Effect of Mammary Adenocarcinoma Supernatant on Hepatic Fibroblast Differentiation

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Abstract

Mammary adenocarcinoma is the most common malignant tumor in women, and the liver is one of the sites with a high incidence of metastasis. This study established a mammary adenocarcinoma-associated fibroblast cell line (ME-iLX-2) that features premetastasis niche based on coculture with the supernatant of hepatic stellate cells (LX-2) and mammary adenocarcinoma cells (TS/A). Multiple experiments, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldimethyltetrazolium bromide (MTT)MTT, Flow Cytometry (FCM), wound-healing, lactic acid, real-time quantitative polymerase chain reaction (qPCR) and WB assays, were carried out to investigate the connection between mammary adenocarcinoma cells and hepatic stellate cells and the source of cancer-associated fibroblasts in the premetastatic microenvironment and to confirm the importance of vascular endothelial growth factor (VEGF) in mammary adenocarcinoma supernatant-induced hepatic fibroblast differentiation. This study determined the following: (1) After long-term coculture with supernatant, LX-2 significantly promoted the proliferation and migration of tumor cells, and massive apoptosis of LX-2 cells occurred; (2) VEGF expression in LX-2 cells was positively correlated with the duration of coculture with supernatant. In addition, with an established linear regression model, high expression of VEGF is suggested to be one of the molecular properties of cancer-associated fibroblasts.

Keywords

Breast cancer, Pre-metasis Niche, Cancer-associated fibroblasts (CAFs)

1. Background

1.1 Tumor niche and premetastasis of breast cancer cells

Breast cancer is the most common malignant tumor, accounting for approximately 30% of female tumor cases. Progress in diagnostic techniques and advances in therapeutic methods have significantly improved the outcome of breast cancer patients and their postoperative quality of life. However, breast cancer metastasis is the leading cause of recurrence and progression. The common sites for metastasis include the bones, lungs, liver, and brain. Among them, the liver is the most common site of solid tumor metastasis and one of the sites with the highest incidence of breast cancer cell metastasis [1].

The tumor cell niche is one of the hot topics of research. To maintain the vigorous anabolism of tumors, cells in the tumor niche supply lactate to feed tumor cells and inhibit the activity of immune cells such as natural killer cells and effector T cells [3]. The paracancerous cells surrounding tumor tissues provide a favorable and indispensable environment for the growth and proliferation of tumor cells. Studies have demonstrated that the removal of fibroblasts induces the atrophy or death of a large number of tumor cells [2].
Breast cancer liver metastases (BCLMs) refer to tumor cells that leave the primary site; invade lymphatic vessels, blood vessels, or the body cavity; and metastasize to the liver to form new lesions. At the metastatic site, tumor cells proliferate despite being disconnected from the primary tumor, although they share the same histologic type. The metastasis of tumor cells always marks the failure of tumor therapy and the immune system [5].

According to the tumor imunoediting theory, the metastasis of tumor cells means that in the fight between tumor cells and immune cells, tumor cells have moved past the elimination and equilibration phases to reach the escape phase [4]. However, an increasing number of recent studies have shown that before escape in the tumor immunoediting theory, the immune system exerts little control over the niche for premetastatic lesions.

In 2006, Jian-Guo CAO et al. proposed the concept of a “premetastatic niche” (PMN), that is, tumor cells in the primary lesions remotely destroy the molecular niche of premetastatic lesions before cancer cell metastasis [6]. At this time, cytokines such as CCL5, IL-17B, FAK, p-P38, and TGF in the PMN become significantly unbalanced and deteriorate, which induces inflammatory reactions and forms immune cell traps [7]. Meanwhile, cancer-associated fibroblasts (CAFs) are recruited to premetastatic lesions, where they become premetastatic-associated fibroblasts and cause further PMN deterioration [8]. This phenomenon is considered common during the metastasis of the overwhelming majority of tumor cells. Despite numerous studies on the source of CAFs, more research is needed on the source and function of premetastatic fibroblasts.

1.2 Angiogenic factors and tumor metastasis

The vascular endothelial growth factor (VEGF) family is an important regulator of angiogenesis and vascular development in embryos and adults and is involved in many physiological and pathological processes. The VEGF family consists of five main molecules, namely, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGF) [9]. It has been suggested that VEGFR-knockout mice die of a vascular proliferation disorder in the embryonic stage (Days 8.5-9.0) [10]. For the VEGF family, an increase in VEGF content causes vascular differentiation and proliferation.

The blood vessels and the lymphoid system in the tumor are directly related to tumor proliferation and metastasis. The recruitment of blood vessels is one of the main means of constructing a favorable niche for tumors.

