S-phase reduction in T47D human breast cancer epithelial cells induced by an S100P antisense-retroviral construct

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Abstract. S100P is expressed in several malignant neoplasms. It was previously demonstrated that S100P is involved in the very early stages of breast carcinogenesis. In the present study we used a retrovirus-mediated transfer of antisense-S100P in order to check whether the decrease in expression of this protein could lead to alterations in the cell cycle of epithelial cells of human breast cancer. The T47D breast carcinoma cell line, a human breast epithelial cell that expresses high levels of S100P, was a tool used in this study to investigate the alteration in cell cycle induced by a retrovirus-mediated transfer of antisense-S100P. First we used the real-time PCR technique to quantify the gene expression. The results showed a reduction of 63% of expression within the T47D-S100P-A/S infected population compared with control T47D-LXSN clones. To determine the impact of the S100P antisense technique on protein expression in T47D cells, we performed immunofluorescence staining and analyzed the resulting images using a confocal microscope. The images showed much less pronounced antibody marking of the S100P protein in the T47D-S100P-A/S compared with control cells. To evaluate whether the antisense approach caused any alteration in the cell cycle, we concluded the study with flow cytometric analysis of the cell distribution. Our findings indicated that, in our model, S100P-antisense cells showed a 23% reduction of cells at the S-phase. Using transduction techniques with an S100P antisense-retroviral construct we were able to demonstrate a significant reduction in S-phase of the T47D cell cycle. To the best of our knowledge, this is the first time that an antisense approach has been used against S100P mRNA in breast cancer epithelial cells. The results showed here seem to further classify S100P as a protein that might be involved in the cell cycle imbalance observed during breast carcinogenesis.

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Introduction

Calcium ions serve as secondary messengers during the control of biochemical pathways. This control of cellular transduction is partly achieved through interactions with Ca-binding proteins. The S100 family consists of Ca^{2+}-binding proteins of the EF-hand type with at least 20 members (1,2).

The functions of these proteins vary widely between individual members. One of the last members of the S100 family is S100P, a 95-amino acid protein first purified from the placenta and with restricted cell distribution in other tissues (3,4). In esophageal epithelial cells S100P expression has been noted during differentiation indicating that it may play a role in normal development (5).

Although the specific function of S100P is unknown, it has been shown to play a role in the carcinogenic process. Indeed, S100P expression has been observed in various cancer cell lines including breast cancer, where it is associated with cellular immortalization, and prostate cancer (6,7). In the CWR22-R prostate cancer xenograft model, S100P expression was decreased following androgen ablation, and subsequently reexpressed upon treatment with androgens (8).

In lung adenocarcinoma, in a gene-expression risk profile based on the genes most associated with patient survival, S100P was correlated with decreased survival (9). Diederichs and co-workers (10) provided evidence that expression of S100P is associated with metastasis and predicts survival in early-stage non-small cell lung cancer. In addition, S100P was also found to be hypomethylated in pancreatic cancer cell lines and primary pancreatic carcinomas (11).

It was previously demonstrated that S100P is involved in the very early stages of breast carcinogenesis (6,12). In the present study we used a retrovirus-mediated transfer of antisense-S100P, in order to check whether the decrease in expression of this protein could lead to alterations in the cell cycle of epithelial cells of human breast cancer.

Materials and methods

Cells and cell culture. The viral packaging cell lines GP+E-86 and GP+*env*Am12 were used for the generation of recombinant retrovirus. Both cell lines were kindly provided by Dr A. Bank, Columbia University (Genetix Pharmaceuticals, Inc., Tarrytown, NY, USA). The T47D breast cancer cell line was a gift from Dr Maria Mitzie Brentani, São Paulo State University, Brazil. All cells were cultured in DMEM (Gibco-BRL), supplemented with 10% FBS (Gibco-BRL), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco-BRL) and glutamine (4 mM), maintained at 37˚C in a humidified 5% CO_2 atmosphere and cultured at subconfluency by passaging with trypsin/EDTA (Gibco- BRL).

