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INVESTIGATION OF CONDITIONED MEDIA-MEDIATED COMMUNICATION BETWEEN PANCREATIC CANCER CELLS AND NEURONS

PANKREAS KANSERİ HÜCRELERİ VE NÖRONLAR ARASINDA KOŞULLU BESİYERİ ARACILI ETKİLEŞİMİN ARAŞTIRILMASI

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ABSTRACT

Objective: Pancreatic cancer (PCa) is one of the deadliest cancers characterized by prominent pathological changes in nerves. PCa cells invade nerves, termed neural invasion, driven by bidirectional interactions between cancer cells and nerves, leading to severe pain and shorter survival. However, understanding of this communication remains limited, particularly in cell culture experiments. Therefore, we investigated the effect of soluble factors derived from neurons and PCa cells on the behavior of each cell type.

Methods: SH-SY5Y cells were differentiated into neurons and the differentiation efficacy was confirmed by immunofluorescence staining and Western blot. PANC-1 and BxPC-3 PCa cells were exposed to conditioned media (CM) from neurons, and changes in migration and invasion were evaluated by wound healing and transwell invasion assays, respectively. Similarly, neurons were treated with CM derived from PANC-1 or BxPC- 3 cells, and changes in migration and neuronal markers were assessed..

Results: Neuron-derived CM showed no significant impact on PCa cell migration and invasion. Similarly, exposure of neurons to CM from PCa cells did not significantly affect their migration, but slightly increased neurite formation.

Conclusion: This study provides preliminary information on the potential effect of CM on the interaction between PCa cells and neurons. However, the absence of other cell types (such as fibroblasts and Schwann cells) in the study design may explain the lack of expected changes. Therefore, different culture models and further research are needed to investigate the role of cells and factors that may contribute to cancer-neuron interactions.

Keywords: Pancreatic cancer, neurons, conditoned media, cell migration, invasion

ÖZ

Amaç: Pankreas kanseri (PKa), sinirlerde belirgin patolojik değişikliklerle karakterize, en ölümcül kanserlerden biridir. Kanser hücreleri ve sinirler arasındaki çift yönlü etkileşimler sonucu PKa hücrelerinin sinirleri istila ettiği bilinmektedir. Nöral invazyon olarak adlandırılan bu durum, hastalarda şiddetli ağrı ve kısa sağkalım süreleriyle ilişkilidir. Sinirler ve kanser hücreleri arasındaki ilişkinin anlaşılmasına yönelik bazı çalışmalar bulunmakla birlikte, özellikle bu kapsamdaki hücre kültürü çalışmaları oldukça sınırlıdır. Bu nedenle, bu çalışmada, nöronlardan ve PKa hücrelerinden salınan faktörlerin her bir hücre tipinin davranışları üzerindeki etkisini araştırılmıştır.

Yöntem: SH-SY5Y hücreleri nöronlara farklılaştırıldı ve farklılaşma etkinliği immünofloresan boyama ve Western blot ile doğrulandı. PANC-1 ve BxPC-3 kanser hücreleri nöronlardan elde edilen koşullu besiyerine (CM) maruz bırakıldıktan sonra migrasyon ve invazyon yeteneklerindeki değişiklikler sırasıyla yara iyileşmesi ve matrijel invazyon deneyi ile değerlendirildi. Benzer şekilde, nöronlar PANC-1 veya BxPC-3 hücrelerinden elde edilen besiyerine maruz bırakıldı ve nöronların migrasyon ve nöronal belirteçlerdeki değişiklikler değerlendirildi.

Bulgular: Nöronlardan toplanan besiyerinin, PKa hücrelerinin migrasyon ve invazyonu üzerinde anlamlı bir etki göstermediği görüldü. Benzer şekilde, PKa hücrelerine ait besiyerinin nöronlara uygulanmasının, nöronların migrasyonunu anlamlı şekilde etkilemediği, ancak nörit oluşumunu arttırdığı bulundu.

