Effect of PDAC-derived conditioned media on endothelial ablation

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is regularly characterized by reduced in overall vasculature with thin collapsed and poor perfusion, known as a hypovascularity which is one of the most challenges of chemotherapeutic drug delivery in PDAC patients. The purpose of this study was to investigate the impact of PDAC cells on vascular endothelial cell (EC) behaviors through paracrine signaling using PDAC-derived conditioned media (CMs). We found that PDAC CMs harvested under both normoxic- and hypoxic-PDAC cultures showed no effect on reducing vascular endothelial cells. Therefore, the ablation of ECs during PDAC progression might be regulated through other communication such as cell-direct manner.

บทคัดย่อ

มะเร็งตับอ่อนชนิดต่อมถูกจัดอยู่ในกลุ่มมะเร็งชนิดที่มีการลดลงของจำนวนหลอดเลือด ร่วมกับขนาดของหลอดเลือดที่ตีบลง หรือที่เรียกว่าภาวะหลอดเลือดน้อย (hypovascularity) โดยการลดลงของหลอดเลือดส่งผลทำให้ประสิทธิภาพการรักษาด้วยวิธีเคมีบำบัดของผู้ป่วยมะเร็งตับอ่อนชนิดต่อมเป็นไปอย่างจำกัด การศึกษานี้มีจุดประสงค์เพื่อศึกษาผลกระทบของการสื่อสารด้วยพาราไครน์จากเซลล์มะเร็งตับอ่อนชนิดต่อมต่อเซลล์หลอดเลือด โดยการเก็บคอนดิชันมีเดีย (Conditioned media, CMs) ซึ่งเป็นอาหารเลี้ยงเชื้อที่คาดว่าจะมีสารคัดหลั่งที่เซลล์มะเร็งตับอ่อนชนิดต่อมหลั่งออกมา ผลการศึกษาพบว่าคอนดิชันมีเดียของเซลล์มะเร็งตับอ่อนชนิดต่อมที่ถูกเก็บในสภาวะปกติ และสภาวะขาดออกซิเจน (Hypoxia) ไม่ส่งผลกระทบต่อการลดลงของเซลล์หลอดเลือด ดังนั้นการลดลงของเซลล์หลอดเลือดในมะเร็งตับอ่อนชนิดต่อมอาจเป็นผลมาจากการสื่อสารระหว่างเซลล์แบบอื่น เช่น การติดต่อโดยตรงของเซลล์

Keywords: Pancreatic ductal adenocarcinoma (PDAC), Hypovascularity, Endothelial ablation

คำสำคัญ: มะเร็งตับอ่อนชนิดต่อม ภาวะหลอดเลือดน้อย การทำลายหลอดเลือด

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) has been considered one of the most devastating malignancies, presenting a significant public health problem. Patients typically exhibit a poor survival rate, about 6-8 months after being diagnosed owing to its aggressiveness and tolerance to conventional therapies (Huang et al., 2021). Despite multiple improved drug therapy, there is research that reported that treatment with anti-angiogenic agents revealed inefficient treatment on advanced PDAC patients implying dismal response toward chemotherapeutic agents of PDAC (Kindler et al., 2010; Yu et al., 2012). Following poor responsiveness to chemotherapeutic drugs, it is caused by its prominent hallmark of PDAC, hypovascularity, which is characterized by reduced overall vessel density with impaired vascular integrity. During PDAC tumor progression, vascular endothelial cells are undergone apoptosis eventually ablated, leaving behind only tumor cells (Nguyen et al., 2019). These incidents might contribute to limit the drug therapeutic delivery to the tumor cells. Furthermore, a cohort of pancreatic cancer patients from the TCGA database showed that the correlation between vascularity and cancer patient survival rates differed based on cancer subtypes. The upregulation of CD31, which indicated the hypervascularized tumor, decreased the overall survival rate in patients with neuroendocrine cancer. PDAC patients with high CD31 expression, on the other hand, have a higher overall survival rate. This is because PDAC patients with high CD31 expression may have a better response to therapeutic treatment (Katsuta et al., 2019).