Previous studies have demonstrated that cells in osteosarcoma tissue or adjacent nontumor tissue can realize neovascularization through hypoxia-inducible factor-1 (HIF-1), regulate hypoxia in the tumor niche, and increase the proliferation rate of tumor cells through the AKT/cyclin D1 signaling pathway. Neovascularization in tumor tissue not only delivers nutrients and oxygen but also provides a channel for and promotes tumor metastasis [13]. Studies by Hongjun ZHU and Shuanglin ZHANG have also shown that neovascularization is indispensable during the growth and metastasis of lung cancer, providing evidence that lung cancer cells and VEGF secreted by cells in the tumor niche have effects that promote tumor metastasis [14].

In view of the above, several drugs have been designed to target VEGF: the VEGF-specific neutralizing antibody bevacizumab [15] and the tyrosine kinase inhibitors sunitinib and sorafenib [16]. When drug resistance to standard-ized cancer therapy occurs, antiangiogenic compounds may be the optimal therapy for cancer patients.

However, bevacizumab was withdrawn from clinical trials due to the lack of clear effects. Existing studies have mostly focused on the primary tumor niche, and the premetastatic niche remains insufficiently studied.

2. Materials and Methods

2.1 Cell lines and cell engineering

2.1.1 Metastasizing mouse cell line (TS/A) and hepatic stellate cell line (LX-2)

The cell lines used in this study, including the metastasizing mouse cell line (TS/A, RRID: CVCL_VQ63) and the hepatic stellate cell line (LX-2, RRID: CVCL_5792), were supplied by the Cell Bank of the Institute of Biophysics, China Academy of Sciences.

TS/A cells, established by Patrizia Nanni et al. in 1983, originated from spontaneous breast cancer in a mouse after giving birth and showed excellent metastasis potential [17]. LX-2 cells originated from hepatic stellate cells and were established by Profession Lieming XU of Shanghai University of Traditional Chinese Medicine through transfection of the SV40T gene, long-term culture under low-serum conditions, and screening.

Cell culture was performed at 37 °C with 5% CO2 and suitable humidity using DMEM (high-glucose) culture medium (HyClone, USA) containing 10% FBS (PAN-Biotech, Germany) and 1% antibiotics (streptomycin and penicillin). Cells were subjected to trypsinization, centrifugation, and passaging.
TS/A cells were used in this study due to their strong metastasis potential. Meanwhile, LX-2 cells, which are commonly used in studies of liver diseases, were also selected.

2.1.2 Preparation of culture supernatant and induction of ME-iLX-2
To establish the relationship between breast cancer and hepatic stellate cells in vitro, supernatants were collected in this study after long-term coculture. The supernatant after coculture contains cytokines secreted by cancer cells, exosomes, and metabolites and therefore is commonly applied in trials to simulate the physiological liquid environment early after metapmetastasis.

After the subculture of TS/A and LX-2 cells for 24 to 48 hours and gentle shaking, the supernatants were removed and filtered with a 0.22 μm cell filter. To avoid nutrient deficiency, the supernatants and DMEM were mixed at a 1:1 ratio to prepare cell culture supernatants that could be stored for one month at 4 °C or three months at -20 °C.

In this study, TS/A cell supernatants and LX-2 cells were cocultured for 43 days to induce significantly typed LX-2 cells, named “ME-iLX-2 cells”, a combination of the names of the mammary adenocarcinoma, TS/A, and author (Enze WANG). Xiangnan ZHANG validated (data not shown) that ME-iLX-2 cells have the properties of CAFs and are considerably more powerful than LX-2 cells at promoting TS/A cells to form spherical aggregates.

2.2 Trial methods

2.2.1 MTT assay for relative cellular activity
Cells were inoculated in a 96-well plate at a density of 5×10³ cells/well. Then, 200 μL supernatant was added, and the cells were cultured at 37°C and 5% CO₂ for 48 h. Next, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to the cells for 4 h at 37°C, followed by the addition of triple solution (10 g SDS, 5 mL isobutanol, and 0.1 mL 10 M HCl in double-distilled water to 100 mL) and incubation overnight to thoroughly dissolve formazan. The absorbance was measured at 570 nm, and the relative cellular activity was determined.

2.2.2 PI single-staining assay of cell apoptosis
Cells cocultured for 72 h with supernatants were digested with trypsin, rinsed with sterile PBS two times, and incubated with propidium iodide (PI) for 30 min. The stained cells were analyzed with a flow cytometer; the forward scatter (FSC) and side scatter (SSC) of cells reflect the size and granularity of the specimens. The effects of cell debris were removed by gating. Finally, a PI fluorescence value of 10² was chosen as the criterion for classification as cell apoptosis, and data were processed with FlowJo (RRID: SCR_008520 Version 10) software.