Sonuç: Nöronlardan toplanan besiyerinin, PKa hücrelerinin migrasyon ve invazyonu üzerinde anlamlı bir etki göstermediği görüldü. Benzer şekilde, PKa hücrelerine ait besiyerinin nöronlara uygulanmasının, nöronların migrasyonunu anlamlı şekilde etkilemediği, ancak nörit oluşumunu arttırdığı bulundu.

Anahtar Kelimeler: Pankreas kanseri, nöron, koşullu besiyeri, hücre migrasyonu, invazyon

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Introduction

Pancreatic cancer (PCa) is one of the most lethal and aggressive cancer types among all major cancers.[1] The close relationship between cancer cells and nerves is recognized as one of the main factors responsible for its aggressive nature. This vicious communication results in neuropathic alterations, including increased nerve density (neural sprouting), nerve size (neural hypertrophy), infiltration of immune cells (neuritis), and invasion of cancer cells (neural invasion).[2]

The invasion of cancer cells into nerves has been demonstrated in many cancer types, such as pancreatic,[3,4] prostate,[5] gastric,[6,7] head and neck cancers.[8,9] Cancer cells use the nerves as an alternative route for metastasis since the nerves provide a less chaotic and stressful environment compared to blood circulation. Thus, the invasion of nerves by cancer cells is closely associated with aggressive tumor growth and metastasis.[10,11] Besides, the interaction of cancer cells with nerves leads to increased pain. The nerve-rich anatomical features of the pancreas facilitate nerve-cancer interaction, making neural invasion a primary cause of PCa-related pain. Furthermore, the number and size of the nerves around tumor tissues are associated with poor overall survival in PCa patients.[12]

Despite the increasing number of studies highlighting the importance of the reciprocal interaction between nerves and cancer cells, most studies focus on only one direction of this interaction, and the mechanisms regulating this interaction remain still unclear. Using neurons in a culture environment is challenging because adult neurons are post-mitotically arrested cells. Using dorsal root ganglion (DRG)-derived mouse neurons is one way to obtain neurons; however, ethical considerations and differences between species (i.e, mouse neurons-human cancer cells) reduce their potential for use in cell culture studies. An alternative approach, the differentiation of neuroblastoma cells into neurons, is commonly preferred due to the high reproducibility of cell lines and the absence of ethical concerns.

Here, we investigated the effect of soluble factors derived from neurons and PCa cells on the migration and invasion abilities of these cells. Briefly, neurons were differentiated from neuroblastoma cells, and the efficacy of differentiation was confirmed by immunofluorescence staining and Western blot. Then, conditioned media (CM) were collected from PCa cells and neurons, and applied to the culture media of each cell type. Following 24 or 48 hours of incubation, changes in cell behaviors, such as invasion and migration, were evaluated. In summary, no significant changes were detected in either cell type after CM exposure. One possible reason for this outcome is that the factors in the CM may be too diluted to induce observable changes. Another possibility is that other cell types, such as pancreatic stellate cells (PSCs) and Schwann cells, may need to be present in the tumor microenvironment (TME) and interact with cancer cells to induce the expected changes. In conclusion, further investigation is needed to elucidate CM-mediated bidirectional interactions between neurons and cancer cells.

Methods

Cell lines and culture conditions

SH-SY5Y human neuroblastoma cell line was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Human PCa cell lines, PANC-1 and BxPC-3, were kindly gifted by Dr. Mumin Alper Erdogan (Izmir Katip Celebi University, Turkey) and Dr. Konstantinos Dimas (University of Thessaly, Greece), respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 supplemented with 10% fetal bovine serum (FBS) and 100-U/ml penicillin-streptomycin solution. All cultured cells were maintained at 37°C with 5% CO2 and 95% air and screened for mycoplasma by PCR.

Neuronal differentiation and characterization

SH-SY5Y cells were treated with all-trans-retinoic acid (ATRA) to induce neuronal differentiation. For this purpose, the cells were cultured in T75 cell culture flasks until they reached 70-80% confluency. The medium was then replaced with differentiation medium* and the cells were cultured for 6 days by refreshing the media every 3 days.

