Original Article

Protumorogenic Potential of Pancreatic Adenocarcinoma-Derived Extracellular Vesicles

(tumour-derived extracellular vesicles / angiogenesis / SCID mice)

M. BAJ-KRZYWORZEKA[1], B. MYTAR[1], K. WĘGLARCZYK[1], R. SZATANEK[1], J. KIJOWSKI[2], M. SIEDLAR[1]

[1]Department of Clinical Immunology, [2]Department of Transplantology, Institute of Paediatrics, Jagiellonian University Medical College, Cracow, Poland

Abstract. Cancer development is a highly complicated process in which tumour growth depends on the development of its vascularization system. To support their own growth, tumour cells significantly modify their microenvironment. One of such modifications inflicted by tumours is stimulation of endothelial cell migration and proliferation. There is accumulating evidence that extracellular vesicles (EVs) secreted by tumour cells (tumour-derived EVs, TEVs) may be regarded as "messengers" with the potential for affecting the biological activities of target cells. Interaction of TEVs with different cell types occurs in an auto- and paracrine manner and may lead to changes in the function of the latter, e.g., promoting motility, proliferation, etc. This study analysed the proangiogenic activity of EVs derived from human pancreatic adenocarcinoma cell line (HPC-4, TEV$_{HPC}$) *in vitro* and their effect *in vivo* on Matrigel matrix vascularization in severe combined immunodeficient (SCID) mice. TEV$_{HPC}$ enhanced prolifera-tion of HPC-4 cells and induced their motility. Moreover, TEV$_{HPC}$ stimulated human umbilical vein endothelial cell (HUVEC) proliferation and migration *in vitro*. Additionally, TEV$_{HPC}$ influenced secretion of proangiogenic factors (IL-8, VEGF) by HUVEC cells and supported Matrigel matrix hae-moglobinization *in vivo*. These data show that TEVs may support tumour propagation in an autocrine manner and may support vascularization of the tumour. The presented data are in line with the theory that tumour cells themselves are able to modulate the microenvironment via TEVs to maximize their growth potential.

Introduction

Extracellular vesicles (EVs) are membrane fragments released by different cell types including platelets, endothelial, or tumour cells. EVs are divided into small (previously called exosomes) and medium/large size vesicles (previously known as microvesicles) released during the life span of the cells (Théry et al., 2018). It has been reported that EVs of different origin may impact progression of tumours (Mezouar et al., 2014; Ren et al., 2016; Whiteside, 2018). Among them, platelet-derived EVs (PEVs) play an important role in tumour progression/metastasis and angiogenesis induction in experimental lung tumours (*in vitro* and *in vivo* models). PEVs stimulated mRNA expression of proangiogenic factors such as metalloproteinase 9 (MMP-9), vascular endothelial growth factor (VEGF), interleukin 8 (IL-8) and hepatocyte growth factor (HGF) in the A549 cancer cell line (Janowska-Wieczorek et al., 2005). Moreover, PEVs promoted proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVEC) (Kim et al., 2004; Sun et al., 2019).

It was also reported that endothelial derived EVs and tumour-derived vesicles (TEVs) may promote basement membrane invasion by carrying metalloproteinases (Dolo et al., 1998; Taraboletti et al., 2002). TEVs are constitutively released by tumour cells and serve as a

vehicle for several tumour determinants such as CD44v, carcinoembryonic antigen (CEA), and extracellular matrix metalloproteinase inducer, CD147 (EMMPRIN) (Baj-Krzyworzeka et al., 2006). They can also transport mRNA for, e.g., proangiogenic IL-8 or VEGF (Baj-Krzyworzeka et al., 2006). It was shown that TEVs derived from lung cancer activate and chemoattract stromal fibroblasts and endothelial cells (Wysoczynski et al., 2009). Others reported that TEVs may exert proangiogenic effects (promoting cell migration, invasion and tube formation) via sphingomyelin-induced neovascularization (Kim et al., 2002).