2.2.3 Wound healing assay
Cells were inoculated in a 6-well plate and cultured with supernatants until the cells reached confluence. The confluent cell monolayer was scratched with a 10 μL pipette tip and rinsed with PBS three times to remove cell debris. Images were taken at 0 and 24 h after scratching. Ten scratches were randomly selected from the pictures, and the width of each scratch was measured to reflect the distance of cell migration.

2.2.4 Lactate concentration determination
Supernatants were collected after 48 h of normal cell culture, and a lactate content detection test kit (Solarbio, Beijing) was used to calculate the lactate concentration in the supernatant.

2.2.5 qPCR detection of relative VEGF mRNA content
Cells were cocultured with supernatants in a 6-well plate for 72 h, and then, total RNA was extracted with TRIzol reagent after precipitation and washing with chloroform–ethanol and reverse transcribed into cDNA using the MLV-reverse Transcriptase System. VEGF primers, PCR-coupled fluorophores, and a real-time quantitative PCR (qPCR) system were used to monitor qPCR in real time. GAPDH was used as a reference gene in this study, and the relative content of VEGF mRNA in specimens was calculated with Rotor Gene 5 software and the ΔΔCt method.

2.2.6 WB assay for VEGFA protein
After culture in a 6-well plate for 120 h, total protein was extracted from cell lysates at low temperatures, and the protein concentration was measured by the BCA method. After PAGE, the GAPDH (36 kDa) and VEGFA (42 kDa) bands were transferred to nitrocellulose filter membranes, which were cut for incubation with primary antibody (Santa Cruz Biotechnology, USA) and secondary antibody (Cell Signaling, USA) before being subjected to chemiluminescence detection, imaging, and analysis.
3. Results

3.1 Induced hepatic stellate cells promoted the growth of tumor cells

Hepatic stellate cells (LX-2), breast cancer cells (TS/A) and long-term-induced hepatic stellate cells (ME-iLX-2) were each cultured with supernatant from the other cell types, and MTT assays were conducted after 24 h of culture with supernatant from the three types of cells.

As shown in Figure 1, the supernatants of breast cancer cells (TS/A supernatants) had a detrimental effect on non-induced hepatic stellate cells (LX-2) and inhibited 16.20% of cell activity; however, TS/A supernatants had no obvious effect on induced hepatic stellate cells (ME-iLX-2). LX-2 supernatants inhibited 16.50% of the activity of TS/A cells, but ME-iLX-2 supernatants increased the activity of TS/A cells by 41.44%.

![Figure 1](image1.png)

**Figure 1.** MTT assays of cells cross-cultured in the supernatants of LX-2, ME-iLX-2, and TS/A cells; the black color denotes the control condition for each group of cells and their own supernatant, the blue color denotes LX-2 cells and supernatant, the yellow color denotes ME-iLX-2 cells and supernatant, and the red color denotes TS/A cells and supernatant. Note: ***, P<0.01; and ns, P>0.1.

![Figure 2](image2.png)

**Figure 2.** PI staining and flow cytometry analysis of cell apoptosis after 72 h of culture of LX-2 and ME-iLX-2 cells with the three supernatants. Note: ***, P<0.01; and ns, P>0.1.
After 72 h of LX-2 and ME-iLX-2 cell culture with the three supernatants, PI staining was performed to detect the proportion of apoptotic cells with a flow cytometer. As shown in Figure 2, TS/A supernatants promoted the apoptosis of LX-2 cells (44.1% apoptosis rate) but had no significant effect on the apoptosis of ME-iLX-2 cells.

3.2 Induced hepatic stellate cells promoted tumor metastasis

TS/A cells were inoculated in a 6-well plate and cultured until they reached confluence. The cell monolayer was scratched with a 10 μL pipette tip, rinsed with PBS, and cocultured with LX-2 or ME-iLX-2 supernatants for 24 h. The cells were imaged as shown in Figure 3. Among the treatment groups, TS/A cells cocultured with ME-iLX-2 supernatants migrated by 836.00 units, and those cocultured with LX-2 supernatants migrated by 627.66 units. After coculture with ME-iLX-2 supernatants, breast cancer cells showed a significant increase in migration (33.19%). Meanwhile, supernatants were removed from each cell group after 48 h of normal culture to determine the lactate content. The results revealed that the lactate content in ME-iLX-2 cell supernatants increased by 28.4%, and the lactate content in TS/A cell supernatants was the highest (1.12 mg/L).

3.3 The duration of coculture with supernatants was positively correlated with VEGF expression

LX-2 cells were cocultured with TS/A supernatants, and the relative expression of VEGF mRNA in LX-2 cells was determined by qPCR. As shown in Figure 4, the relative expression of VEGF mRNA in LX-2 cells was significantly and positively correlated with the duration of coculture with TS/A supernatants. As shown in Table 1, linear regression analysis revealed that the relative expression of VEGF mRNA during 0 to 15 days of coculture was governed by the following empirical formula: \( C_{\text{VEGF}} = 10.17t + 4.07 \), where \( C_{\text{VEGF}} \) denotes the relative expression of VEGF mRNA and \( t \) denotes the coculture time.