The differentiation efficiency of the cells was assessed by evaluating the cells for the presence of neurite structures via phase-contast microscopy and immunofluorescence staining for β-III tubulin. Additionally, changes in the expression of neuronal markers such as neuron-specific enolase (NSE), neuronal nuclear protein (NeuN), and Nestin were assessed via Western blot.

**Differentiation medium composition; DMEM:F12 medium-containing 10 µM ATRA, supplemented with 1% FBS, 1X B27 supplement, and 1% P/S.*

Conditioned media (CM) collection

To evaluate the effect of soluble factors released from both PCa cells and neurons on cell behaviors, CM were collected from cancer cells and neurons. PANC-1 or BxPC-3 cells were cultured in T75 flasks until they reached 80% confluency. Then, the media were replaced with FBS-free medium, and the cells were incubated for an additional 24 hours. Following the incubation period, the media were collected and centrifuged at 2000 rpm for 30 minutes at

+4°C to remove cellular debris. The supernatant was carefully transferred to fresh tubes without disturbing the pellet, and the tubes were kept at +4°C until use.

For collection of neuron-conditioned media, SH-SY5Y cells were first differentiated into neurons as explained above. The media were then replaced with FBS-free media (without ATRA). After 24 hours of incubation with FBS-free media, conditioned media were collected via centrifugation. All conditioned media were freshly prepared and immediately used for the experiments avoiding freeze-thaw cycles.

Wound healing assay

To measure changes in cell motility and migration, an in vitro wound healing assay was performed. Briefly, PANC-1 and BxPC-3 cells were seeded into 24-well plates and cultured until they reached ~80% confluency. Similarly, SH-SY5Y cells were seeded into 24-well plates and differentiated into neurons as mentioned above. Wounds were then carefully made on the cell layer using 200 µl sterile pipette tips. Immediately after scratching, the wells were carefully washed with PBS, and the cell culture media were replaced with conditioned media. Wounds were photographed using a phase-contrast microscope (Nikon Instruments Inc., USA) to determine the wound width at 0 hours. Cells were exposed to CM for 48 hours, and wound areas were photographed at 24 and 48 hours. Wound closure was calculated by comparing the wound distances at 0 hours with those taken at 24 and 48 hours using ImageJ software (National Institutes of Health, Bethesda, MD). All experiments were performed at least twice in duplicate.

Transwell invasion assay

PCa cells or neurons were seeded on the top of transwell inserts (8-µm-pore size) coated with Matrigel. The lower chambers of the inserts were filled with 500 µl of 50% CM containing 10% FBS as chemoattractant. After 24 hours, the invading cells were fixed and stained with 0.5% crystal violet prepared in methanol. Cells remaining on the upper side of the membrane were removed by gently wiping with a cotton swab, and the membranes were photographed. The number of invaded cells was quantified by counting at least six fields per membrane using ImageJ software. Results were expressed as the mean number of invaded cells from duplicate measurements.

Immunofluorescence staining

The efficacy of neuronal differentiation was evaluated by immunofluorescence staining. SH-SY5Y cells were seeded onto round coverslips pre-coated with Poly-L-lysine and differentiated into neurons as described above. For the staining procedure, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Subsequently, the cells were blocked with 10% normal goat serum. Following 24 hours incubation with β-III tubulin antibody (1:200 dilution), a secondary antibody conjugated with FITC was added, and the cells were visualized under a fluorescence microscope. Undifferentiated SH-SY5Y cells were used as a control group for comparison with differentiated cells.

Western blotting

To confirm neuronal differentiation and evaluate the effects of PCa cell-derived CM on the expression of neuronal proteins, Western blot analysis was performed. First, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. Total protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific, USA). Equal amounts of protein (30 µg) were loaded onto 4-15% gradient SDS-PAGE gels and separated by electrophoresis. Subsequently, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA). The membranes were probed with specific primary antibodies against β-III tubulin, NSE, NeuN, and Nestin, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, USA). β-actin was used as the loading control.

Statistical analyses

The data are expressed as the mean ± standard error from at least two independent experiments. Statistical analysis was performed using one way ANOVA to determine statistical significance. P values indicate the probability of the means compared, being equal with *P<0.05, **P<0.01, ***P <0.001, and ****P <0.0001. ANOVA calculations were conducted using GraphPad software (version 10).

