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Expression of Preprotachykinin-I (PPT-I), Neurokinin-1 (NK-1) and Neurokinin-2 (NK-2) in Breast Cancer Cells Improves Tumor Cell Survival in Bone Marrow in the Early Stage of Metastasis

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OBJECTIVE To study the potential relationship between the expression of PPT-I, NK-1, NK2 and the development of breast cancer cells in bone marrow stroma and to provide evidence of potential molecular mechanisms of bone metastasis in early stage of breast cancer patients.
METHODS The cocultures of breast cancer cell line T-47D and marrow-derived mesenchymal stem cells (MSC) were established with equal numbers. T-47D cells were separated from the coculture system at 48 h and 96 h after coculture by MACS magnetic cell sorting (MicroBeads). The expression of PPT-I, NK-1, NK-2 in T-47D was then examined before and after coculture by real-time PCR and by Western blot. Alterations in cellular ultrastructure of T-47D cells were detected before and after coculture under electron microscope. Finally, changes in cell cycle distribution were examined by flow cytometry, and growth curves from before and after coculture were drawn and analyzed.
RESULTS Following coculture of T-47D and MSC, the expression of PPT-I mRNA and protein was significantly upregulated, while the expression of NK-1 and NK-2 mRNA and protein was greatly downregulated. The analysis of cell cycle distribution by flow cytometry showed that the proportion of T-47D during S phase was increased, and the duration of the G_2/M phase was sharply decreased. Under electron microscope, we observed that the synthesis of hereditary material was increased, but the hepatin granules were shown prominent stacking in T-47D cells. These results suggest that although the synthesis of DNA was increased, the proliferation of cells was inhibited after coculture. The cell growth curve confirmed the findings from the observation under the electron microscope and flow cytometry.
CONCLUSION Tumor cells could survive through the upregulation in expression of preprotachykinin-I gene during early bone metastasis in breast cancer. The phenomenon of growth suppression in breast cancer cells after coculture in the current study could be induced by downregulation in expression of NK-1 and NK-2.

KEY WORDS: breast cancer, bone metastasis, tachykinin, mesenchymal stem cells.

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Introduction

Breast cancer remains a clinical dilemma despite the improvement of treatment, education, early detection and awareness efforts[1]. Among women in China, breast cancer (BC) continues to be one of the main causes of cancer deaths. Bone marrow (BM) metastasis of breast

cancer cells (BCCs) correlates with poor prognosis[1,2]. Many studies have described the biology of BC at a point in time when the tumor burden is evident. However, markers to detect BCCs in the BM during the early stages of diagnosis have yet to be elucidated. In particular, BC studies have failed to address the period when the frequencies of BCCs in the BM might be at a single cell level and perhaps lower than the frequency of other cells in the BM. We hypothesize that BCCs could become a part of the BM microenvironment at a time long before clinical diagnosis. In terms of this supposition, it is important to understand the mechanisms of early entry and existence of BCCs in the BM. On the basis of this thought, it is not surprising that chemotherapy frequently plays an unsuccessful role in the prevention of bone marrow metastasis[3]. Although it is clear that BCCs maintain a preference for BM[4,5], the following remain undefined: the signature of the cancer cells that evade the innate immune system and enter the BM at a period before clinical detection or early during cancer development; the area in the BM where the BCCs are located; and the mechanisms that allow the BCC to survive within the BM microenvironment.