TEVs present in the tumour bed may interact with tumour and endothelial cells as well as with tumour-infiltrating leukocytes (Maia et al., 2018). Here, we focus on TEV interactions with tumour and endothelial cells as a model of interactions in the tumour bed. The present study determined the proangiogenic effect of TEVs derived from a human pancreatic adenocarcinoma cell line (HPC-4) and presented evidence to support the theory about the autocrine loop in tumour growth.

Material and Methods

TEV isolation

TEVs were obtained from the HPC-4 (human pancreatic adenocarcinoma, TEV_{HPC}) cell line as previously described (Baj-Krzyworzeka et al., 2006). Cells were cultured by bi-weekly passages in RPMI 1640 (Sigma, St. Louis, MO) with 5% foetal bovine serum (FBS, PAA Laboratories, Pasching, Germany) centrifuged at 50,000 $\times g$ for 1 h at 4 °C. The cell line was regularly tested for *Mycoplasma sp.* contamination by using a PCR-ELISA kit according to the manufacturer's procedure (Roche, Mannheim, Germany). Supernatants from well-grown cell cultures were collected (app. 400 ml), centrifuged at 2,000 $\times g$ for 20 min to remove the cell debris and centrifuged again at 50,000 $\times g$ (RC28S, Sorvall, Newton, CT) for 1 h at 4 °C. Pellets were washed with serum-free RPMI 1640 medium and then spun down at 50,000 $\times g$. This step was repeated for the total of four times, after which the pellets were finally resuspended in serum-free medium (100 µl). Quantification of TEV protein content was evaluated by the Bradford method (BioRad, Hercules, CA). TEVs were tested for endotoxin (LPS) contamination by the Limulus test according to the manufacturer's instructions (Charles River Laboratories, Inc., Wilmington, MA) and stored at –20 °C.

Isolation of HUVEC cells

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as previously described (Gimbrone et al., 1974). Cells were suspended in medium X Vivo10 (BioWhittaker, Cambrex, Baltimore, MD) supplemented with 10% FBS, 1 mg/ml bovine brain extract, 1 ng/ml human epithelial growth factor, 1 mg/ml hydrocortisone, 10 U/ml heparin (BioWhittaker) and seeded in plastic Petri dishes (Costar Corning, Cambridge, MA) covered with 0.1% gelatin (BioRad, Hercules, CA).

Effect of TEVs on the proliferation of HUVEC and HPC-4 cells in vitro

Proliferation of HUVEC and HPC-4 cells was determined by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay performed according to the manufacturer's description (MTS, Promega, Madison, WI). Briefly, cells were suspended in serum-free RPMI 1640 or endothelial cell medium (EGM, BioWhittaker) at the concentration of 1×10^5 cells/ml and cultured in 96-well microplates (BD Falcon, Bedford, MA) with or without TEV_{HPC} (30 µg/ml). After 48 h, 20 µl of CellTiter 96 Aqueous One Solution reagent was added, after which the plates were incubated for 2–4 h at 37 °C, 5% CO_2 and then absorbance at 490 nm was measured in the EL_x800NB microplate reader (Bio-tek Instruments, Inc., Winooski, VT).

Chemotactic assay

The chemotactic assay was performed using BD Falcon cell culture inserts with 8-µm size pore filter (translucent polyester (PET) membrane) placed over 24-well plates (BD Falcon). HUVEC or HPC-4 cells were resuspended in RPMI 1640 medium supplemented with 0.5% bovine serum albumin (BSA, Sigma) at the concentration of 1×10^5 cells/ml. Pre-warmed serum-free medium alone or supplemented with TEV_{HPC} (30 µg/ml) was added to the lower chambers of the transwell plate (in triplicates). Cell suspension (HUVEC or HPC-4, respectively) was added to the upper chambers and incubated overnight at 37 °C, 5% CO_2, 95 % humidity. Next, cells from the upper chambers were carefully removed and those present on the lower (reverse) part of the membrane were stained with haematoxylin/eosin (Merck, Darmstadt, Germany) for 10 min. The cells present in five randomly selected fields at 400× magnification were counted under a light microscope (BX 40, Olympus, Tokyo, Japan). Mean values calculated as the number of cells crossing the membrane were presented.