Results

Neurons were differentiated from neuroblastoma cells

The increased number and size of nerves in tumor are known to be associated with poor overall survival. As shown in Figure 1A, PDAC patients with high TUB3 expression, a neural marker, have shorter overall survival compared to patients with low TUB3 expression. We therefore aimed to investigate bidirectional interaction between PCa cells and neurons to understand the effect of each cell type on the other.

Briefly, SH-SY5Y neuroblastoma cells were differentiated into neurons via ATRA treatment, as illustrated in Fig. 1B. The efficacy of differentiation was confirmed by morphological evaluation under phase-contrast microscope. While SH-SY5Y neuroblastoma cells typically grow as cluster-like structures without cell extensions, differentiated neurons have small extensions, called axons, indicating they acquired neuronal morphology (Fig. 1C). The presence of β-III tubulin-positive axons was further confirmed by immunofluorescent staining (Fig. 1C). Besides, cells were observed to lose their proliferation ability when they differentiated into neurons. Additionally, the expression of neuron-related proteins, NSE and NeuN, was evaluated by Western blot (Fig. 1D). As shown in the figure, differentiated neurons have higher expression of NSE and NeuN than neuroblastoma cells. Consistently, the expression of the neuroblastoma- specific protein, Nestin, was evaluated. While neuroblastoma cells displayed high expression of Nestin, differentiated neurons did not exhibit Nestin expression (Fig. 1D).

Neuron-derived CM slightly increased cell migration in PANC1 cells but not in BxPC-3 cells

To investigate the effect of neuron-derived factors on the migration and invasion of PCa cells, CM was collected from neurons. PANC-1 and BxPC-3 cells were then

exposed to neuron- derived CM, and changes in their migration ability were assessed by wound healing assay.