The activity of endothelial ablation, one of the contributors of hypovascular PDAC, might be regulated by tumor-endothelial communication likely through two basic cell signaling including paracrine signaling and cell-cell direct contact (Reece et al., 2011). However, the molecular mechanism for the apparent endothelial ablation remains unclear. In this study, we aim to investigate the role of PDAC regulating endothelial cell behavior through paracrine signaling by using a conditioned medium collected from normoxic- and hypoxic- cultured PDAC cells. This study provided basic information for further study of the cause of endothelial ablation in PDAC. Understanding PDAC-ECs communication will provide an important insight to further study on tumor hypovascularity through another model.

Objectives of the study

The aim of this study was to investigate the impact of PDAC cells on endothelial cell behaviors through PDAC-derived conditioned media.

Methodology

Cell culture

The primary cell, human umbilical vein endothelial cells (HUVECs), were isolated from umbilical cords, obtained from Ramathibodi Hospital. The HUVEC cells were cultured in Endothelial Cell Growth Medium-2, EGM-2 (PromoCell, Heidelberg, Germany) in an incubator at 37 °C under 5% CO_2.

There are two human PDAC cell lines, including PANC-1 and MIA-PaCa-2, used in this study obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium, DMEM (Gibco, Thermofisher, USA) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-streptomycin for a final concentration of 100 U/ml Penicillin-streptomycin in culture media (Gibco, USA). They were incubated in an incubator at 37 °C under a 5% CO_2.

Cancer conditioned media (CM) preparation

The conditioned media were collected from PDAC cells grown upon normoxic and Cobalt chloride-induced hypoxic conditions. The 50% confluent cultures of PANC-1 and MIA-PaCa-2 cell lines in 10-cm plates were rinsed with1x PBS and then incubated in the presence of 10 ml of DMEM containing 2.5% Fetal bovine serum (FBS) and 1% Penicillin-streptomycin for a final concentration of 100 U/ml Penicillin-streptomycin in culture media with or without $CoCl_2$ (0 or 25 μM, respectively) for 48 hours in a humidified incubator at 37 °C under a 5% CO_2. A separate dish without cultured tumor cells was also prepared with the same amount of DMEM (2.5% FBS and 1% Penicillin-streptomycin for a final concentration of 100 U/ml) containing with and without $CoCl_2$ (0 or 25 μM, respectively) for 48 hours to serve as a normal medium (NM) under normoxia and hypoxia, respectively. The collected culture medium was centrifuged at 5000 rpm for 5 minutes to remove the cell debris and was then filtered with a 0.45-μm filter (BIOFIL). The PDAC-conditioned media was preserved at -80 °C for the later experiment.

As DMEM medium was not designed specifically for HUVECs, the treatment of HUVEC with conditioned media (CM) required the addition of EGM-2 medium in their mixture to avoid the negative effects of DMEM's improper culture conditions on HUVEC growth. According to prior data from our laboratory investigation, the eligible ratio of CM and EGM-2 was 1:1, which did not interfere with HUVEC cell growth.

