RESEARCH ARTICLE

Effects of Tissue Factor, PAR-2 and MMP-9 Expression on Human Breast Cancer Cell Line MCF-7 Invasion

Zeng-Mao Lin, Jian-Xin Zhao*, Xue-Ning Duan, Lan-Bo Zhang, Jing-Ming Ye, Ling Xu, Yin-Hua Liu

Abstract

Objective: This study aimed to explore the expression of tissue factor (TF), protease activated receptor-2 (PAR-2), and matrix metalloproteinase-9 (MMP-9) in the MCF-7 breast cancer cell line and influence on invasiveness. Methods: Stable MCF-7 cells transfected with TF cDNA and with TF ShRNA were established. TF, PAR-2, and MMP-9 protein expression was analyzed using indirect immunofluorescence and invasiveness was evaluated using a cell invasion test. Effects of an exogenous PAR-2 agonist were also examined. Results: TF protein expression significantly differed between the TF cDNA and TF ShRNA groups. MMP-9 protein expression was significantly correlated with TF protein expression, but PAR-2 protein expression was unaffected. The PAR-2 agonist significantly enhanced MMP-9 expression and slightly increased TF and PAR-2 expression in the TF ShRNA group, but did not significantly affect protein expression in MCF-7 cells transfected with TF cDNA. TF and MMP-9 expression was positively correlated with the invasiveness of tumor cells. Conclusion: TF, PAR-2, and MMP-9 affect invasiveness of MCF-7 cells. TF may increase MMP-9 expression by activating PAR-2.

Keywords: Breast cancer - tissue factor - protease activated receptor-2 - matrix metalloproteinase-9

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Introduction

Tissue factor (TF) is an important clotting promoter that is involved in intracellular signal transduction and tumorigenesis. TF is also closely related to tumor growth, invasion, and metastasis (Forster et al., 2006; Kashthuri et al., 2009; Ruf et al., 2010). The combination of TF with factor VIIa, as well as the combination of TF with factor VII and protease activated receptor-2 (PAR-2) (Hjortoe et al., 2004; Morris et al., 2006) induces tumor cells to form new blood vessels and promote metastasis. In addition, TF changes the expression of cancer extracellular matrix proteins through a variety of signaling pathways. Matrix metalloproteinases (MMPs) are enzymes important for degrading the extracellular matrix (ECM) that can promote cancer cell invasion and metastasis by degrading ECM proteins, thereby allowing tumor cells to penetrate the basement membrane (Tryggvason et al., 1987). MMP-9 (gelatinases B) decomposes the type IV collagen in the basement membrane, and is attracting increasing attention as a specific enzyme for degrading the basement membrane (Wieczorek et al., 2012). Although the invasion and metastasis of breast cancer is a major cause of death, its etiology remains unclear. Previous clinical studies (Zhao et al., 2008) found that TF expression is significantly correlated with MMP-9 expression in breast cancer, and that MMP-9 serves as an independent prognostic factor. TF has been found to regulate MMP expression through PARs in human colon cancer cells (Hu et al., 2013; Wu et al., 2013a; Wu et al., 2013b); however, no corresponding research has been conducted on breast cancer cells. Therefore, this study investigates TF, PAR-2, and MMP-9 expression in the MCF-7 breast cancer cell line and its influence on tumor cell invasiveness.

Materials and Methods

Cell culture

Human MCF-7 breast cancer cells (Union of Basic Medical Cell Center, Beijing, China) were seeded in 50 ml culture flasks with Dulbecco’s modified Eagle’s medium (DMEM) complete medium (containing 10% fetal bovine serum (FBS) and 10% green streptomycin) and incubated at 5% CO$_2$ and 37 °C until the cells covered the bottom. The cells were digested with 0.25% trypsin, harvested, and placed in a 12-well cell culture plate. After adding 1 ml of complete medium, the culture medium was replaced with serum-free medium, and the cells were incubated for 24 h.