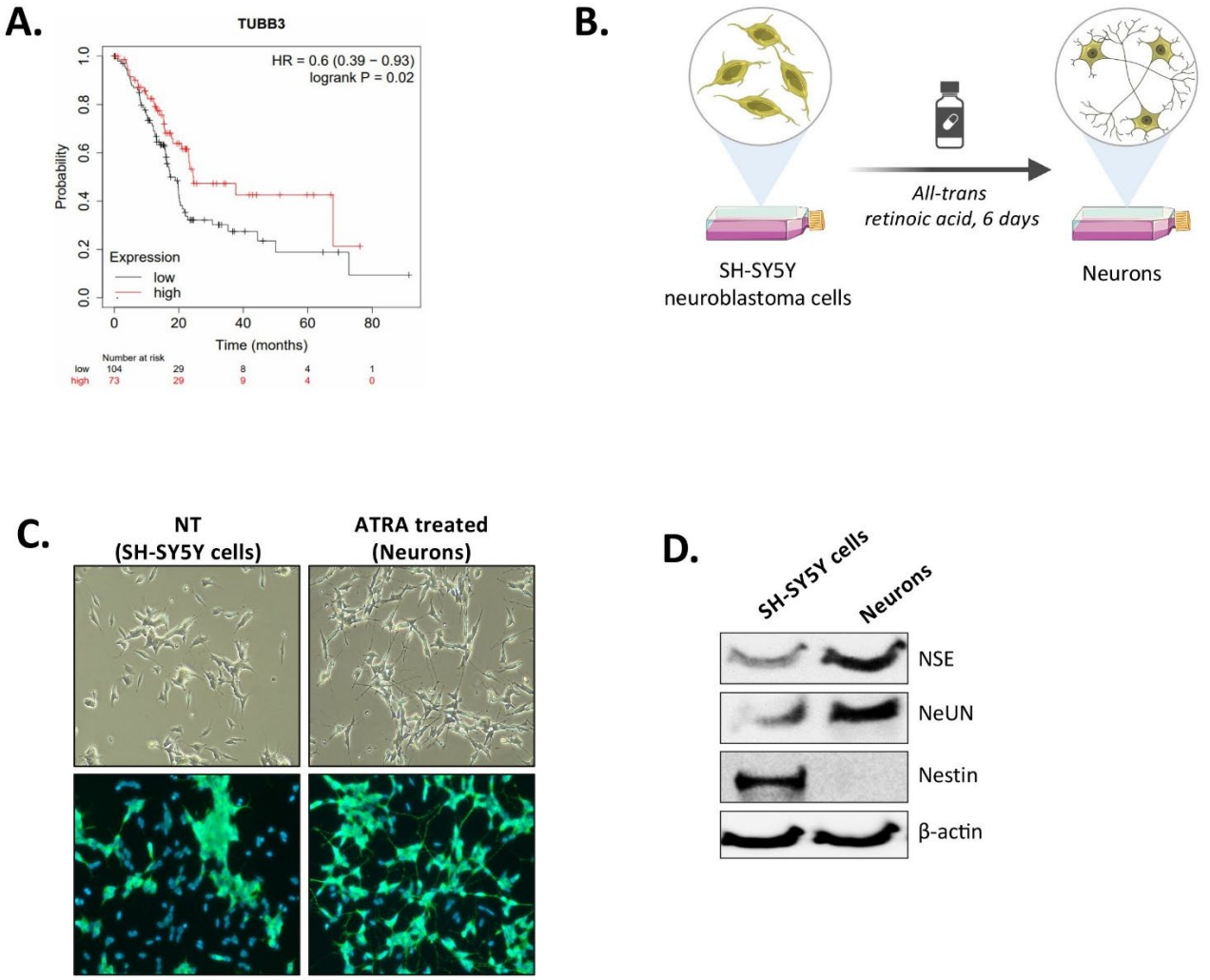

Figure 1. Neuronal differentiation and characterization. (A) The high levels of neuronal marker, TUB1, is associated with shorter overall survival in PCa patients. Kaplan-Meier survival analysis of the TCGA PCa database showed the overall survival rates of PCa patients with high and low expression of TUB1. (B) Schematic illustration for differentiation of SH- SY5Y neuroblastoma cells into neurons. (C) Phase-contrast images and β-III tubulin staining for confirmation of neuronal differentiation. The phase image shows presence of neuronal extensions, neurites, in neurons. Similarly, β-III tubulin positive neurites were observed in neurons. (D) Western blot analysis for neuron-specific proteins. ?-actin was used as loading control. ATRA; All-trans retinoic acid, NSE; neuron specific enolase, NeuN; neuronal nuclear protein.

The results showed that neuron-derived CM slightly increased the migration of PANC-1 cells but did not significantly affect the migration of BxPC-3 cells (Fig. 2A). Besides, 24 hours of incubation of PCa cells with neuron-derived CM did not alter the invasion capability of either cell type (Fig. 2B, C).

PCa cell-derived CM did not alter migration and invasion of neurons but slightly increased neurite formation

In the next step, to evaluate the effect of PCa cell-released factors on neurons, we collected CM from both PANC-1 and BxPC-3 cells. We then applied CM from each cell type to neurons, and assessed changes in migration and neurite formation. As shown in Fig. 3A, neither PANC-1 CM nor BxPC-3 CM resulted a change in the migration ability of neurons. Surprisingly, both PANC- 1 and BxPC-3 CM caused a slight induction in the formation of new neuronal extensions from neurons, known as neurites (Fig. 3B, C).

Discussion

PCa is one the most lethal cancers among among major solid tumors, with a 5-year survival rate barely above 10%.[1] Survival after diagnosis is around 4-6 months for the majority of patients.[13] Patients are generally diagnosed in the middle to late stage of disease during clinical consultation, as PCa tumors often lack specific symptoms.[14] Thus, the majority of patients are diagnosed at an advanced stage with metastatic tumors, leading to a survival rate of approximately 3%.[15] The unique TME of PCa is responsible for the aggressive and lethal nature of this cancer. In addition to cancer cells, the TME consist of various components such as fibroblasts, immune cells,

adipocytes, blood and lymphatic vessels and the extracellular matrix. There is a dynamic and reciprocal relationship between cancer cells and the TME, which contributes to all hallmarks of cancer.[16]