Research on neuroimmune-related molecules in breast cancer development is relatively limited when compared to other molecular studies in breast cancer research. Reports in the literature on basic, translational, and clinical research have shown that neurotrophic factors and neurotransmitters could be involved in tumorigenesis[6-9]. Preprotachykinin-I (PPT-I) is a single copy gene with seven exons that is conserved by evolution[10]. Through alternate splicing and post-translational modification, the PPT-I gene produces several peptides that belong to the tachykinin family[10]. Tachykinins interact with neurokinins-1 and -2 (NK-1, NK-2), both G protein–coupled, seven-transmembrane receptors[11], which leads to autocrine proliferation. Recent research has shown that NK-1 and PPT-I peptides could be linked to disease progression in cancer[11-14]. Mesenchymal stem cells (MSCs) play an important role in hematopoiesis in BM. Additionally, MSCs surround the abluminal vasculature of BM and are therefore poised to interface the periphery and BM cavity[15]. Furthermore, MSCs are immune suppressors and could thus prevent immune clearance of few BCCs, which would be expected during an early period and with low tumor burden. To this end, our study focused on the relationship between the expression of PPT-I, NK-1 and NK-2 and the metastasis of breast cancer cells in early BM metastasis. In this research, we first established a cellular model in BM by coculture of BCCs and mesenchymal stem cells to imitate the microenvironment of early BM metastasis.

Materials and Methods

Cell lines and reagents

The MSC line was established from BM aspirates of healthy individuals as described previously[16]. MSCs were cultured in DMEM/F12 with 10% Hyclone FCS. The T-47D breast cancer cell line and the HL-100 normal breast cell line were kindly provided by the Department of Immunology at the Tianjin Medical University Cancer Institute and Hospital Trizol (Invitrogen Life Technologies, Lot 1121070, USA)

Antibody

Preprotachykinin-I: goat polyclonal IgG, 200 µg/ml (sc-14104, Santa Cruz); NK-1R: goat polyclonal IgG, 200 µg/ml B (sc-14116, Santa Cruz); NK-2R: goat polyclonal IgG, 200 µg/ml (sc-14121, Santa Cruz). Superscript Reverse Transcription Kit (Invitrogen Life Technologies, USA). PCR amplification kit (Biological Engineering Technology Company, Shanghai, China). SYBRGreen PCR Kit (TaKaRa Biotechnology CO., LTD, Dalian, China). CD326 (EpCAM) MicroBeads Human MACS (Miltenyi Biotec, Germany). GeneAmp 5700 Sequence Detection System and GeneAmp 5700 SDS Software (Applied Biosystems, USA).

Coculture of BCCs and MSCs/growth curve

In accordance with the procedure well described in the literature, equivalent numbers of BCCs and MSCs were cocultured in stromal media with daily replacement of 50% culture media. At different times (before coculture, 48 h and 98 h after coculture), cells were trypsinized, and the BCCs were positively selected (twice) with Dynabead conjugated anticytokeratin (Fig.1), as described previously[17]. Both cell populations (MSCs and BCCs) were counted, and the purity (> 99% +) of each verified by flow cytometry with PE-cytokeratin mAb and prolyl 4-hydroxylase mAb-PE-rat antimouse κ IgG for stroma. Finally, a growth curve over the time following coculture was generated.

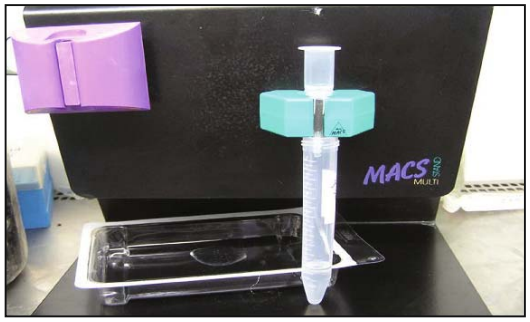

Fig.1. MACS magnetic cell sorting (MicroBeads).

mRNA expression of PPT-I, NK-1 and NK-2 in T47D cells before and 48 h and 96 h after coculture, detected by real-time PCR

Cells were plated in 96-well plates, and real-time PCR was performed. Total cellular RNA was extracted by using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity and concentration were determined by measuring the absorbance

