EXTRACELLULAR VESICLES FROM ORAL SQUAMOUS CARCINOMA CELLS
DISPLAY PRO- AND ANTIANGIOGENIC PROPERTIES

Running title: Angiogenic properties of OSCC-derived EVs

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ABSTRACT

BACKGROUND: A new intercellular communication mode established by neoplastic cells and tumor microenvironment components is based on extracellular vesicles (EVs). However, the biological effects of the EVs released by tumor cells on angiogenesis are not completely understood. Here we aimed to understand the biological effects of EVs isolated from two cell lines of oral squamous cell carcinoma (OSCC) (SCC15 and HSC3) on endothelial cell tubulogenesis.

METHODS: OSCC-derived EVs were isolated with a polymer-based precipitation method, quantified using nanoparticle tracking analysis and verified for EV markers by dot-blot. Functional assays were performed to assess the angiogenic potential of the OSCC-derived EVs.

RESULTS: The results showed that EVs derived from both cell lines displayed typical spherical-shaped morphology and expressed the EV markers CD63 and Annexin II. Although the average particle concentration and size were quite similar, SCC15-derived EVs promoted a pronounced tubular formation associated with significant migration and apoptosis rates of the endothelial cells, whereas EVs derived from HSC3 cells inhibited significantly endothelial cell tubulogenesis and proliferation.
CONCLUSION: The findings of this study reveal that EVs derived from different OSCC cell lines by a polymer-based precipitation method promote pro- or antiangiogenic effects.

Keywords: extracellular vesicles; angiogenesis; oral cancer.

INTRODUCTION
Tumor microenvironment consists of a specialized interface of non-cancerous cells from diversified lineages, such as fibroblasts, immune and inflammatory cells and endothelial cells, in addition to signaling factors and extracellular molecules, which may induce neoplastic progression (Azmi et al., 2013). All these elements favor tumor growth and invasion by promoting therapeutic resistance, defending the tumor from immune threats, and supplying nutrients, oxygen and pathways of metastatic spread through vascular neoformation (Folkman, 1995; Bergers and Benjamin, 2003; Ziyad and Iruela-Arispe, 2011; Azmi et al., 2013). In fact, solid tumors progression can only occur in the simultaneous presence of angiogenesis.

Over the last years, essential pleiotropic roles associated with tumor development and progression have been attributed to extracellular vesicles (EVs) (Tauro et al., 2013). Due to the heterogeneity of the secreted vesicle sets, the nomenclature of their members is still conflicting and the term EV encompasses all of them (Gould and Raposo, 2013). EVs are a group of secreted membrane-enclosed vesicles (50–1000 nm) produced by all cell types, under pathological and physiological processes. These vesicles are loaded with proteins, lipids, microRNAs, RNAs and DNAs and operate by releasing information in order to control the activity of recipient cells (D’Asti et al., 2012; Lemoinne et al., 2014; Lötvall et al., 2014; Minciacchi et al., 2015). EVs participate in tumor angiogenesis through the release of angiogenic proteins, such as transforming growth factor (TGF-β), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF),

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or specific microRNAs, which may directly affect angiogenesis (Carmeliet, 2005; Zhang et al., 2015).

It has been recently shown that vesicles obtained from gastric cancer cells increase angiogenesis and tumor burden in mice (Stec et al., 2015). EVs derived from colorectal carcinoma cells carrying miR-1246 induce angiogenesis by activating of SMAD cascade (Yamada et al., 2014), and EVs derived from renal carcinoma cells expressing CD105, a mesenchymal stem cell marker, not only collaborate with the activation of the angiogenic switch, but also guide tumor growth and metastatic spread (Grange et al., 2011). Sento et al. (2016) have revealed that oral squamous cell carcinoma (OSCC) cells-derived EVs promoted neoplastic growth and expansion, nonetheless, the events triggered by these vesicles on endothelial cells are still incompletely understood.

Angiogenesis, as well as inductors and inhibitors of this process, can be evaluated in vitro through a series of assays involving proliferation, migration, apoptosis and differentiation (Tahergorabi and Khazaei, 2012). Expansion and migration of precursor cells prior to differentiation are essential for vessel formation. The formation of capillary-like tubes in vitro on basement membrane matrix, also known as tube formation assay, represents the later stage of the angiogenic process in which the endothelial cells differentiate into tubes simulating in vivo situation. In this study we analyzed the biological roles of EVs derived from two OSCC lines, displaying low (SCC15) and high (HSC3) invasive behavior in the induction of angiogenesis in vitro.