In addition to blood and lymph vessels, the presence of nerves within tumors has been observed in many cancer types, particularly in PCa.[12,17] The pancreas is a well-innervated organ by autonomic nervous system, and during tumorigenesis this nerve-rich nature facilitates nerve-cancer interactions. The close interaction between nerves and cancer cells is associated with poorer overall survival in PCa patients, making the presence of nerves in PCa tumors an independent prognostic factor.[4] In a study conducted in 2021, PCa patients were categorized into nerve-positive (+) and nerve-negative (-) groups based on the presence or absence of nerves in their tumors. The results revealed that the survival rate of nerve (+) patients was significantly lower compared to nerve (-) patients. Additionally, nerve size was compared between normal pancreas and cancer tissues, showing a significant increase in nerve size in cancer tissues. Furthermore, patients with tumors containing larger nerves had shorter survival rates than those with relatively smaller nerves.[12]

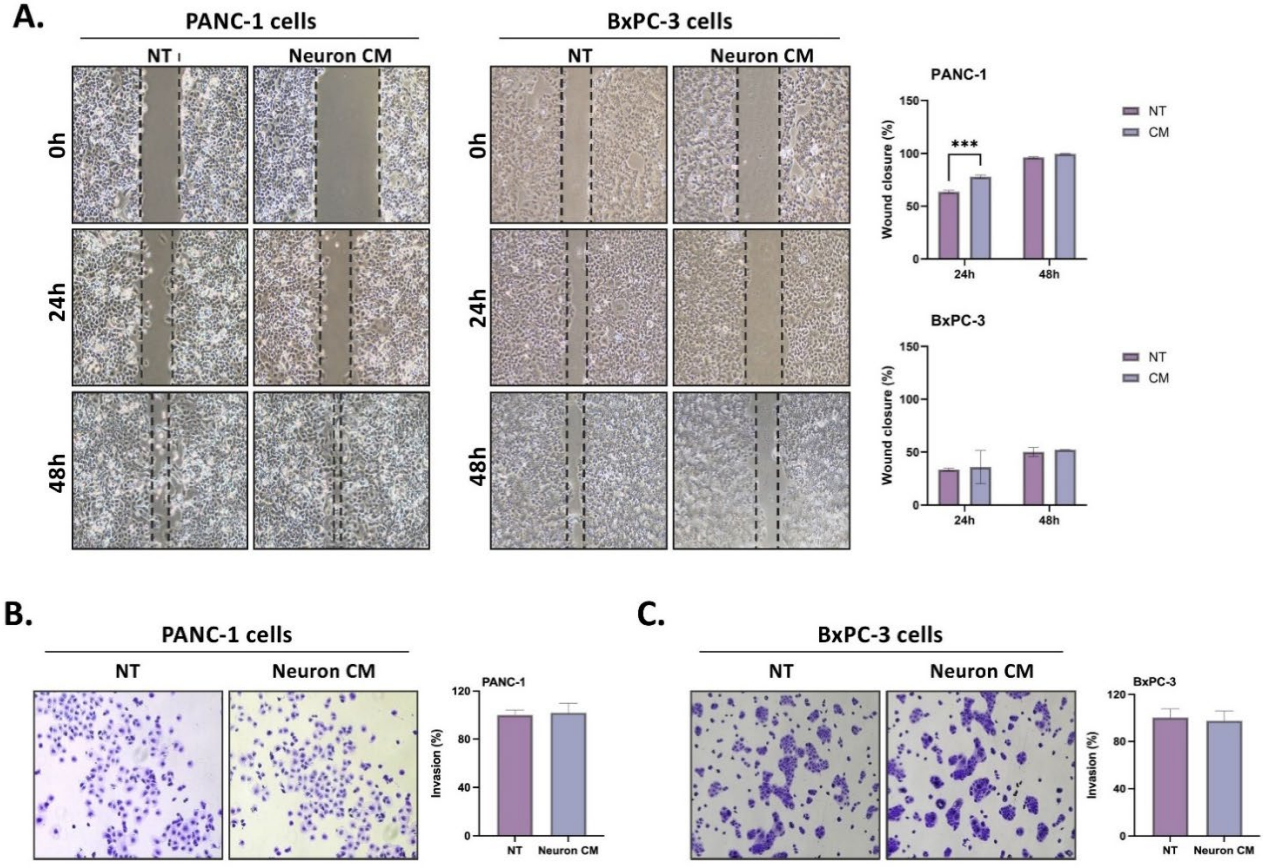

Figure 2. Neuron-derived conditioned media (CM) did not significantly affect migration and invasion of PCa cells. (A) Two different PCa cells, PANC-1 and BxPC-3, were exposed to CM-derived from neurons and the migration of cells were assessed by wound healing assay. Representative images of migrated cells and the quantified data after 24 and 48 hours of CM exposure. (B, C) Changes in the invasion ability of PANC-1 and BxPC-3 cells after 48 hours CM exposure.

In addition to the presence of nerves within the tumor, cancer cells are known to invade nerves, a phenomenon referred as neural invasion, which is observed in 80-100% of PCa cases. Neural invasion is considered an alternative route for metastatis and is correlated with increased pain and poor prognosis.[2,3] So far, several factors involved in the vicious interactions between nerves and cancer cells have been identified. Nerve-derived molecules, such as neurotransmitters and chemokines, have been reported to promote aggressive characteristics of PCa, including proliferation, migration, and invasion. A study performed in mouse models of PCa showed

that neuron-derived chemokines, CXCL10 and CCL21, increased the migration of tumor cells toward neurons.[18] Moreover, various neurotransmitters, including norepinephrine, serotonin, GABA, substance P, and glutamate, released by tumor-infiltrating nerves, have been shown to induce the proliferation, migration, and invasion of PCa cells.[19-24] PCa cells, on the other hand, secrete neuromodulatory molecules, such as neural growth factor (NGF) and glial cell-derived neurotrophic factor (GDNF), to protomote neural remodeling and facilitate cancer-nerve interactions.[2]