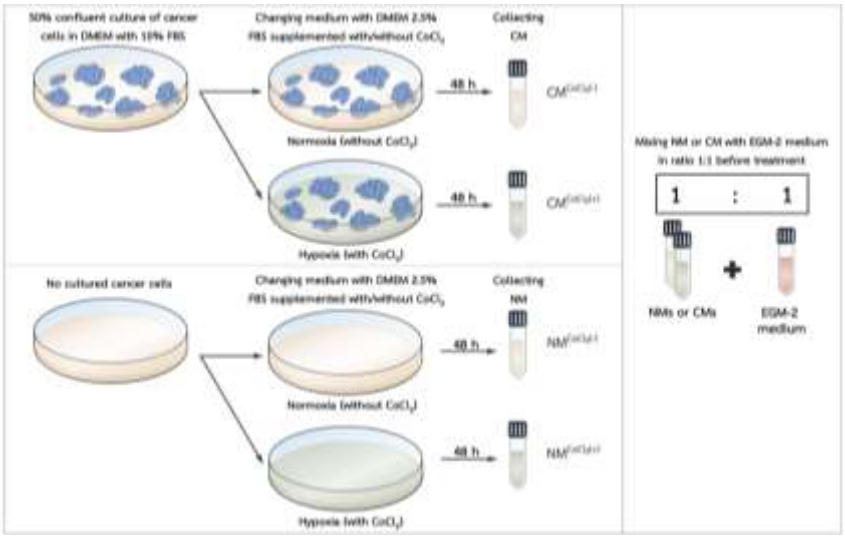


Figure 1 Graphical experiment of preparing conditioned medium under normoxia and hypoxia.

Cell proliferation assay

HUVEC cells were seeded into 24-well plates at 1 x 10^4 cells/well in 500 μl EGM-2 medium for 8 hours to allow cell attachment. Then, the culture medium was replaced with a mixture of EGM-2 and control medium (NM) or PDAC-derived conditioned media (CMs) in a 1:1 ratio and was then incubated in an incubator at 37 °C under a 5% CO_2 for 24, 48, and 72 hours. After the incubation period, cell proliferation was assessed by using the MTT assay with 0.5 mg/ml MTT solution. Following incubation for 3 hours, the supernatant was removed, and the violet formazan crystal was dissolved with DMSO. The absorbance was measured at 540 nm by using a microplate reader (Thermo, Multiskan EX).

Cell viability assay

PANC-1 and MIA-PaCa-2 were seeded into 24-well plates at 1 x 10^4 cells/well. After seeding for 24 hours, cells were treated with different concentrations of cobalt chloride (0, 25, 50, 100, 200 μM) to induce hypoxia incubated in an incubator at 37 °C under a 5% CO_2. The 48-hour following treatment with $CoCl_2$, cell viability was detected with MTT assay, as described in the section of cell proliferation assay.

Wound healing assay

HUVEC cells were seeded into 24-well plates at 1 x 10^5 cells/well. The following day, cells were scratched by using a sterile P200 pipette tip. Then, the culture medium was replaced with a mixture of EGM-2 and control medium (NM) or different PDAC-derived conditioned media (CMs) in the ratio of 1:1. Image of the scratches was taken every three hours by using a microscope at 100x magnification. The percentage of relative wound recovery was determined by ImageJ software (NIH).

Matrigel-based tube formation assay

HUVEC cells at 2 x 10^4 cells/well were loaded on top of a 50 μl of Matrigel Matrix Basement Membrane (Corning, MA, USA) in 96-well plates and was then cultured for 1 hour allowing cells to invade into the gel. The supernatant was replaced with a mixture of EGM-2 and control medium (NM) or different PDAC-derived conditioned media (CMs) in the ratio of 1:1. The tube formation of HUVEC was imaged under a microscope at 0, 2, 4, 6, 8, 10, and 24 h after conditioned-medium treatment. The photographs were evaluated through total tubes, total branching point, and total loops, using the Wimasis AI software.

Western blot analysis

The protein lysates were extracted using TENT lysis buffer supplemented with protease inhibitors. Then, 50 μg of each sample protein was fractioned by 8% SDS-PAGE followed by transfer to Nitrocellulose Membrane 0.45 μm (Bio-Rad, Germany). Following blocking in 5% BSA at room temperature for 1 hour, membrane was incubated overnight at 4 °C with the primary antibody, anti-β-actin or anti-HIF-1α mouse antibody (1:1000, sc-13515, Santa Cruz Biotechnology) and then incubated with secondary antibody, anti-mouse IgG HRP-linked antibody (1:1000, Cell signaling) and anti-rabbit IgG HRP-linked antibody (1:1000, Cell signaling) at room temperature for 1 hour. The expression of the protein was detected by chemiluminescence with an ECL reagent.