(A) at 260 nm and 280 nm (A_{260}/A_{280}). Amplification of human β-actin served as an internal control. The primers of PPT-I used were 5'-GTC GAA TTC ATG AAA ATC CTC GTG-3' (forward) and 5'-GGT CTC GAG TTA ACG TCT TCT TTC ATA ATT CTG-3' (reverse). The amplification products were 354 bp in length. The primers for NK-1 were 5'-AAA TGA AAT CCA CCC GGT ATC-3' (forward) and 5'-TTA CTC GAG CTA GGA GAG CAC ATT G-3' (reverse). The amplification products were 230 bp long. The primers for NK-2 were 5'-ACA CCC ACC AAG GAA GAT AAG-3' (forward) and 5'-CCG GAC TCT AGA TCA AAT TTC AAC ATG AG-3' (reverse). The amplification products were 225 bp (Table 1). SYBR Green PCR amplification was performed in a GeneAmp 5700 sequence detection system (PE Applied Biosystems). The reactions were carried out in a 96-well plate with a 25-μl reaction volume containing 7.1 μl of 2 × SYBR Green Master Mix (PE Applied Biosystems), a 0.24 μM concentration of each forward and reverse primer, and 1 ng of total cellular DNA. The thermal profile for all SYBR Green PCR was 95°C for 10 min, followed by 40 cycles at 95°C for 5 sec, 55° C-58°C for 10 sec and 72°C for 12 sec.

The baseline adjustment method of the Gene Amp 5700 SDS (Applied Biosystems, USA) software was used to determine CT (threshold cycle) in each reaction. A melting curve was plotted for each primer pair to verify the presence of 1 gene specific peak and the absence of primer dimers. All samples were amplified in 3 independent reactions, and the mean was used for further analysis.

PPT-I, NK-1 and NK-2 protein expression detected by Western blot

Twenty micrograms of protein was incubated in loading buffer (125 mmol/L Tris-HCl, pH 6.8, 10% b-mercaptoethanol, 4.6% SDS, 20% glycerol and 0.003% bromophenol blue) for 5 min at 100°C, and then separated by sodium dodecyl sulfate polyacrylamode gel (SDS-PAGE) and finally electroblotted to PVDF membrane (BioRad). After blocking non-specific binding sites for 1 h with 5% skimmed milk in TPBS (PBS contained 0.05% Tween 20), the membrane was incubated overnight at 4°C with primary antibody. After washing 3 times in TPBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 2 h at room temperature, and washed twice with TPBS. Detection of the immunoblot was performed by autoradiography using an enhanced chemoluminescence detection kit.

Observation of cell ultrastructure in T47D cells before and after coculture

The cell suspension was collected into an agar tube and then centrifuged at 2,000 r/min to conglobate the cells. The supernatant was discarded, and the cell conglobation was fixed with 2% glutaraldehyde at 4°C for 30 min. The cell conglobation was stripped and then fixed with 1% osmic aceticaldehyde for 1-2 h. Ultra-thin sections were prepared. Finally, the changes at different time points (before and 96 h after coculture) in the ultrastructure of the cells were observed under a transmission electron microscope.

Flow cytometric analysis of the T47D cell cycle before and after coculture

T47D were collected before and 48 h and 96 h after coculture, and washed with pre-cooled PBS twice, and then fixed with cold 70% ethanol. Then, 1 ml 20 μg/ml PI with 50 μg/ml Rnase was added, and this was incubated at 37°C for 30 min. Flow cytometric analysis was conducted, and 10^5 cells were used in cell cycle analyses.

Results

Coculture of BCCs (cell lines) and MSCs

A coculture method was developed to mimic a model that represents early metastasis of BCCs to the BM. The model assumes that the BCCs will be located in a region BM with stromal cells. Due to the infrequency of stromal cells in the BM, and as the current study hypothesizes that few BCCs enter the BM before clinical detection, cultures were initiated using 100 stromal cells/25-cm^2 flasks and equivalent numbers of BCCs. The cocultures did not require growth supplements in

Table 1. Four target genes primer sequences, annealing temperatures, and PCR product lengths.