Construction of retroviral vector. The S100P-expressing replication-deficient retrovirus vector was constructed as follows: S100P cDNA was released from pET 3a vector by the PCR technique using Platinum Pfx DNA polymerase (Invitrogen) and primers with *Xho*I (5') and *Eco*RI (3') restriction sites; forward P1 5'-CTA CTC GAG CAT ATG ACG GAA CTA G-3' and reverse P2 5'-TTA GAA TTC GGA TCC AGG GCA TCA T. cDNA and pLXSN vector were digested with *Eco*RI and *Xho*I enzymes (Promega) and the products were used in the ligation reaction sites of the pLXSN enzymes. After confirmation of insert antisense orientation with *Sac*I (Promega) digestion, this construction was called LS100PSN-AS.

Production of replication-defective retrovirus. GP+E-86 ecotropic retroviral packaging cells were transfected with retroviral vector (LS100PSN-AS or LXSN) separately, by the $CaCl_2$ precipitation method. Subsequently, GP+E-86 medium was used to infect the amphotropic GP+*env*Am12 packaging cell line, and individual G418-resistant clones were selected as described (13). For each isolated clone, the viral titer was determined by fibroblast infection (13) and the clones of packaging cell lines with highest viral titers were used to infect T47D cells.

Transduction of T47D cells. By using the supernatants of the above packaging cells, T47D cells ($4x10^5$) were infected in 100-mm plates. After 48 h the media was changed and supplemented with 0.5 mg/ml G418 and selected for resistance at 2 weeks when individual clones were isolated (14,15). Clones containing the antisense were named S100PA/S.

RNA extraction. Total RNA was extracted using TRIZOL® reagent (Invitrogen), followed by RNA extraction with 200 μl chloroform and precipitation with 500 μl isoprophanol. The pellets were washed in 75% ethanol and re-suspended in 50 μl RNase-free water. RNA content and quality were assessed by agarose/formaldehyde gel eletrophoresis and spectrophotometry at 260 nm (16).

Reverse transcription. Prior to the transcription, RNA was treated with DNase I (Invitrogen) at 25˚C for 15 min. The isolated RNA was reverse transcribed into cDNA with 2 μl oligo-dT primer (Invitrogen), at 65˚C for 10 min, 1 μl BSA (Promega), 10 μl 5X First-strand buffer, 10 μl deoxynucleotide triphosphate (dNTP) 10 mM, 2.5 μl $MgCl_2$ and 2 μl M-MLV reverse transcriptase, and incubated for 1 h at 37˚C (17,18).

Real-time PCR. cDNA was amplified by using SYBR® Green PCR master mix (Applied Biosystems). The real-time PCRs were performed in an ABI PRISM model 7700 sequence detector (Perkin-Elmer Applied Biosystems) under the following conditions: 95˚C for 10 min, followed by 40 cycles at 94˚C for 15 sec, 55˚C for 30 sec, and 72˚C for 30 sec. The optimal concentration of primers was determined in preliminary experiments. The sequences of primers are as follows: forward 5'-AAT TGC TCA AGG ACC TGG ACG-3' and reverse: 5'-GCA TCA TTT GAG TCC TGC CTT C-3'. Cyclophilin A was used as a reference housekeeping gene with the following primers: forward 5'-CAA ATG CTG GAC CCA ACA CA-3' and reverse 5'-TTG CCA AAC ACC ACA TGC TT-3'. Real-time PCR was performed in triplicate (19).

Flow cytometric analysis. For the flow cytometric analysis, $1x10^6$ cells from each clone were harvested in trypsin, washed with cold PBS, fixed in 1 ml cold 70% ethanol, and stored at 4˚C. At the day of analysis, cells were washed twice with cold PBS, treated with 100 μg/ml RNase A, and stained with 50 μg/ml propidium iodide (Sigma). Analysis of DNA content was performed in a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences) with 10.000 events. Results were analyzed with ModFit software (version 2.0, Verity Software, Inc., Topsham, ME, USA). The experiment was repeated three times in duplicate (20,21).