Statistical analysis

The quantitative data were shown as the mean ± standard deviation (SD). The statistical significance was calculated using the two-tailed unpaired Student's t-test and one-way ANOVA. $P < 0.05$ was considered to be a statistically significant difference.

Results

Condition medium derived from MIA-PaCa-2 cultured under normoxia promoted cell proliferation of HUVECs, whereas PANC-1 conditioned medium had no considerable effect.

According to hypovascularity and impaired-vascular integrity of PDAC, we hypothesized that PDAC cell lines, PANC-1 and MIA-PaCa-2 might have inhibitory effect on endothelial cell growth through paracrine signaling. Hence, we observed the effect of PDAC-derived conditioned medium on endothelial cell proliferation by performing an MTT assay. HUVECs were cultured in the presence of the conditioned medium derived from PANC-1 and MIA-PaCa-2 for 24, 48, and 72 hours. Unexpectedly, the result illustrated that there was no difference in HUVEC proliferation between the control medium (NM) and conditioned medium (CM) derived from PANC-1. While MIA-PaCa-2 CM showed significantly induced the HUVECs proliferation at 24, 48, and 72 hour-post treatments compared to control medium (NM) (Figure 2). This could be suggested that PDAC-derived conditioned medium might not provide an inhibitory effect on HUVEC cell growth. However, we found that conditioned media from MIA-PaCa-2 promoted HUVECs proliferation.

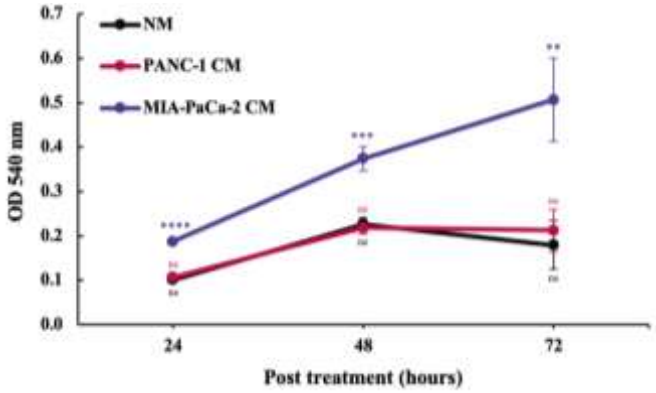
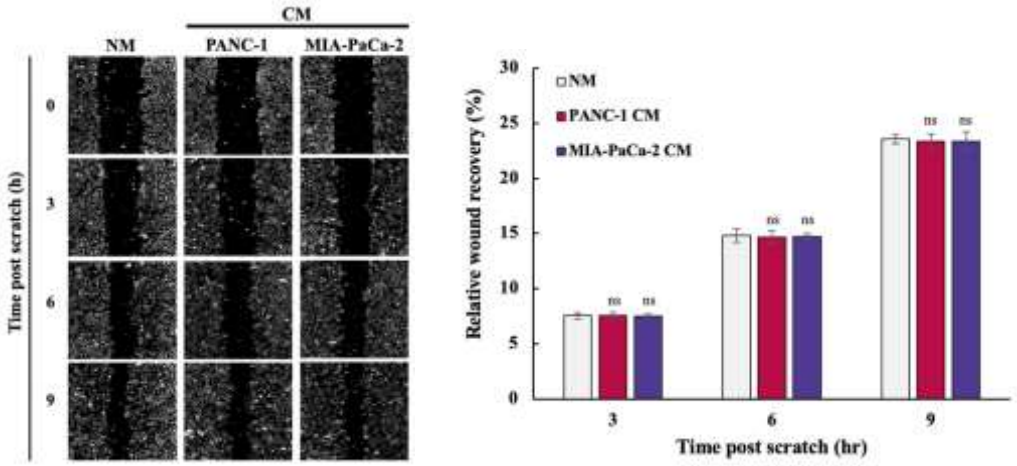


Figure 2. HUVEC cell proliferation after conditioned medium (CM) treatment for 24, 48, and 72 hours. Control medium (NM), medium without cultured PDAC cells (Black), PANC-1 CM (red), and MIA-PaCa-2 CM (violet). The cell growth of HUVEC under PANC-1 CM did not significant to NM, whereas MIA-PaCa-2 CM obviously stimulated HUVEC proliferation in three time periods. The statistical analysis was tested by a Two-tailed student's t-test. Error bars represented the mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ indicated statistical significance compared to control medium (NM), indicating the 1:1 of EGM-2 medium and DMEM (2.5% FBS) without cultured tumor cells. ns, not significant.