Gene	Sequence	Length (bp)	Temperature (°C)
PPT-I	Forward: 5'-GTC GAA TTC ATG AAA ATC CTC GTG-3' Reverse: 5'-GGT CTC GAG TTA ACG TCT TCT TTC ATAATT CTG-3'	354	58
NK-1	Forward: 5'-AAA TGA AAT CCA CCC GGT ATC-3' Reverse: 5'-TTA CTC GAG CTA GGA GAG CAC ATT G-3'	230	55
NK-2	Forward: 5'-ACA CCC ACC AAG GAA GAT AAG-3' Reverse: 5'-GGA CTC TAG ATC AAA TTT CAA CAT GAG-3'	225	55
β-actin	Forward: 5'-CCT GGG CAT GGA GTC CTG TG-3' Reverse: 5'-AGG GGC CGG ACT CGT CAT AC-3'	305	59.7

contrast to the BCCs cultured alone. At cell confluence, both the stromal cells and BCCs stopped dividing and remained viable, as detected by trypan blue exclusion. The cocultures were photographed from day1 to day 5 following cell confluences. Each cell line was studied with at least 3 different BM donors. Unlike the contact inhibition observed in the cocultures, when BCCs were cultured alone, the cells formed foci of confluence and cell death happened.

Growth curves of T47D cells in cocultures with MSCs

We observed that the cocultures of T47D and MSC achieved confluence at a slower rate when compared with T47D cells cultured alone. To this end, we made growth curves of the T47D cells in cocultures and in culture alone. The results showed that the number of T47D cells in cocultures was decreased by more than 2-fold over the number of T47D cells in separate cultures. The cocultures did not contain other growth factors compared with the supplement for the BCCs. Therefore, we established growth curves for BCCs in the presence of MSCs and then compared the growth with BCCs cultured in regular culture media. The results showed obviously slower growth of the BCCs in coculture than that of the cells cultured alone (Fig.2).

Cell cycle changes in T47D cells before and after coculture

The analysis of cell cycle distribution by flow cytometry showed that the proportion of T-47D cells in S phase was increased, and the number in G_2/M phase was sharply decreased. However, the number of T-47D cells in G_0/G_1 phase had no obvious changes in the 2 groups (Table 2 and Figs.3,4).

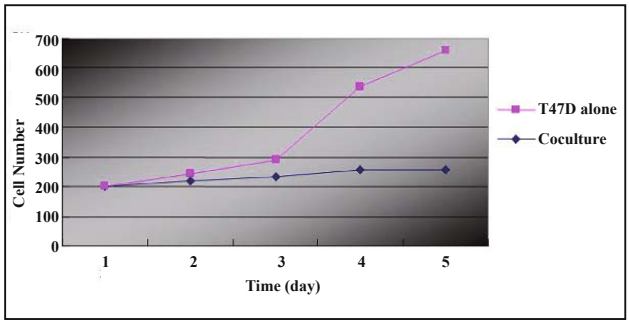

Fig.2. The growth of T-47D was greatly inhibited after coculture with MSC. The growth of T-47D in coculture was slower than T47D alone as a control group. However HBL-100 could not survive in coculture system (not described in the curve).

mRNA and protein expression of PPT-I, NK-1 and NK-2 in T47D cells before and after coculture

The expression of PPT-I mRNA in T-47D showed significant change before and after coculture. At 48 h after coculture, the relative expression of PPT-I in T-47D was increased 6.32 times compared with that before coculture. At 96 h after coculture, the relative expression of PPT-I in T-47D was increased 16.27 times (Table 3). The results showed that the expression of PPT-I mRNA in T-47D after coculture was greatly increased in com-

Table 2. The influence of the T-47D cell cycle following coculture (%).

Group	G_0/G_1	S	G_2/M
Control	47.3	32.0	20.7
Coculture	45.6	54.4	0

Table 3. The relative expression of PPT-I mRNA in T-47D before coculture and at 48 h, 96 h after coculture.