Confocal microscopy. To determine the impact of S100P antisense on protein expression in T47D cells, immunofluorescence staining was performed. Cells were seeded at a density of $5x10^4$ cells on chamber glass slides (Nalge, NUNC) and cultured overnight. Cells were washed twice with PBS, fixed with 4% cold paraformaldehyde at 4˚C for 10 min after blocking with bovine serum albumin and 0.1 M glicine, and then permeabilized with 0.01% saponin for 10 min and subsequently incubated with monoclonal anti-S100P antibody (BD Transduction Laboratories) overnight at 4˚C in the dark. The cells were then incubated with polyvalent FITC-conjugated immunoglobulin (Sigma) and DAPI (4,6-diamidino-2-phenylindole) for 30 min at room temperature. After several washes with PBS, coverslips were mounted and the immunofluorescence images were obtained in a Zeiss inverted fluorescence microscope Axiovert 100 M. For data collection and image analysis LSM 510 software was utilized (22).

Statistical analysis. The data are presented as mean ± SD for the number of experiments. Statistical significance was $P<0.05$.

Results

Real-time PCR. Real-time PCR quantification was based on threshold PCR cycle number (C_T) values, when the increase in fluorescent signal of the PCR product showed exponential amplification. The target gene mRNA was normalized to the housekeeping gene cyclophilin A. This gene is stable in

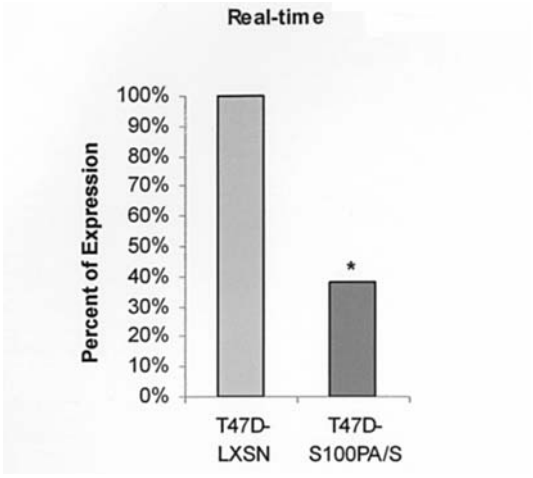

Figure 1. Percent expression of S100P gene in T47D mammary tumor cell line. Data are expressed as percentage of expression. The expression of control is considered 100% and the expression of T47D-S100P/As represents 37% of the control expression. Each point represents the mean ± standard error of triplicate measurements from 2 independent experiments. *Significantly different, control vs. T47D-S100P/Antisense; Student's t-test, P<0.001.

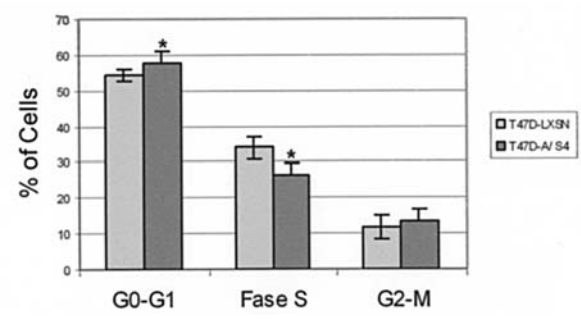

Figure 2. Cell distribution according to the phase of the cell cycle. The fluorescence of individual cells was measured by flow cytometry. Cell debris and aggregates were excluded as described in Materials and methods. Each bar represents the mean ± standard error of at least 3 separate experiments. Statistical significance was determined using the Student's t-test; *P<0.01, significantly different, control vs. T47D-S100P/Antisense.

