Basic Research Paper

Expression of TNF-like weak inducer of apoptosis (TWEAK) and its relationship to microvessel density in breast cancer

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Background and Objective: The expression of TNF-like weak inducer of apoptosis (TWEAK) in breast cancer remains disputable. This study sought to investigate the expression of TWEAK in breast cancer tissues and breast cancer cell lines with different invasive abilities, and the relationship between TWEAK and microvessel density (MVD). Methods: Immunohistochemical S-P method was adopted to detect the expression of TWEAK in 70 specimens of breast cancer and 30 specimens of adjacent normal breast tissues. The protein expression of TWEAK was determined by western blot in a poorly invasive breast cancer cell line MCF-7 and a highly invasive breast cell line MDA-MB-231. Secretion of TWEAK was measured by ELISA assay in MCF-7 and MDA-MB-231 cells. Results: The expression of TWEAK was higher in breast cancer (60%) than in adjacent normal breast tissues (6.67%) (p < 0.05), and was higher in infiltrating ductal carcinoma of the breast (76.67%) than in breast ductal carcinoma in situ (42.85%) (p = 0.003). MVD was higher in infiltrating ductal carcinoma of the breast than in breast ductal carcinoma in situ (p < 0.05). The expression of TWEAK correlated significantly with MVD in infiltrating ductal carcinoma of the breast (r = 0.611), but not with breast ductal carcinoma in situ (r = 0.015). The expression of TWEAK and secretion of soluble TWEAK were higher in MDA-MB-231 cells than in MCF-7 cells (t = 4.259, p = 0.007; t = 3.6504, p = 0.006). Conclusion: TWEAK expression is related to the metastatic ability of breast cancer.

TWEAK (TNF-like weak inducer of apoptosis) is a member of the tumor necrosis factor ligands superfamily and can be hydrolyzed to produce active soluble protein. TWEAK has multiple biological functions: inducing apoptosis, mediating cell-killing activity, inducing cell proliferation, promoting the secretion of cytokines and being involved in angiogenesis. TWEAK expresses at a high level in multiple types of tumors, however, many different views exist in regards to its expression in breast cancer and its relationship with microvessel density (MVD). This study uses immunohistochemical S-P method, western blot assay and ELISA assay to detect the expression status of TWEAK in human breast cancer and to explore the relationship between TWEAK and MVD.

Materials and Methods

Materials. The tissues from 35 cases of invasive breast ductal cancer and 35 cases of intraductal carcinoma were obtained from the First Affiliated Clinical Hospital of China Medical University after surgery-resection of breast cancer between January, 2003 and December 2004. All patients were female, aged 35–70 years old, with the median age of 52. No patients received any radiotherapy or chemotherapy before the surgery. Adjacent noncancer tissues were taken from 30 cases at the time of surgery and served as controls. Tissues were fixed by 4% paraformaldehyde and then embedded in paraffin. Human estrogen receptor dependent breast cancer cell line MCF-7 (low-metastatic) and human estrogen receptor-independent cell line MDA-MB-231 (high-metastatic) were purchased from Chinese Academy of Sciences Shanghai Cell Culture Center. MCF-7 was cultured by DMEM medium and MDA-MB-231 was cultured by RPMI 1640 medium in 5% CO₂ at 37°C.

Major reagents and source. Rabbit anti-human polyclonal anti-TWEAK antibody (sc-5558) was purchased from Santa Cruz; mouse anti-human anti-CD34 monoclonal antibody (sc-M716529) was purchased from Daco company; S-ultrasensitive immunohistochemistry kit (KIT-9706, KIT-9709, KIT-9710) and DAB enzyme substrate color development kit (DAB-0031) were purchased from Fuzhou Maixin Biotechnology Development Corporation. ELISA kit was purchased from Bender Company (Lot: 15879003).

Methods. Immunohistochemistry. Immunohistochemical staining on tissues from 35 cases of invasive breast ductal cancer, 35 cases of intraductal carcinoma and 30 cases of adjacent non-cancer controls was done using anti-TWEAK polyclonal antibody and
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anti-CD34 monoclonal antibody. The staining method followed the instruction in Streptomyces anti-biotin protein-Peroxidase (SP) staining kit. Briefly, slices were dewaxed, repaired under high pressure, pretreated to block the endogenous peroxidase, blocked by serum, incubated by first antibody, stained by DAB, and then contrast stained by HE method.