	Ct_{PPT-I}	Ct_{actin}	ΔCt	-ΔΔCt	$2^{-\Delta\Delta Ct}$
Before	21.45 ± 2.33	15.89 ± 1.77	5.87 ± 0.88	0	1.00
48 h	22.26 ± 2.44	18.98 ± 2.87	3.21 ± 0.87	2.66	6.32
96 h	20.95 ± 2.49	19.16 ± 2.67	1.76 ± 0.97	4.11	17.27

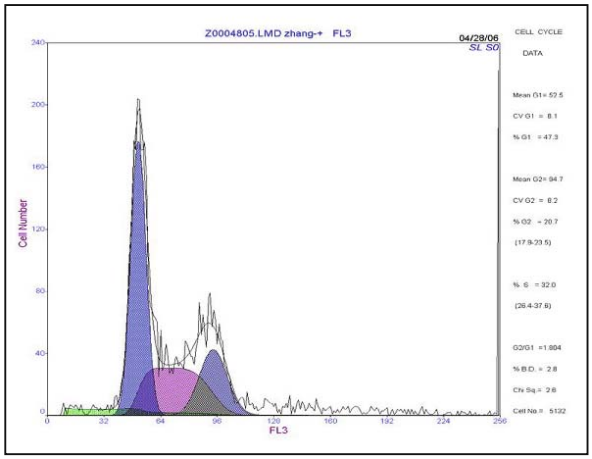

Fig.3. The cell cycle of T47D at 48 h after culture alone.

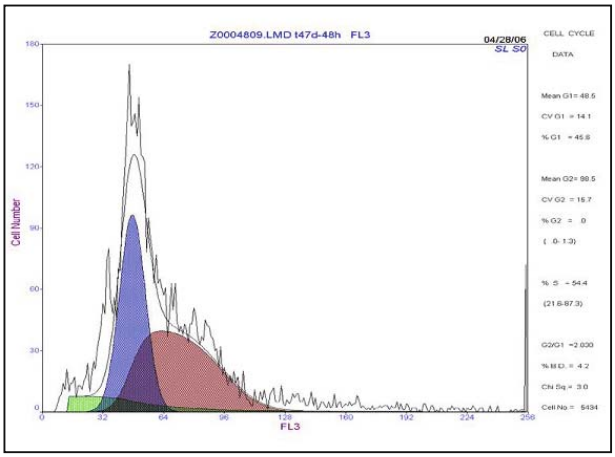

Fig.4. The cell cycle of T47D at 48 h after coculture.

Table 4. The relative expression of NK-1 mRNA in T-47D before coculture and at 48 h, 96 h after coculture.

	Ct_{NK-1}	Ct_{actin}	ΔCt	-ΔΔCt	$2^{-\Delta\Delta Ct}$
Before	25.43 ± 2.63	16.49 ± 1.97	8.87 ± 0.48	0	1.00
48 h	37.26 ± 2.47	16.34 ± 2.86	20.98 ± 1.77	-12.11	2.26×10^{-4}
96 h	38.95 ± 2.21	15.23 ± 2.37	23.77 ± 1.95	-14.9	3.27×10^{-5}

Table 5. The relative expression of NK-2 mRNA in T-47D before coculture and at 48 h, 96 h after coculture.

	Ct_{NK-2}	Ct_{actin}	ΔCt	-ΔΔCt	$2^{-\Delta\Delta Ct}$
Before	29.45 ± 2.57	16.49 ± 1.97	13.45 ± 1.58	0	1.00
48 h	35.34 ± 2.87	16.34 ± 2.86	19.23 ± 1.66	-5.78	0.018
96 h	37.56 ± 2.12	15.23 ± 2.37	23.12 ± 2.15	-9.67	0.0012

parison to the expression before coculture. The expression of NK-1 and NK-2 mRNA in T-47D before and after coculture also had obvious changes. After coculture, the relative expression of NK-1 and NK-2 in T-47D was decreased sharply compared with the level before coculture (Tables 4, 5).

Similar to the interesting findings seen with mRNA, changes in protein expression of PPT-I, NK-1 and NK-2 in T-47D also occured before and after coculture most notably at 96 h. At 96 h after coculture, PPT-I protein expression in T-47D was greatly increased compared with the levels before coculture. In contrast, protein expression of NK-1 and NK-2 in T-47D at 96 h after coculture was decreased in comparison to expression before and 48 h after coculture (Fig.5).