many physiological and pathophysiological conditions (19). The relative expression level of the target gene compared to that of the housekeeping gene was calculated as $2^{-\Delta CT}$, where ΔCt = Ct S100P gene - Ct cyclophilin gene. The ratio of relative expression of the T47D-S100P/AS gene to that of T47D-LXSN was then calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta Ct = \Delta Ct_{T47D\text{-}S100P/AS} - \Delta Ct_{T47D\text{-}LXSN}$ (23). Fig. 1 shows the results of T47D-S100P/AS and cyclophilin A assay in two independent experiments in triplicate (n=6). All triplicate amplification resulted in very similar C_T values; the standard deviation did not exceed 0.35 for any sample. The results showed a reduction of 63% in the expression of T47D-S100PA/S.

Cell cycle analysis. We cultured the T47D-LXSN and T47D-LS100PA/S clones under the same conditions, and observed a decrease in cellular proliferation in the antisense clones compared to the control without any significant cell death. We decided to analyze the cell cycle by flow cytometry, and this growth inhibition was in agreement with an increase in the percentage of cells in the G_0/G_1-phase of the cell cycle and with a decrease of the S-phase fraction (Table I) of T47D-S100P/AS cells. The proportion of cells in the S-phase decreased significantly (P<0.01 compared with control); once the S-phase fraction (SPF) is considered a prognostic factor in breast cancer, this result seems to be noteworthy.

The data obtained with flow cytometry were stored and the ModFit program was used for analysis of cell cycle distribution. As shown in Fig. 2, 34.04% control cells were at the S-phase whereas 26.40% from the transducted cells were at the same phase. These findings indicated that, in our model, S100P-antisense cells showed a 23% reduction of cells at the S-phase.

Confocal microscopy. The next step was to prove if, after the reduction of transcription, efficiency of the S100P-A/S gene also reflects a decrease in protein expression. Four T47D-S100P/AS clones were grown on chamber slides, formalin-fixed and examined by immunofluorescent microscopy for detection of S100P (shown in green). The nuclei were stained with DAPI (shown in blue) (Fig. 3). We examined the cellular distribution of S100P in the T47D-S100P/AS and T47D-LXSN clones using antiS100P antibody and, to note, S100P remained less intensive in the transfected T47D cells.

Discussion

In the present study, by using a functional retroviral delivery system, we were able to demonstrate that the S100P calcium-binding protein seems to be an important element favoring cell division in human breast cancer cells.

Indeed, our antisense strategy against S100P was able to significantly reduce, not only cellular protein levels, but

Table I. Effect of antisense transduction on cell cycle distribution of T47D cells.

G_0/G_1-phase (%)		S-phase (%)[a]		G_2/M-phase (%)	
T47D-mock	T47D-antisense	T47D-mock	T47D-antisense	T47D-mock	T47D-antisense
54.07±2.17	57.73±1.25	34.04 ±3.17	26.40±3.35	11.89±3.47	13.26±3.67

Each point represents the mean ± standard error of at least 3 separate experiments. Statistical significance was determined using the Student's t-test; [a]P<0.01, significantly different, control vs. T47D-S100P/AS.