The results of the WB experiment also verified that the VEGF content increased with the duration of coculture time.

Figure 3. Effects of the long-term induction of hepatic stellate cells on the metabolism and metastasis of breast cancer cells:
Note: ***, \( P<0.01 \).

Figure 4. VEGF mRNA levels after coculture of LX-2 cells with TS/A supernatants.
4. Discussion and analysis

1) TS/A supernatants provide a selective pressure for LX-2 cell differentiation to ME-iLX-2 cells to induce niche deterioration
2) ME-iLX-2 cells promote the activity, migration, and dissemination of TS/A cells
3) VEGF mRNA levels in ME-iLX-2 cells increase to generate blood vessels and promote metastasis

4.1 Tumor cells were activated by CAF-like cells in the screened coculture systems

The MTT assay and cell migration results showed that ME-iLX-2 significantly promoted the activity of TS/A cells, as indicated by the increased proliferation and migration rates. TS/A supernatants had no significant effect on the activity of ME-iLX-2 cells but greatly accelerated the apoptosis of LX-2 cells. In the coculture system, there was competition between normal fibroblasts (LX-2) and ME-iLX-2 CAFs induced by breast cancer cell supernatants. Therefore, the degree of inhibition of LX-2 apoptosis was equivalent to that of the promotion of ME-iLX-2 apoptosis. Meanwhile, ME-iLX-2 also contributed to the proliferation of cancer cells.

This interaction relationship confirmed that primary tumors signal remotely to cause cell niche deterioration through cytokines and exosomes. In parallel, CAFs also interact with tumor cells in a remote manner. Thus, a positive feedback loop forms between tumor cells and CAFs, which promotes a more favorable niche for the migration and growth of tumor cells.

4.2 Selection of tumor cells during the typing of hepatic stellate cells

Existing studies have suggested that tumor cells induce and transform fibroblasts in the tumor niche into CAFs through induced dedifferentiation and redifferentiation, during which apoptosis and the inhibition of cell activity are seldom or scarcely involved. In the MTT assays and FCM analysis of cell apoptosis, normal fibroblasts (LX-2) commonly underwent apoptosis and showed reduced activity after coculture with supernatants, with increasingly smaller populations over time. After the induced typing of CAFs (ME-iLX-2), the ability of TS/A supernatants to inhibit cellular activity and proliferation was barely detectable.

These findings indicate that during the induced typing of normal fibroblasts, in addition to the induced dedifferentiation and redifferentiation, there is also a process of selection by tumor cells: tumor cells apply selective pressure on colonies of fibroblasts to promote tumor cell proliferation and metastasis.

The tumor niche generally involves the direct killing of CAFs. The findings in this study may provide a new direction of research on the tumor niche: Can the malignant progression of the tumor niche be blocked by inhibiting the ability of tumor cells to promote the apoptosis of normal fibroblasts? Does the tumor niche deteriorate after exposure to specific drug treatments that increase the proliferation of normal fibroblasts?

4.3 VEGF content can be a molecular marker of CAFs

The secretion of VEGF in the tumor niche promotes the generation of new blood vessels in tumor tissue, enabling an abundant nutrient and oxygen supply for tumor cells to create favorable conditions for anabolism and proliferation. In addition, VEGF can promote the redistribution of actin by activating the HSP27, FAK, and P13K pathways, strengthen the migratory ability of tumor cells to allow migration to new blood vessels and lymphatic vessels, and finally lead to tumor metastasis.

Previous studies have reported similar effects during tumor premetastasis. In the qPCR assays in this study, VEGF mRNA levels in ME-iLX-2 cells increased with induction time in a significant linear manner. Meanwhile, the lactate content in ME-iLX-2 supernatants after induced typing was significantly higher than that in normal fibroblast LX-2 supernatants; the increase in lactate concentration in the niche not only weakened the identification of tumor cells by immune cells and their tumoricidal capacity but also triggered somatic cells to secrete VEGF, providing further...
evidence that the VEGF content significantly increased in the premetastatic niche simulated by the supernatant co-culture system.

At present, it is a common opinion that cytokines and cell signaling pathways, such as chemotactic factor-5 (CCL5), interleukin-17 (IL-17B), focal adhesion kinase (FAK), the mitogen-activated protein kinase pathway (P38-MAPK pathway) and transforming growth factor (TGF-β), are significantly changed in the premetastatic tumor niche. However, the findings in this study indicate that VEGF, a molecular marker, is involved in the process of constructing a premetastatic niche for tumors.

References