Transfection of MCF-7 cells using pcDNA3.1/ZeoTF (+)

Single cell suspensions of MCF-7 cells were prepared by adding 7.5 × 10$^6$ cells into a 25 ml cell culture flask with DMEM complete culture medium until 70% to 80% confluence. A total of 4 µg of successful cloning plasmid pcDNA3.1/ZeoTF (+) (Colorectal Cancer Laboratory, Breast Disease Center, Peking University First Hospital, Beijing, China * For correspondence: zjxcn@aliyun.com

Transfection of MCF-7 cells using TF-ShRNA

Single cell suspensions of MCF-7 cells were prepared by adding 7.5 × 10⁴ cells into a 25 ml cell culture flask with DMEM complete culture medium until 70% to 80% amalgamation was achieved. A total of 4 μg of successful cloning plasmid pGCsi/TF (-) shRNA (Beijing Yixin Industrial Co., Ltd., Beijing, China) (Figure 1B) was diluted, mixed with Lipofectamine 2000 (Invitrogen, New York, USA) dilution, and incubated at room temperature for 20 min to form DNA/liposome complexes. The complexes were then added to the medium. The cells were incubated at 37 °C and 5% CO₂ for 24 h. The filter medium was replaced with DMEM containing FBS and Zeocin (50 μg/ml) (Invitrogen, New York, USA). The cells were cultured at 37 °C and 5% CO₂, and the screening medium was replaced every 2 d to 3 d until the cell clones appeared. Screening was performed for 1 wk to 2 wk to maintain stable growth and allow the subculturing of the cells. The cells were observed under an inverted fluorescence microscope (Leica DM IL LED, Wetzlar, Germany). Under fluorescence microscopy, successfully transfected cells would emit green fluorescence. The transfection efficiency was also calculated.

Cell invasion assay

The treated cells were rinsed with serum-free medium to produce a single cell suspension with a cell density of 5 × 10⁴ cells/ml. Then, 400 μl (2 × 10⁵) of the treated cells was allowed to react with the dose-saturated primary antibodies (mouse anti-human TF monoclonal antibodies at 1:100 dilution, mouse anti-human PAR-2 monoclonal antibodies at 1:200 dilution, mouse anti-human MMP-9 monoclonal antibodies at 1:100 dilution from the Santa Cruz Corporation, Dallas, USA) in an ice bath for 40 minutes, centrifuged, and washed twice. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Beijing Zhongshan Golden Bridge Technology Co., Ltd., Beijing, China) were added. The cells were placed on ice. After 40 min, the cells were centrifuged, washed, and fixed. A flow cytometer (BD Company, Franklin Lakes, USA) was used to detect the fluorescence of 10,000 counts per tube of cells, with three replicates.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. The cell counts were recorded as mean ± standard deviation (SD). The differences between groups were analyzed using t-tests. The correlations among factors were determined using regression analysis. Differences with P < 0.05 were considered statistically significant.
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Table 2. The Invasive Experiment Result in 4 Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Result (/400×)</th>
<th>7±SD</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>15</td>
<td>17</td>
<td>21</td>
<td>17.67±3.06</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>68</td>
<td>69</td>
<td>69.67±2.08</td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td>109</td>
<td>116</td>
<td>108.00±8.54</td>
</tr>
<tr>
<td>4</td>
<td>95</td>
<td>101</td>
<td>100</td>
<td>98.67±3.21</td>
</tr>
</tbody>
</table>

*P < 0.05

Figure 2. The TF Expression among TF (+) Group, TF (-) Group and Control Group. A: The TF expression (Western blot); B: The TF expression (comparative absorbance).

Results

**TF protein**

Lane 1 in Figure 2A represents MCF-7 human breast cancer cells, lane 2 represents the MCF-7 cells transfected with TF cDNA, and lane 3 represents the MCF-7 cells transfected with TF shRNA. The results show the TF expression in each group. Compared with the TF expression in MCF-7 cells, that in MCF-7 cells transfected with TF cDNA increased, whereas that in MCF-7 cells transfected with TF-shRNA decreased. The TF relative absorbance intensities of the three groups are shown in Figure 2B. The differences were analyzed using a t-test. The T value was 20.967, and the P value was 0.040.