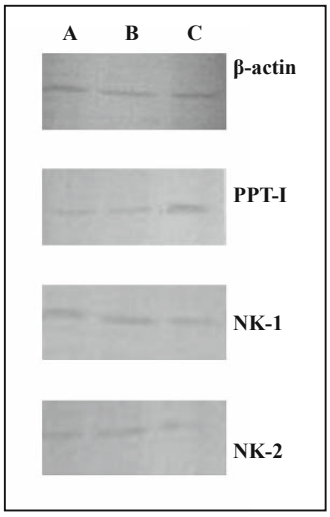

Fig.5. The protein expression of PPT-I, NK-1 and NK-2 in T-47D before and after coculture (A is designated protein expression before coculture. B, C is designated protein expression at 48 h and 96 h after coculture, respectively). At 96 h after coculture, protein expression of PPT-I in T-47D was greatly increased compared to expression in T-47D before coculture. However, at 96h after coculture, protein expression of NK-1 and NK-2 in T-47D was decreased compared to expression in T-47D before and 48 h after coculture.

Ultrastructural changes in T47D cells before and after coculture

T-47D cells at 96 h following coculture had obvious increases in cellular size, several times larger than the cells in the control group. In the coculture group, T47D cells took on an irregular polygonal or flask-shaped conformation, and the nucleus had an irregular appearance. In some cells, especially large cells, the nucleus was divided into several parts. Nucleoli were loose and inconsistent in size, and were marginated. Within the nucleus, the proportion of euchromatin was increased significantly. Additionally, the nucleus was bright with a low electron density and was filled with free ribosomes. The mitochondria were small in size, oval, and with an expansion of the space within the ridge. They appeared as structures with small amounts of bubbly cytoplasm. In some cells, a cavity similar to microcapsules could be seen. The stacking of glycogen granules in the cytoplasm was a prominent characteristic in the coculture group. Beneath the cellular membrane and in the cleavage sites of the cell, numerous microfilaments were present. Within some cells, the structures of cell-cell links could be seen.

Discussion

Recent studies have suggested that bone marrow metastasis occurring in patients with breast cancer is always indicative of poor prognosis. Due to developments in diagnostic measures, it is not usually difficult to discover bone metastasis in cases when the tumor burden is evident. To date, most BC studies have not presented an explicit description of the period when frequencies of BCCs in the BM might be at the single cell level and perhaps lower than the frequency of BM stem cells. We conclude that BCCs can play a role in part of the BM microenvironment at a time long before clinical detection. In the BM, BCCs find a microenvironment that is conducive to survival while evading treatment. Because BCCs may enter the BM before clinical detection, it is very important to discover the potential mechanisms of early entry and existence of BCCs in the BM. Such studies are particularly significant because of the limitations, e.g., false negatives in BC screening by mammogram and self-examination[18]. Despite seemingly curative therapy, cancer could resurge in the BM even 10 years after cancer remission[19]. Furthermore, BCCs could metastasize from the BM to other tertiary sites.

The BM limits the dose of current cancer treatments, mostly because of potential toxicity to the finite number of hematopoietic stem cells that supplement the adult immune system[20]. The disappointing outcome of autologous BM transplantation in BC patients[21] is compounded by CD34 expression on other cancer cells that also show preference for the BM[22]. Perhaps BCCs could also express CD34, a marker that is used clinically to select hematopoietic cells for BM transplantation. Thus, if BCCs do express CD34 it is not surprising that autologous BM transplantation would be unsuccessful[23]. Without the option of hematopoietic repopulation, protection of the resident hematopoietic stem cells limits the doses of chemotherapeutic agents. Limited cancer