Despite recent studies focusing on understanding the molecular mechanisms underlying PCa cell-nerve interactions, there are still missing points that need to be addressed. Therefore, the use of cell culture models to mimic cancer-nerve interaction is a need for conducting functional and molecular analyses. Here, we therefore focused on the bidirectional interaction between PCa

cells and neurons, and evaluated the effects of this interaction on the behavior of both cell types. For this purpose, neurons were differentiated from SH-SY5Y neuroblastoma cells, and the success of differentiation was confirmed by immunofluorescent staining and Western blot.

In the literature, mature neurons are known to have β-III tubulin positive axons.[25] Consistently, we observed the formation of new extensions from cell bodies, termed neurite formation (or axonogenesis), using β-III tubulin staining. Additionally, the expression of neuron-specific markers, neuron specific enolase (NSE) and neuronal nuclear protein (NeuN),[26,27] was confirmed by Western blot analysis. Furthermore, Nestin expression is known to downregulated during neuronal differentiation.[28] Consistent with this information, we observed a dramatic decrease in Nestin expression in the differentiated cells.

Then, we collected CM from both PCa cells and neurons and applied CM to the culture media of each respective cell type. After 24 or 48 hours of incubation, we evaluated changes in cell behavior, such as invasion and migration. Although some studies have examined the effect of CM on PCa cells, most research focuses on only one direction of this bidirectional interaction, typically focusing on changes in PCa cells.[29-31] In contrast, our study aimed to obtain preliminary data on the effects of factors released from one cell type on the other.

As a result of our CM experiments, no significant changes were observed in either cell type after CM exposure. There might be some reasons for this outcome. Firstly, the factors in CM may be too diluted to induce observable changes. To address this, we plan to reproduce our data using more concentrated CM, obtained through specific filters and ultracentrifugation. Secondly, the presence of other cell types in the TME, such as PSCs and Schwann cells, might be required to stimulate changes through interaction with the cells. PSCs are one of the most common cell types in the PCa TME and play critical roles in the aggressive nature of PCa.[32] Although the effect of PSCs on PCa cells are quite well-documented, their impact -either directly or indirectly- on nerve-cancer interactions remains unknown. Therefore, incorporating these cells into cell culture experiments may more accurately mimic the TME.