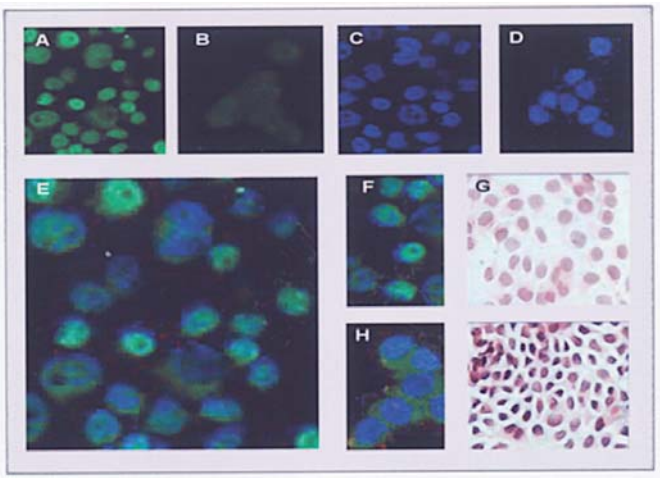

Figure 3. Immunofluorescent microscopy for S100P detection in T47D-LXSN cells (A,C,E,F) and T47D-S100P/Antisense (B,D,H). Nuclear DAPI staining for T47D-LXSN and T47D-S100P/Antisense, respectively (C,D). Superimposed double staining (E,F,H). Confocal microscopy shows different staining intensity patterns: T47D-LXSN/AS (A) shows intense immunofluorescent detection of S100P when compared to the antisense cells T47D-S100P/AS (B). Changes in nuclear morphology (H&E staining, x400) can also be seen when comparing T47D cells before (G) and after antisense therapy for S100P (I).

more importantly, the number of cancer cells in S-phase when compared to controls.

These findings are in agreement with our previous results showing that the overexpression of this protein is associated with immortalization of human breast epithelial cells *in vitro* and early stages of breast cancer development *in vivo* (6).

S100P was first purified from the placenta (4), and is associated with a large number of diseases. It has been detected in esophageal epithelial cells during differentiation (5), with doxorubicin resistance in colon cancer cells (24), and with androgen independence in prostate cancer (8). S100P levels are also significantly associated with the disease progression of prostate and pancreatic carcinomas (11,25-27) and with the aggressiveness of pancreatic cancer (28). It has been described to be upregulated in intraductal papillary mucinous neoplasms (29), and a decreased survival of patients with lung cancer has also been shown to correlate with S100P overexpression (9).

Although the biological effects of S100P remain to be fully elucidated, Arumugam and colleagues (30) have demonstrated an interaction between S100P and the receptor for activated glycation end products (RAGE) that acts in an autocrine manner to stimulate cell proliferation and survival. Our results are in complete agreement with these findings.

Regarding our choice of T47D cells as a model, it is important to mention that this breast cancer cell line was found to overexpress S100P in our previous study (6). However, in order to analyze the overexpression in human breast tissues in a larger sample of patients, we recently assessed, by immunohistochemistry, a total of 155 samples from patients submitted to stereotactic vacuum-assisted core biopsy due to breast microcalcifications (12). Results showed a positive association between estrogen receptor (ER) and S100P overexpression as well as a clear positive association between S100P overexpression and the risk of breast cancer. The strong association of S100P and ER expression highlights our hypothesis about the possible role played by S100P in the very early stages of breast carcinogenesis (12).

It seems that S100P is able to transport calcium out of the cell with two consequences: the reduction of intracytoplasmatic calcium concentration and calcium precipitation in the extracellular environment. Since increments in intracellular calcium concentration are responsible for mechanisms which can lead to senescence and programmed cell death in breast epithelial cells (31,32), apoptosis would be blocked by S100P.

Regarding the ability of S100P to elicit proliferation, we found an important positive correlation between the proliferation marker MIB and S100P expression in our preinvasive breast carcinoma clinical samples (12). Still, regarding breast carcinoma, Wang *et al* (33) recently demonstrated that survival of patients with S100P-positive carcinomas is significantly reduced ~7-fold than for those with negatively stained carcinomas.

Concerning the mechanism through which this protein is upregulated, it seems that the methylation status of S100P may answer this question. Indeed, S100P was found to be aberrantly hypomethylated in a large fraction of pancreatic cancer cell lines and primary pancreatic carcinomas (11).

In conclusion, to the best of our knowledge, this is the first time that an antisense approach was used against S100P mRNA in breast cancer epithelial cells. The results demonstrated here further classify S100P as a protein that may be involved in the cell cycle imbalance observed during breast carcinogenesis.

Acknowledgements

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