**Expression of TF, PAR-2, and MMP-9 proteins**

Each group was treated with a PAR-2 agonist. Indirect immunofluorescence was used to detect TF, PAR-2, and MMP-9 protein expression. The results are shown in Figure 3A. The PAR-2 agonist (group 1 and group 2) significantly increased the MMP-9 expression rate (P = 0.002). However, TF and PAR-2 expression was not significantly different. Adding the PAR-2 agonist to the control group was without significant affect.

**Cell invasiveness**

Invasiveness was tested by treating the cells in each group with the PAR-2 agonist. The results are shown in Table 2. Group 1 exhibited the weakest invasiveness (17.67 ± 3.06/400× field). The PAR-2 agonist significantly increased the invasiveness of group 2 compared with group 1. The Transwell assay revealed that group 2 had 69.67 ± 2.08 cells/400× field, which is significantly greater than that in group 1 (P < 0.05). The invasiveness of group 3 (108.00 ± 8.54 / 400 × field) did not significantly differ from that of group 4 (98.67 ± 3.21 / 400 × field).

Table 3. The Relationship Between Protein Expression and Invasive Experiment Result

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Regression coefficient</th>
<th>t</th>
<th>P</th>
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<tbody>
<tr>
<td>TF</td>
<td>7.91±0.45</td>
<td>10.89±0.46</td>
<td>53.18±2.91</td>
<td>55.47±2.91</td>
<td>0.439</td>
<td>2.473</td>
<td>0.039*</td>
</tr>
<tr>
<td>PAR-2</td>
<td>47.48±2.13</td>
<td>49.99±1.56</td>
<td>52.79±2.88</td>
<td>50.67±3.13</td>
<td>0.074</td>
<td>0.463</td>
<td>0.656</td>
</tr>
<tr>
<td>MMP-9</td>
<td>15.03±4.65</td>
<td>33.57±4.58</td>
<td>32.85±3.79</td>
<td>36.91±4.91</td>
<td>0.551</td>
<td>3.916</td>
<td>0.004*</td>
</tr>
<tr>
<td>The invasive result</td>
<td>17.67±3.06</td>
<td>69.67±2.08</td>
<td>108.00±8.54</td>
<td>98.67±3.21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05

TF, PAR-2, and MMP-9 expression

The TF, PAR-2, and MMP-9 protein expression in groups 1 and 3 (both control groups) was compared. The results are shown in Figure 3B. The TF (+) group expressed higher TF and MMP-9 protein level than the TF(-) (P = 0.000 and 0.001, respectively). PAR-2 expression significantly differed between the two groups.

**Relationship of TF, PAR-2, and MMP-9 protein expression with cell invasion**

The relationship of the protein expression of TF, PAR-2, and MMP-9 with cell invasion in the all four groups is shown in Table 3. The PAR-2 agonist significantly increased MCF-7 and MMP-9 expression in the TF (+) group, and the protein expression consistent changed with invasion assay cell counts. Meanwhile, the positive rate of TF and PAR-2 slightly increased. The PAR-2 agonist slightly increased TF and MMP-9, but slightly decreased the cell count. The regression analysis showed that the invasion assay cell counts were correlated with TF and MMP-9 expression. The difference had statistical significance (P = 0.039 and 0.004, respectively).

Discussion

TF is an important factor in various malignant tumors (van den Berg et al., 2012). TF has become one of the targets of targeted therapy for breast cancer cells (Cole et al., 2013), and is considered an important aspect of breast cancer research (Santos et al., 2013). Studies have shown that TF and MMP-9 expression is significantly correlated with breast cancer cells in humans and dogs (Zhao et al., 2008; Hu et al., 2013). In human colon cancer cells (Hu et al., 2013), and is considered an important aspect of breast cancer research.
pathways capable of inducing cartilage degradation: a basic science study. *Arthritis Res Ther*, 9, R121.


References