PDAC-derived conditioned medium exhibited no influence on HUVEC cell migration

Not only proliferation and differentiation but migration is also one of the crucial features in the process of constructing a vascular network (Ausprunk et al., 1977), we also examined the effect of PDAC-derived conditioned medium (CM) on the migration of HUVEC by using wound-healing assay. It was shown that the treatment of PANC-1 and MIA-PaCa-2 CM did not show any effect on HUVEC migration as compared to the control medium (NM) (Figure 3a and 3b). This finding could be indicated that PDAC-derived conditioned medium might be likely inadequate impact on the migration capability of HUVECs.



(a) (b)

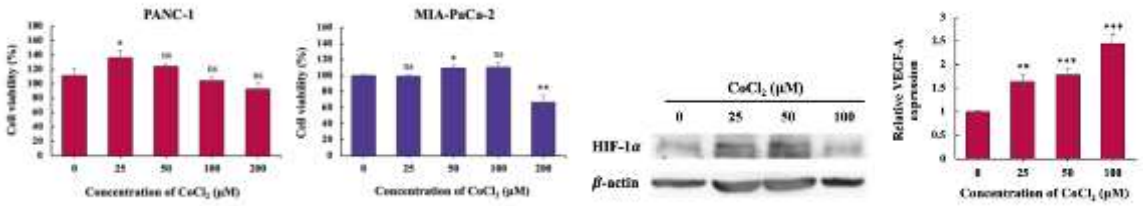
Figure 3. PDAC-derived conditioned medium exhibited no considerable impact on HUVEC migration. Control medium (NM, Gray), PANC-1 CM (blue), and MIA-PaCa-2 CM (violet) **(a)** Relative micrographs of the wound recovery area of HUVEC under different CM at various time points. **(b)** The quantification of percent relative wound recovery of HUVECs. The statistical analysis was tested by Two-tailed student's t test. Error bars represented mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 indicated statistical significance compared to control medium (NM), indicating the 1:1 of EGM-2 medium and DMEM (2.5% FBS) without cultured tumor cells. ns, not significant.

Effect of $CoCl_2$ on PDAC cell viability and hypoxia induction

We further validated the tube network formation capability of HUVECs culture in CM derived from PDAC cells cultured under normoxic and hypoxic conditions. To generate the hypoxic condition in the tumor cell, Cobalt chloride ($CoCl_2$) was used to provide a hypoxia environment, which mimics the usual environment of cancer (Muñoz-Sánchez et al., 2019; Chen et al., 2018). We investigated the effect of $CoCl_2$ at the various concentration on PDAC cell viability. PANC-1 and MIA-PaCa-2 cells were exposed to different concentrations of $CoCl_2$ (0, 25, 50, 100, 200 μM) for 48 hours to observe cytotoxicity by

performing an MTT assay. Data demonstrated that $CoCl_2$ exposure showed an inducible effect on cancer cell viability but differ maximum concentrations in each PDAC cell line. PANC-1 strongly elevated cell proliferation when treated with 25 μM of $CoCl_2$, whereas MIA-PaCa-2 cell growth was significantly highest increased at 50 μM $CoCl_2$. Once the concentration reached 200 μM, it markedly decreased the cell viability of MIA-PaCa-2 as compared to untreated cells (0 μM of $CoCl_2$). This can be suggested that $CoCl_2$ at a concentration between 25 to 100 μM did not create a toxicity effect on the cell viability of PDAC cell lines (Figure 4a, and 4b). Therefore, concentrations of $CoCl_2$ at 25, 50, and 100 μM were selected for further study to test their ability of hypoxia-induced contributor on PDAC cell lines.