Western blot. Western blot was used to test the expression of TWEAK in MCF-7 and MDA-MB-231 cells. We collected MCF-7 and MDA-MB-231 cells in their logarithmic growth phase, added lysis buffer at 4°C, let stand still for 30 min, centrifuged at low temperature and high speed (4°C, 20,050 xg, 0.5 hours) after sonication to break the cells, and saved supernatant containing total cell proteins. We quantified protein by Folin phenol reagent method, took equal amount protein, separated by SDS polyacrylamide electrophoresis, transferred, blocked by non-fat milk, incubated by anti-TWEAK (1:200) at 4°C overnight, then incubated by secondary antibody (1:500) at room temperature for two hours, developed DAB color for 3–10 minutes, rinsed with double-distilled water twice, dried and preserved in dark. Experimental results were collected by automatic gel electrophoresis imaging analysis instrument, measured densitometry and compared TWEAK expression levels between two cell lines. Experiments were repeated three times.

ELISA assay. ELISA assay detected the secretion of TWEAK in MCF-7 and MDA-MB-231 cells. We collected the culture medium of MCF-7 and MDA-MB-231 cells in logarithmic growth phase, centrifuged at 2,695 xg at 4°C for ten minutes and saved supernatant. Procedures followed the instruction brochure of the ELISA instant kit. Experimental results were collected by ELISA reader, the protein content was quantified and the difference in TWEAK expression was compared between two cell lines. Experiments were repeated three times.

Results characterization. Characterization of the immunohistochemical results. Results characterization was done blindly and each slice was counted separately by two pathologists. Under light microscope, five fields under high magnification were selected on each slice, 100 cancer cells were counted within each field, and the percentage of TWEAK positive cancer cells was calculated. TWEAK is a membrane/cytosol expressing protein, so positive staining shows the plasma membrane and cytosol stained with a brownish yellow color. The positive staining on vascular endothelium and vascular smooth muscle cells can be used as an internal positive control. The cancer cells were categorized by their TWEAK positive rate: TWEAK positive cancer cells >30% was considered positive, while ≤30% was considered negative expression.

Characterization of western blot results. Clear, specific bands on transferred membrane denoted a positive result. Experimental results were quantified by densitometry measurement.

Microvessel (MVD) counting. Judging criteria for CD34 was: strip or lacuna-shaped, isolated or clustered structures formed by endothelial cells stained with brownish yellow color; if lumen presented, lumens containing less than eight red blood cells was counted as one blood vessel. CD34 positive lumens were considered as MVD. Under low magnification, we first selected fields containing tumor MVD at highest density (hot spot), counted the number of MVD within three fields under 200X magnification and took averages of the MVD numbers. MVDs were re-counted if the number counted by two physicians differed by 10% or more. Where the lumen size was larger than eight red blood cells, or there was inflammation, granulation, necrosis or thick muscular layers, the area was not counted.

Statistical analysis method. Using SPSS software for Windows 13.0, we adopted χ² test, t test and Pearson correlation to analyze the relationship between TWEAK expression and MVD. p < 0.05 was used to determine statistical significance.

Results

The expression of TWEAK in invasive breast ductal cancer and intraductal carcinoma. TWEAK is a membrane/cytosol expressing protein. It does not express in normal breast tissue or expresses very weakly. The positive expression rate of TWEAK in invasive breast ductal cancer and intraductal carcinoma were 76.67% (27/35) and 42.85% (15/35) respectively, which was significantly higher than the 6.67% (2/30) expression rate in the adjacent non-cancer tissues. Meanwhile, the expression in invasive breast ductal cancer was significantly higher than that in intraductal carcinoma (p = 0.003) (Fig. 1A–C).

The expression of membrane-type TWEAK in breast cancer cell lines. Western Blot results showed that in high-metastatic breast cancer cell line MDA-MB-231 the expression level of membrane-type TWEAK was significantly higher than in the low-metastatic breast cancer cell line MCF-7 (t = 4.259, p = 0.007) (Fig. 2).

The secretion of soluble TWEAK in breast cancer cell lines. ELISA results showed that in high-metastatic breast cancer cell line MDA-MB-231, the secreted TWEAK level was higher than in the low-metastatic breast cancer cell line MCF-7 (t = 3.504, p = 0.006) (Fig. 3).

The relationship between TWEAK and MVD. In invasive breast ductal cancer, the MVD number was significantly higher than in intraductal carcinoma (p = 0.04) (Fig. 4A and B). In invasive breast ductal cancer, TWEAK expression positively correlated (r = 0.611) with MVD. However, in breast intraductal carcinoma, TWEAK expression and MVD had no significant correlation (r = 0.015).