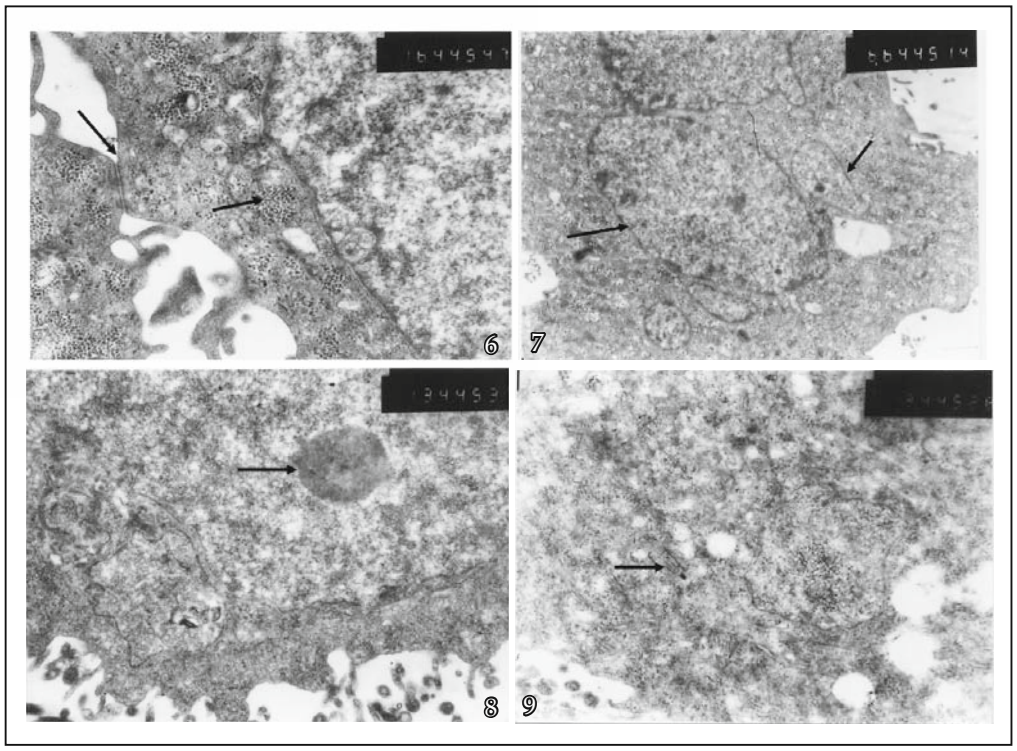

Fig.6. The ultrastructure of T-47D at 96 h after coculture with MSC × 24000. Cell-cell links were increased in comparison to control cells. There was prominent stacking of hepatin granules in the cell cytoplasm. Vesicles of different sizes were seen in the superficial cytoplasm. The contents were homogenous and of low electronic density.

Fig.7. The ultrastructure of T-47D at 96 h after coculture with MSC × 24000. The size of the nucleus was larger than that of control cells, and the shape was abnormal. The karyotheca was round, and in some cells, especially the larger cells, the nuclei were divided into several parts. The nucleoli were more numerous, varied in size and were centralized relative to the karyotheca. The proportion of chromatin in the nuclei was greatly increased. The nuclei were bright and of low electron density.

Fig.8. The ultrastructure of T-47D in the control group × 19500. The nuclei were similar and round. Most of the cells had only 1 nucleus, few had a double nucleus. The nuclei were large, and the proportion of nucleus to cytoplasm was increased. The karyotheca appeared light to moderate and circular. A few karyotheca showed depressed wrinkles.

Fig.9. The ultrastructure of T-47D in control group × 19500. Mitochondria were sparse. The shape of the mitochondria was oval, and the electron density of the stroma was high. The mitochondria were of dark cristae-type. Beneath the cell membrane and in cleavage sites of the cells, numerous microfilaments were present. Small bundles of microfilaments were seen in the cytoplasm. Vesicles were also found, located in the superficial cytoplasm. Cells were separate, and cell-cell links were not seen.

treatment would provide an advantage for BCCs in the BM. Due to the current controversy, it is important to address the notion that cancer treatments with BM stem cell replacement could be improved if the mechanisms of early entry and survival of BCCs in the BM were understood.