Similar to PSCs, Schwann cells are key components of peripheral nerves. They play critical roles in the development, maintenance, function, and regeneration of peripheral nerves. In addition to their physiological functions, Schwann cells are known to have critical roles in neuron-cancer interactions. Recent studies indicate that Schwann cells serve as the first contact point in the neural invasion process. These cells have been shown to recruit and guide cancer cells toward nerves.[33] Considering their role in neural invasion, incorporating Schwann cells into culture experiments, alongside neurons, may provide additional insights.

In summary, our results provide preliminary data for the effect of CM derived from PCa cells and neurons on the migration of both cell types. As we discussed above, further modifications and optimizations may be required to obtain more significant results. Therefore, additional investigation is needed to fully elucidate the bidirectional interaction between neurons and cancer cells.

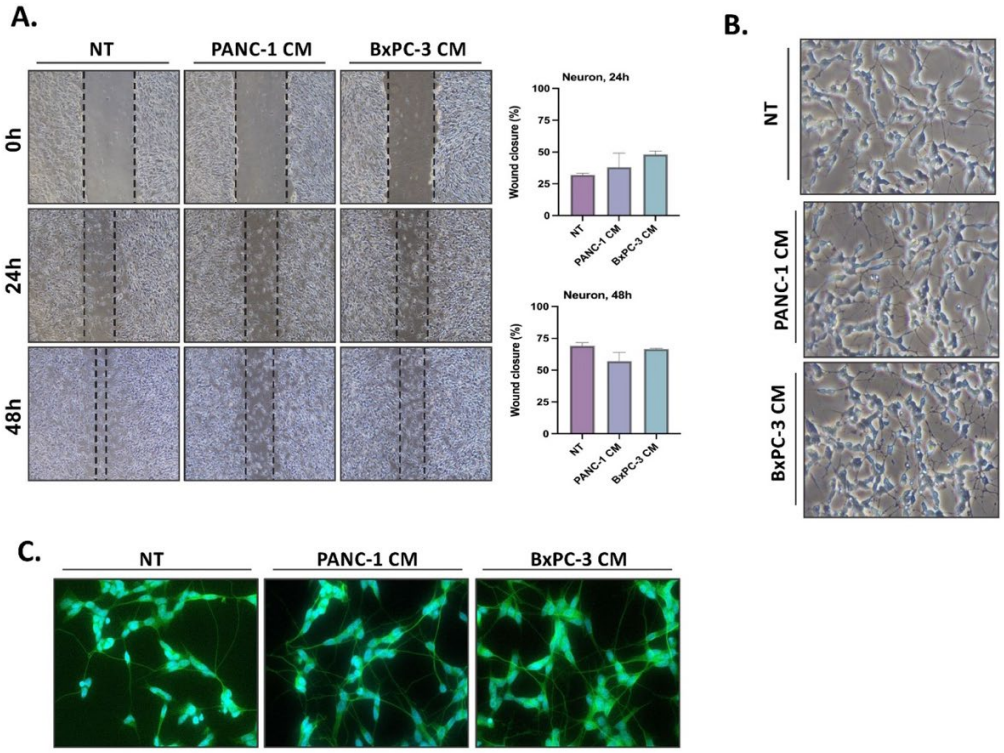

Figure 3. CM derived from PCa cells did not change migration, but induced neurite formation in neurons. (A) Neurons were treated with CM-derived from either PANC-1 and BxPC-3 cells and changes in their migration ability were evaluated. (B) The changes in the cellular extensions, neurites, axons, were evaluated by phase-contrast microscope. (C) The effect of PCa CM on neurite formation were also evaluated by immunofluorescent staining with β-III tubulin antibody.

Ethical Approval

The study does not require ethics committee approval, since no animal or human material were used in the study.

Conflict of Interest

The author declares no conflicts interests.

Author Contributions

DK: Concept-Design; Data Collection and/or Processing; Analysis and/or Interpretation; Literature Review; Writer; Critical Review

Financial Support

None

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