To elucidate the $CoCl_2$-induced hypoxia stimulation in PANC-1 and MIA-PaCa-2, HIF-1α protein expression was detected by western blot where β-actin served as an internal control. It was observed that $CoCl_2$-increased HIF-1α protein expression has been found in PANC-1 after treatment of $CoCl_2$ at 25 μM and 50 μM as compared to control (0 μM of $CoCl_2$) (Figure 4c), similar result was also found in MIA-PaCa-2 (the data was not shown). As VEGF-A is the key downstream molecule of hypoxia response, the level of VEGF-A was then investigated through qRT-PCR to confirm the hypoxia induction during $CoCl_2$-induced hypoxia treatment (Rana et al., 2019). Data indicated that expression of VEGF-A in PANC-1 treated with 25, and 50 μM of $CoCl_2$ was strongly significant increase as compared to control (Figure 4d) and MIA-PaCa-2 cells were shown the result likewise (data was not shown), supporting the hypoxia stimulation by $CoCl_2$ addition. Together, these data suggested that 25 μM of $CoCl_2$ was the most suitable concentration triggering hypoxia condition in both PDAC cell lines. Therefore, we decided to use the concentration of $CoCl_2$ at 25 μM in the next experiment.



(a) (b) (c) (d)

Figure 4. Effect of $CoCl_2$-induced hypoxia on PDAC cell viability. **(a-b)** PDAC cell viability after addition of various $CoCl_2$ concentrations for 48 hours was analyzed by using MTT assay; PANC-1 (a, red), and MIA-PaCa-2 (b, violet). **(c)** The expression level of HIF-1α protein and β-actin in PANC-1cell treated with different concentrations of $CoCl_2$ for 48 hours were determined by western blot analysis. **(d)** The VEGF-A expression in mRNA level in $CoCl_2$-treated PANC-1 cells was evaluated by qPCR compared to untreated PANC-1 cell. The statistical analysis was tested by a Two-tailed student's t-test. Error bars represented the mean ± SD of three independent experiments. ns, not significant. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 indicated statistical significance compared to control group (0 μM of $CoCl_2$ treatment).

Conditioned medium under both normoxia and $CoCl_2$-induced hypoxia did not affect the tube network formation capability of HUVECs

The new blood vessel formation in most tumor tissues was largely established through the well-known process called angiogenesis. Newly formed vascular-like structure lined with endothelial cells was organized by several sequences of cellular events, including endothelial cells (ECs) proliferation, migration toward the angiogenic stimulus, subsequently, ECs reassembly to form new vascular lumen, followed by pericyte recruitment for vessel stabilization and finally initiation of blood flow (Stryker et al., 2019).

Due to poor vascularized PDAC tumor, it was conjectured that PDAC-derived conditioned media might provide a negative effect on the tube-forming capacity of HUVEC. Therefore, we performed the Matrigel-based tube formation assay. According to Matrigel matrix containing a solubilized basement membrane and extracellular matrix protein as collagen IV, laminin, and other growth factors, it has been considered as a powerful utility for evaluation of angiogenic activity owning to its closely mimic *in vivo* trusted environment. To observe the effect of cancer conditioned medium on angiogenic properties, HUVECs were grown in Matrigel culture with normal medium (NM) or various cancer conditioned medium (CMs) harvested under normoxia and hypoxia. Next, the capillary-like structure formation was imaged under the microscope at various time points. Then, total tubes, which was a parameter representing the tube-forming capacity, were analyzed by using Wimasis AI software.

For the results of the tube-forming capacity of HUVECs determined under normoxic condition (without $CoCl_2$ addition), we found that the number of total tubes under culture HUVECs in both PDAC-derived conditioned medium (PANC-1, and MIA-PaCa-2 $CM^{CoCl2(-)}$, blue bar) showed not significantly different with culture in normal medium ($NM^{CoCl2(-)}$, gray bar) (Figure 5). Additionally, the total branching point and total loops were also evaluated which also showed a similar result (data not shown). It could be indicated that CMs derived from PDAC cell lines, PANC-1 and MIA-PaCa-2, cultured under normoxia might have no influence on the tube network formation of HUVECs.