Soluble factors such as neurohormones and neurotransmitters interact to maintain biological homeostasis[24]. Neuroendocrine functions are associated with the development of BC[25-27], the second leading cause of cancer death. The molecular pathways within the neuroendocrine system that lead to BC are complex[28,29] and need dissection. In this research, we studied the potential role that PPT-I, NK-1 and NK-2 could play during the early metastasis stage in BM. PPT-I is associated with the neuroendocrine system, and is constitutively expressed in some cancer cells and, interestingly, in BM stromal cells[30,31]. Because PPT-I peptides are also hematopoietic modulators, their implication in BC may be relevant to integration in the BM, a preferred site of metastasis[12]. Finding common mechanisms in BC development and metastasis would lead to novel therapeutic strategies. Communication among different organs may partly explain neuroendocrine influence on cancer development. PPT-I peptides, such as substance P (SP) and neurokinin A, are examples of soluble factors that may mediate this cross-talk among different organs. Through various mechanisms, PPT-I may be involved in cancer development and metastasis[32]. These include angiogenesis, enhancement of cell invasiveness, metastasis, and promotion of cancer cell survival. Receptors for PPT-I peptides are targets in experimental cancer treatment[13,32]. In addition, PPT-I expression could be affected by neuroendocrine-related events that are implicated in cancer development.

In this report, we investigated a role for PPT-I and its receptors, NK-1 and NK-2, because it may lead to identification of novel therapeutic targets. We showed

a potential role of PPT-I and its receptors, NK-1 and NK-2, in BC. Considering that these genes are involved in neuroendocrine functions, this study may help to explain the molecular link between the neuroendocrine system and BC. The literature on cancer development indicates complex, multifold mechanisms. Regardless of the mechanism, a commonality among cancers is the inappropriate expression of several genes. The basic question addressed in this study is the extent of PPT-I involvement in BC development. Inference from these findings supports PPT-I peptides as a possible link between the neuroendocrine system and BC. The relevance of increased production of neuropeptides to tumorigenicity would be supported by the simultaneous expression of its respective receptor.

The aim of our study is to explore the potential relationship between the expression of PPT-I, NK-1, NK2 and early events for bone marrow metastasis in breast cancer. In this research, we first established cocultures of breast cancer cell line T-47D and marrow-derived MSC with equal numbers of cells to imitate the similar microenvironment of bone marrow metastasis in early stage. At 48 h and 96 h after coculture, we collected positive T-47D cells from the coculture system by MACS magnetic cell sorting (MicroBeads). Moreover, we studied the expression changes of PPT-I, NK-1, NK-2 in T-47D before and after coculture by real-time PCR and Western blot. In addition, we observed the changes of cellular ultrastructure of T-47D before and after coculture under electron microscope. We also studied the changes of cell cycle distribution by flow cytometric analysis and by drawing the growth curves of cells before and after and coculture. After coculture of T-47D and MSC, the expression of both PPT-I mRNA and protein was significantly upregulated, and the expression of NK-1 and NK-2 mRNA and protein was greatly downregulated. The analysis of cell cycle distribution by flow cytometry showed that the proportion of T-47D in S phase was increased and the number of T-47D in G_2/M phase was sharply decreased. The results suggested that the cell cycle of T47D cells were blocked after coculture. Under electron microscope, we observed that the synthesis of hereditary material was increased, but that hepatin granules were prominently stacked in T-47D cells. These results demonstrated that although the synthesis of DNA was increased, the proliferation of cells was inhibited after coculture. The cell growth curves also confirmed the outcome of observation under the electron microscope and flow cytometry. In terms of these discoveries, we concluded that breast cancer cells could sustain themselves for survival by the upregulation of the expression of the preprotachykinin-I gene during early bone metastasis in breast cancer. The suppression in breast cell growth after coculture could be induced by the downregulation of the expression of NK-1 and NK-2 receptors.

As we know, PPT-I also is overexpressed in solid tumors other than breast cancer. Therefore, future studies involving other cancer cells would be important as this may be relevant to BM metastasis, a common phenomenon in cancer. Furthermore, the cancers, e.g., lung cancer, that showed overexpression of PPT-I also metastasize to the BM. Overall, our and other recent studies all suggest that further research on the neuroendocrine-tumorigenic pathway may prove that it is crucial for preventively therapeutic interventions in BC development and metastasis to its preferential site, BM.

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