Determination of VEGF and IL-8 mRNA expression in HUVEC and HPC-4 cells after TEV_{HPC} stimulation

Total RNA was extracted from control or stimulated with TEV_{HPC} (30 µg/ml, for 2 h at 37 °C) HUVEC or HPC-4 cells by a single-step isolation method using TRI-ZOL reagent (Gibco-BRL, Grand Island, NY) according to the manufacturer's protocol. The first strand cDNA was obtained from the total RNA samples (2 µg) with Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Sigma) and oligo-dT (Gibco) primer as specified by the manufacturer's protocol. The quantitative polymerase chain reactions (PCR) for vascular endothelial growth factor (VEGF) and IL-8 were performed using the LightCycler system (Roche Diag-

nostics, Mannheim, Germany) as described before (Baj-Krzyworzeka et al., 2006). β-Actin was used as an internal control of each LightCycler PCR run to control equivalency of cDNA in each sample. The fluorescent signals generated during the informative log-linear phase were used to calculate the relative amount of mRNA using the $2^{-\Delta\Delta Ct}$ method. Melting curve analysis was performed to verify the specificity of the amplified products.

Evaluation of VEGF and IL-8 secretion by HUVEC and HPC-4 cells after TEV$_{HPC}$ stimulation

HUVEC or HPC-4 cells were cultured for 18 h in 96-well microplates (1×10^5 cells/well) at 37 °C, 5% CO_2 in humidified atmosphere in the presence of TEV$_{HPC}$ (30 µg/ml). Supernatants were collected and the concentration of VEGF and IL-8 was measured by the Cytokine Bead Array (CBA) method using the Flex Set system (BD Biosciences, San Diego, CA) and FACSCanto cytometer (BD Bioscience, Immunocytometry Systems, San Jose, CA). The specified beads were discriminated in the FL-4 and FL-5 channels, while the concentration of individual chemokines was determined by the intensity of FL-2 fluorescence. The amount of chemokines was computed using the respective standard reference curve and FCAP Array software (BD Biosciences). The detection level was 10 pg/ml.

Matrigel implants in SCID mice

The angiogenesis assay was carried out by injecting 6–8-week old SCID mice (CB-17/IcrCrl) in the abdominal midline with 100 µl of TEV$_{HPC}$ (30 µg/ml) or HPC-4 cells (1×10^6) mixed with 400 µl of precooled Matrigel matrix (BD Falcon, Bedford, MA). HPC-4 cells were also preincubated *in vitro* with TEV$_{HPC}$ for 2 h, mixed with Matrigel matrix and injected into SCID mice. Five mice per group were used in each experiment. Matrigel alone was used as a background control. After six days, mice were euthanized, Matrigel implants were harvested and suspended in 500 µl of the BD Recovery Solution

(BD Falcon) to enable recovery of haemoglobinized cells. For the next seven days, Matrigel plugs were kept in a refrigerator and vortexed every day. After that, the Matrigel plugs were spun down and the supernatants were mixed with the Drabkin's reagent (Sigma) (Passaniti et al., 1992; Janowska-Wieczorek et al., 2002; Gong et al., 2017). One-hundred µl of this mixture was placed into a 96-well plate. The haemoglobin content was measured by the colorimetric assay (absorbance measurement at 540 nm in a U-1800 spectrophotometer (Hitachi, Tokyo, Japan). Animal experiments were approved by the Local Ethics Committee (Krakow, Poland) by decision number 5/OP/2005.

Statistical analysis

Statistical analysis was performed by the paired Student's *t*-test in all experiments except for mouse experiments where nonparametric Mann-Whitney and one way analysis of variance (ANOVA) tests were used. Differences were considered significant at P values < 0.05.

