its 5' flanking region that may be responsible for the second band observed in our Northern blot. Steroid hormones via the response element at position 189 from the 5' proximal promoter region of the CD36 gene may exert important regulatory effects on the modulation of CD36 expression (17).

A multiplicity of interactions and functions have been ascribed to CD36. This glycoprotein acts as a receptor for the extracellular matrix...
glycoproteins thrombospondin-1 (18-21), and collagen types I and IV (20, 21). Thrombospondin-1 is a trimeric high molecular weight glycoprotein known by its ability to mediate cell adhesion. It also seems to be involved in the modulation of phenomena such as hematogenous tumor spread, tumor cell adhesion, invasion, and angiogenesis (18-21). Therefore, the down-regulation of CD36 expression observed in TAM-treated patients might explain the antitumorigenic effect of this antiestrogen (Fig. 4).

CD36 has also been implicated in the binding to ox-LDL. Recent evidence strongly implicates ox-LDL in the pathogenesis of atherosclerosis (22). The oxidation of low-density lipoprotein in the arterial wall is thought to contribute to human atherosclerosis development, in part by the high-affinity uptake of ox-LDL by macrophages, resulting in foam cell formation (23, 24). Several receptors that bind and internalize ox-LDL have been identified, but their relative importance in vivo is unclear. Only CD36, which has been implicated as a putative receptor for ox-LDL, has been shown to be relevant (Fig. 4; Refs. 23 and 24). Therefore, the low incidence of myocardial infarction associated with TAM therapy (25, 26) might be attributed to the down-regulation of CD36 expression during treatment with TAM reported in the present study; this observation clearly needs further evaluation.

An important additional finding from our data base search was a very high homology of AP5-1 sequence to the gene that codifies for an M, 85,000 cell membrane protein overexpressed in Adriamycin-resistant cells (27). Because TAM seems to antagonize the resistance against anthracyclines and vinblastine (28), it is possible to postulate that this might be one of the mechanisms through which TAM exerts a reversal in multidrug-resistant cells (Fig. 4). The multiple functions affected by down-regulation of CD36 by TAM warrant the need for additional studies.

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