For further findings in hypoxic conditions, the culture of PANC-1 and MIA-PaCa-2, had been stimulated hypoxic environment by $CoCl_2$ (25 μM) addition. Following hypoxic conditions, the CMs were collected to culture HUVECs on Matrigel. To determine whether the $CoCl_2$, a hypoxia-mimetic agent, did not directly act on tube-forming capacity, we first compared two conditions of normal medium with $CoCl_2$ ($NM^{CoCl2(+)}$, violet bar) and without $CoCl_2$ ($NM^{CoCl2(-)}$, gray bar). We found that $CoCl_2$, a hypoxia-mimetic agent, did not do any effect on the tube-like structure formation of HUVECs, ensuring the $CoCl_2$-affected tube forming independence. However, the CMs collected from PDAC cells grown under hypoxic condition ($CM^{CoCl2(+)}$, red) did not show any significant changes in tubular network formation when compared to normal medium under hypoxia ($NM^{CoCl2(+)}$, violet) (Figure 5) which provided similar observation in normoxia. Together with normoxic results, we proposed that CMs derived from cancer cells cultured under both normoxic and hypoxic conditions showed no inhibitory effect on the vascular-like formation of HUVECs.

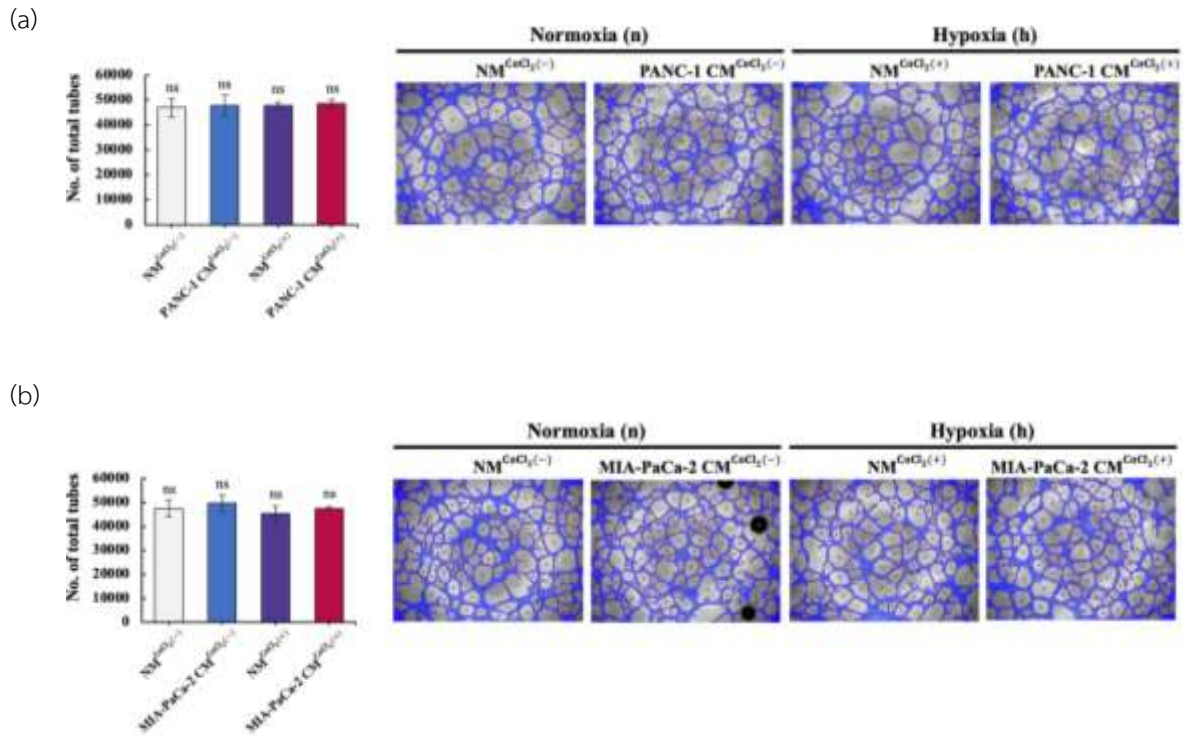


Figure 5. Independent effect of PDAC CMs collected under both normoxia and $CoCl_2$-induced hypoxia on tube formation capability of HUVECs. Quantification of total tubes, analyzed by using Wimasis AI software. Representative image of tube network formation of HUVEC cultured upon NM and CMs at time point 6-hour post treatment. **(a)** PANC-1 CM. (**b**) MIA-PaCa-2 CM. The statistical analysis was tested one-way ANOVA. Error bars represented mean ± SD of three independent experiments. ns, not significant. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 indicated statistical significance compared to $NM^{CoCl2(-)}$, normal medium without $CoCl_2$ addition).

Discussion

PDAC has poor vascularization, in contrast to many solid tumors that commonly exhibit rich vasculature (Longo et al., 2016). The hypovascularity in PDAC could be caused by its high intense stromal extracellular matrix deposition in the tumor microenvironment. It has been implicated in the raised interstitial fluid pressure (IFP) and solid stress (SS) allowing vascular compression with dismal drug delivery (Provenzano et al., 2012; Chauhan et al., 2013). Furthermore, the study of the 3D organotypic model of PDAC also reported the disappearance of endothelial cells due to the invasion of PDAC cells into vascular endothelial cells, which was considered as a process of endothelial ablation (Nguyen et al., 2019). In our study, we investigated the role of PDAC cells on endothelial ablation through paracrine signaling by using PDAC-derived conditioned medium. However, our findings have shown that both PDAC