Results

TEV$_{HPC}$ enhance proliferation of HPC-4 and HUVEC cells

TEV$_{HPC}$ obtained from the HPC-4 cell line significantly stimulated proliferation of HPC-4 and HUVEC cells *in vitro* (Fig. 1A and 1B, P < 0.05). TEV$_{HPC}$ in the dose of 30 µg/ml induced migration of HPC-4 and HUVEC cells (Fig. 2A and 2B); however, the increase in the number of migrating cells was not statistically significant.

Effect of TEV$_{HPC}$ on mRNA expression and production of proangiogenic factors by HUVEC and HPC-4 cells

Experiments undertaken to determine the impact of TEV$_{HPC}$ on the secretion of IL-8 and VEGF by HUVEC and HPC-4 cells showed an increase in the secretion of
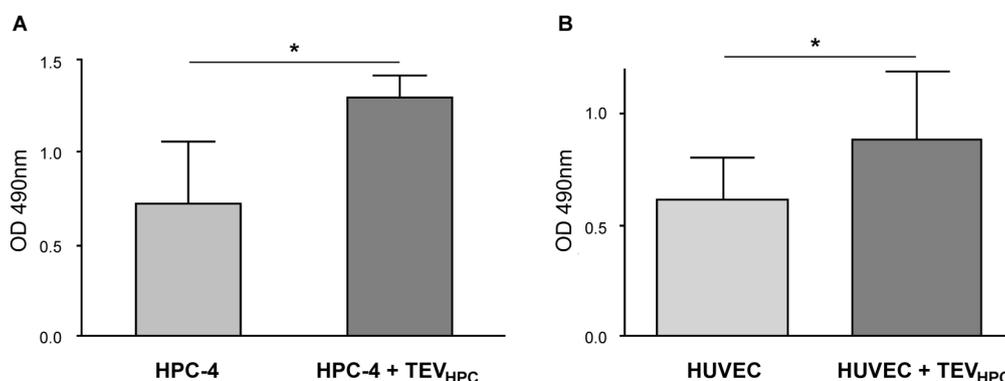
Fig. 1. Proliferation of HPC-4 (**A**) and HUVEC cells (**B**) in the presence of TEV$_{HPC}$. Proliferation was determined by MTS in 48 h culture alone or with TEV$_{HPC}$. Data represent mean ± SD of six independent experiments. *P < 0.05
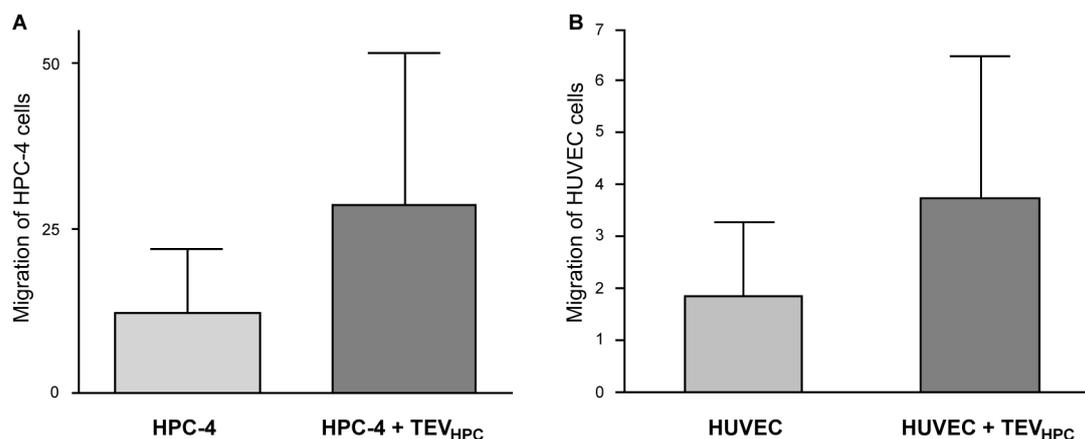
Fig. 2. Migration of HPC-4 (**A**) and HUVEC (**B**) cells to TEV$_{HPC}$ (30 μg/ml). Migration was analysed after an overnight incubation by staining the membrane of the inserts with haematoxylin/eosin. Results are presented as the number of migrating cells found in five fields at 400 × magnification under the light microscope.
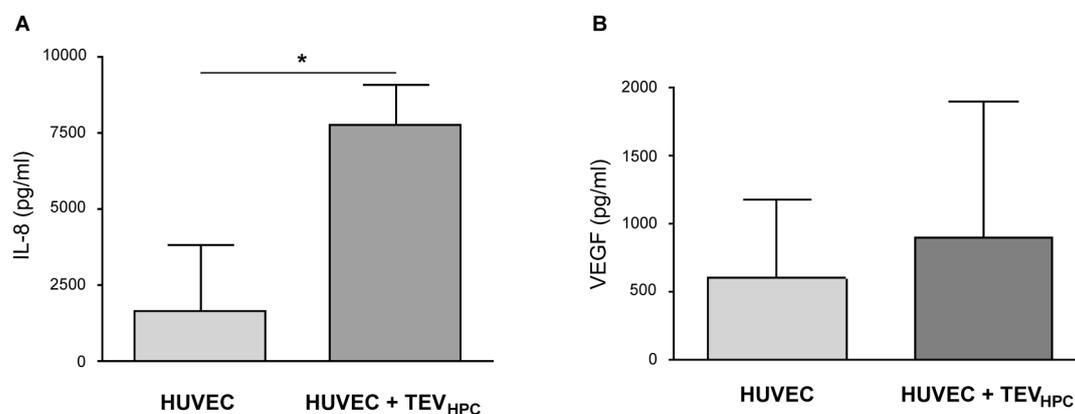
Fig. 3. Secretion of IL-8 (**A**) and VEGF (**B**) by HUVEC stimulated with TEV$_{HPC}$ for 18 h. Cells were incubated for 18 h with TEV$_{HPC}$ (30 μg/ml) and IL-8 and VEGF levels were determined in culture supernatants by the FlexSet method. Data represent mean ± SD of six independent experiments. *$P < 0.05$