Discussion

TWEAK (TNF-like weak inducer of apoptosis) is a member of the tumor necrosis factor superfamily. It expresses in a wide variety of human normal tissues and non-lymphoid tumor cell lines. It is a type II membrane-anchor protein with a molecular weight of 30 ku. It can be hydrolyzed to produce an 18 ku soluble factor with multiple biological activities for executing biological effects at long-distance. Some studies have discovered that in certain tumor cell lines soluble TWEAK does not strongly induce cell apoptosis. Its major functions are to induce cell proliferation, migration, differentiation, to promote cytokines secretion and endothelial cell proliferation and angiogenesis. At the same time, it is involved in the regulation of the innate immune system. However, the exact mechanisms by which TWEAK is involved in and induces angiogenesis is not clear at the present.
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Figure 1. Expression of TWEAK in human breast tissues (SP x400). (A) TWEAK is not expressed or is weakly expressed in human adjacent normal breast tissues. (B) TWEAK is moderately expressed (in yellow) in cytoplasm and on the membrane of human breast ductal carcinoma in situ. (C) TWEAK is strongly expressed (in brown) in cytoplasm and on the membrane of infiltrating ductal carcinoma of the breast.

Figure 2. Expression of TWEAK in breast cancer cell lines. (A) MDA-MB-231; (B) MCF-7.

Figure 3. The level of soluble TWEAK in the supernatants of human cancer MCF-7 (low metastasis) and MDA-MB-231 (high metastasis) cells detected by ELISA. All values are presented as mean ± SD of three independent experiments. *p < 0.05 vs. MCF-7 cells.

Figure 4. Microvessel density in tissues of infiltrating ductal carcinoma of the breast (A) and human breast ductal carcinoma in situ (B) (SP x400).
Studies have found that TWEAK expression increases in many tumor tissues such as liver cell carcinoma and colon cancer, etc. Currently most people believe that TWEAK expression increases in tumor by binding to its receptor Fn14 (Fibroblast growth factor-inducible 14), and the latter conjugates with different receptor protein or cytosolic protein containing TRAF (TNF receptor-associated Factor) domain, activating NFκB and MAPK signal transduction pathways and triggering the activation of a series of genes. NFκB is an important downstream signaling molecule of TWEAK: its activity directly determines TWEAK function. Previous studies have found that tumor necrosis factor is involved in angiogenesis, however, this function is fulfilled indirectly through upregulating vascular endothelial growth factor. Contrarily, the regulating role of TWEAK on angiogenesis and endothelial cell proliferation does not depend on vascular growth factor. Its acting mechanism, however, is still unknown. Therefore, we explored the expression of membrane-type TWEAK in different invasive stages of breast cancer tissues and the secretion status of soluble TWEAK in breast cancer cell lines with different invasion abilities, and the relationship between TWEAK and MVD density.

This study shows that TWEAK is highly expressed in invasive breast ductal cancer and intraductal carcinoma, and its expression in invasive breast ductal cancer is significantly higher than in intraductal carcinoma. Western Blot results showed that in high-metastatic breast cancer cell line, the expression of TWEAK is significantly higher than in low-metastatic breast cancer cell line. ELISA results showed that in high-metastatic breast cancer cell line the secreted soluble TWEAK level is significantly higher than in low-metastatic breast cancer cell line. These results suggest that the increase of membrane-type TWEAK in breast cancer and increase of soluble TWEAK secretion may relate to the growth of breast cancer and its invasion ability.

Some studies have found that Fn14 expresses highly in vascular endothelial cells and smooth muscle cells. TWEAK acts on receptor Fn14 and affects the migration and proliferation of vascular endothelial cell migration and corneal angiogenesis. There are also reports that low concentration of soluble TWEAK promotes cell proliferation and migration, and participates in angiogenesis, however, at a relatively high concentration, TWEAK can induce cell apoptosis instead. Our study found that in invasive breast ductal cancer, the TWEAK expression is closely correlated with MVD. The secreted TWEAK amount in either high- or low-metastatic breast cancer cell lines are within the range of low concentration, suggesting that TWEAK may be involved in tumor angiogenesis.

In summary, our study found that TWEAK has high expression level in breast cancer while its expression in invasive breast ductal cancer is significantly higher than in intraductal carcinoma. Meanwhile, in high-metastatic breast cancer cell line, the secreted soluble TWEAK is significantly higher than in the low-metastatic breast cancer cell line. In addition, TWEAK expression in invasive breast ductal cancer is closely related with MVD. The above results suggest that TWEAK may play certain roles in promoting tumor growth, invasion and angiogenesis. Therefore, inhibiting TWEAK function may provide a new target for treating breast cancer.

References