CMs, PANC-1 and MIA-PaCa-2 CM, did not show inhibitory regulation on HUVEC behavior. Conversely, MIA-PaCa-2 CM promoted HUVEC cell proliferation but not found this evident in PANC-1 CM. This result might be due to the fact that MIA-PaCa-2 expressed a higher level of some secreted factor than PANC-1, such as interleukin-6 (IL-6), which was found in their culture media (Feurino et al., 2007). Paracrine IL-6 has been reported that promoted upregulated angiogenesis and metastasis, particularly through the upregulation of vascular endothelial growth factor (VEGF), a key mediator for endothelial cell proliferation (Masui et al., 2002; Ellis et al., 2008). However, we observed that PDAC-derived conditioned medium did not show any effect on HUVEC migration as compared to control medium (NM) as well as no considerable impact on the vascular-like structure formation of HUVECs in both under normoxia and $CoCl_2$-induced hypoxia condition. Perhaps, PDAC-derived conditioned media might be a probably inadequate impact on HUVEC behaviors. Although the result of paracrine signaling under our condition did not provide an inhibitory impact on HUVEC function, dissecting the mechanism of endothelial ablation through cell direct contact is interesting for further study. A better understanding of the role of PDAC cells on endothelial ablation may lead to the development of new PDAC therapy.

Conclusions

In this study, PANC-1 CM and MIA-PaCa-2 CM, collected under normoxic and $CoCl_2$-induced hypoxic PDAC culture, which were prepared under our conditional methodology, did not provide an inhibitory effect on cell proliferation, migration, and tube network formation capacities of HUVECs. Nevertheless, MIA-PaCa-2 CM could promote HUVEC proliferation as compared to control condition media. This could suggest that the process of endothelial ablation, one of the causes of hypovascular PDAC, might not be regulated through paracrine signaling. Therefore, further investigations into the cause of endothelial ablation in PDAC progression through other communication, such as cell direct contact was warranted.

Acknowledgments

This study was financially supported by National Research Council of Thailand (NRCT), and Biochemistry Department, Faculty of Science, Mahidol University, Thailand.

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