IL-8 by HUVEC (Fig. 3A, $P < 0.05$) and HPC-4 (not significant, not shown) cells. These results correlated with IL-8 mRNA expression in HUVEC and HPC-4 cells stimulated with TEV$_{HPC}$ (5- and 3-fold increase, respectively). VEGF production was observed when HUVEC cells were stimulated with TEV$_{HPC}$ (Fig. 3B, not significant). Untreated HPC-4 cancer cells secreted quite a high level of VEGF (620 pg/ml), which was not affected by the TEV$_{HPC}$ stimulation (not shown). The protein secretion level results were in concordance with the VEGF mRNA expression, where exposure to TEV$_{HPC}$ caused a 10-fold increase in HUVEC but not in HPC-4 cells.

Induction of (neo)angiogenesis in vivo by TEVs

Since TEV$_{HPC}$ stimulated proliferation of HUVEC cells *in vitro*, we asked ourselves whether they were capable of inducing angiogenesis *in vivo* as assessed by vascularization of Matrigel matrix implants in SCID mice. TEV$_{HPC}$ alone induced vascularization of Matrigel matrix (Fig. 4). Cancer cells (HPC-4) also induced Matrigel matrix vascularization, which was significantly increased after their exposure to TEV$_{HPC}$ (Fig. 4, $P < 0.003$, 1-way ANOVA $P < 0.001$).

Discussion

The present study demonstrates that TEVs may be involved in angiogenesis, as they seem to exert an angiogenic effect on endothelial and autologous tumour cells both *in vitro* and *in vivo*. It was shown that TEVs induced HUVEC cell proliferation and migration. These observations are in concordance with data of others, which also suggested that TEVs (of different origin such as prostate, ovarian, glioblastoma cell lines) stimulated migration (Kim et al., 2002) and proliferation (Millimaggi
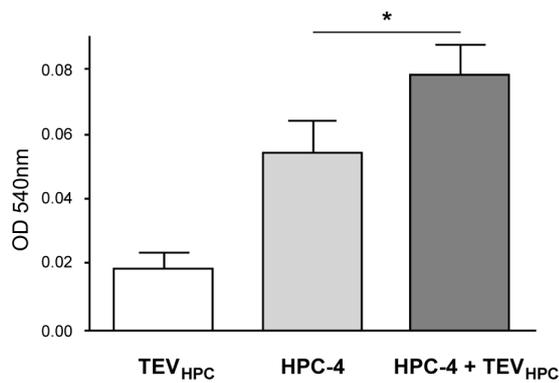
Fig. 4. Vascularization of Matrigel implanted in SCID mice. Left bar represents administration of Matrigel with TEV$_{HPC}$ (30 µg/ml) alone, middle bar with HPC-4 cells (1 × 10^6/mouse) alone, right bar with HPC-4 cells preincubated with TEV$_{HPC}$ (30 µg/ml). Data represent mean ± SD of three independent experiments. *P < 0.003, 1-way ANOVA P < 0.001

et al., 2007; Monteforte et al., 2017) of endothelial cells. It was shown in the current study that TEV$_{HPC}$ increased secretion of proangiogenic IL-8 by HUVEC cells. The biological activity of TEVs was confirmed by *in vivo* experiments where TEV$_{HPC}$ implanted into SCID mice alone enhanced Matrigel matrix vascularization. Tumour-induced angiogenesis was also enhanced by autologous TEVs. HPC-4 cells induced strong vascularization of Matrigel matrix, which was further increased by preincubation of tumour cells with TEV$_{HPC}$. The mechanism of the observed synergism is not clear; however, the role of biological factors (e.g., IL-8, TGF, amphiregulin, miRNAs) transferred by TEVs as well as proliferation of tumour cells induced by TEVs may play a role (Ratajczak et al., 2006; Taraboletti et al., 2006; Baj-Krzyworzeka et al., 2011; Higginbotham et al., 2011; Shang et al., 2020). Our results obtained in the simple chamber chemotaxis assay indicated a slight chemoattractant activity of TEVs. This is in concurrence with the study by Szatanek et al. (2020), who have shown, by using a more sophisticated cell movement tracking analysis, that tumour cells migrate towards their increasing auto-TEV gradient *in vitro* (the tumour cell movement was more orderly and faster in the presence of TEVs).

In the current study, it was presented that TEVs significantly enhanced proliferation of a pancreatic cancer cell line (in an autocrine manner). Contrary to our results, Ristorcelli et al. (2008) reported that nanoparticles derived from pancreatic tumour cell lines induced apoptosis in tumour cells. These authors defined nanoparticles as structures in the size range of 20–75 nm. Currently, such small structures are called exomers or small exosomes (Zhang et al., 2018). Exomers are characterized by distinct proteomic content and cellular

functions other than exosome subpopulations, which may cause the observed differences. Ristorcelli et al. (2009) reported that poorly differentiated carcinoma cell line MiaPaCa-2 was much less sensitive to nanoparticle-induced apoptosis than the differentiated cell lines. This correlates with our observation, as HPC-4 cells strongly proliferate and form tumours in SCID mice composed of undifferentiated cells (Siedlar et al., 1995).

Ristorcelli et al. (2009) concluded that nanoparticles derived from tumour cells exert pleiotropic effects on cells, as they may activate the phosphatidylinositol 3-kinase/ protein kinase B (PI3K/Akt) survival pathway or induce phosphatase and tensin homolog deleted on chromosome ten (PTEN) and glycogen synthase kinase 3 (GSK-3) activities leading cells towards apoptosis. We fully agree with such statements, as an autocrine loop in tumour growth is still unclear and further studies focusing on the role of TEVs in this process are necessary (Szatanek et al., 2020).

To summarize, the data from the current study show that TEVs induced proliferation of endothelial cells and tumour cells (in an autocrine manner). TEVs enhanced production (protein and mRNA) of proangiogenic factors such as IL-8 and VEGF by HUVEC. The observed impact of TEVs on autologous tumour cells and endothelial cells may facilitate tumour vascularization.

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