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MATERIALS AND METHODS

Cell cultures

Human umbilical vein endothelial cells (HUVEC) and the OSCC cell line SCC15 were purchased from American Type Culture Collection (ATCC, USA), whereas HSC3 was obtained from Japan Health Sciences Foundation (Osaka, Japan). SCC15 and HUVEC cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s media and Ham’s F12 media (DMEM/F12; Invitrogen, USA) with 400 ng/ml hydrocortisone (Sigma-Aldrich, USA), 10% fetal bovine serum (FBS) and antibiotics. HSC3 media contained DMEM/F12 with 400 ng/ml hydrocortisone, 10% FBS, antibiotics and 50 μg/ml ascorbic acid. All cell lineages were maintained in a humidified incubator with 5% CO₂ at 37ºC.

EV isolation

OSCC cells (8,500 cells/cm²) were plated and cultured in a media without serum for 24 h. Conditioned-media was harvested, cleared by centrifugation and EVs were purified with ExoQuick™ TC solution (SBI, Mountain View, California) in accordance with the instructions issued by the manufacturer. Purified EVs were conditioned at -80ºC in phosphate-buffered saline (PBS) for nanoparticle tracking analysis (NTA) and transmission electron microscopy or were subjected to protein extraction in 30 µl of urea buffer (50 mM Tris-HCl pH 7.5, 8 M urea, 2 M thiourea, and 1 mM DTT) (Winck et al, 2015).

Nanoparticle tracking analysis (NTA)

The characterization system (number and size) of OSCC-derived EVs was determined with the NanoSight NS300 instrument (NanoSight, Amesbury, United Kingdom). After 1:100 dilution with PBS, the samples were introduced into the 532 nm laser chamber with a constant and controlled flow at room temperature. Video capture and data analysis were performed with the software NTA 2.3 Build 0013. The concentration and size of individual
particles were ascertained by their Brownian motion velocity. Particles with dimensions in the range of 100-1000 nm were characterized as EVs (Winck et al, 2015).

**Dot-blot assay**

The presence of the EV markers CD63 and Annexin II was verified by dot-blot. A 96-well microfiltration unit was prepared using a nitrocellulose membrane (GE Healthcare, WI, USA) with a pore size of 0.45 μm. A suspension of 20 µl/well of EVs (3x10^{10} particles) was added and vacuum dried. The membrane was then blocked by addition of 10% nonfat dry milk in a 0.1% Tween-20 PBS solution for 2 h at room temperature. The incubation with the mouse anti-CD63 antibody (1:500, clone TS63, Invitrogen, USA) or mouse anti-annexin II antibody (1:1000, clone 5/Annexin II aa. 123-328, BD Biosciences, USA) was followed by the incubation with secondary anti-mouse IgG fluorescein-conjugated antibody (1:1000, Abcam, USA). Proteins were captured with an Alliance 9.7 instrument (UVITEC, Cambridge, UK).

**Transmission electron microscopy**

EV preparations (1x10^8 vesicles) suspended in PBS were placed on copper grids covered by an ultrathin carbon film and positively charged (15 mA for 25 s). A 2% solution of uranyl acetate dissolved in distilled water was used to perform a negative stain. The preparations were examined in a JEM-3010 transmission electron microscope with an operating power of 300 kV and representative images were obtained (Winck et al, 2015).
Treatment

After purification and quantification, EVs were stored at -80°C. To assess the effects of the EVs on endothelial cell phenotypes, cells were cultured in culture media containing $5 \times 10^7$ or $5 \times 10^8$ EVs/ml. To comparison, cells were cultured in the same manner in the absence of EVs (negative control).

Tube formation assay

Cells were seeded in 96-well plates previously covered with 50 μl of Matrigel®-GFR (BD Biosciences) in the presence or absence of OSCC-produced EVs for 12 h and at a density of 40,000 cells/well. An inverted microscope (Eclipse Ti-S, Nikon, Tokyo, Japan) was used to observe tube formation and photomicrographs were acquired with the NIS Elements F 3.0 software (Nikon, Tokyo, Japan) and analyzed with the Motic Images Plus 2.0 software (Motic China Group Co.). The perimeter of the tubes was evaluated by delimiting and measuring the length of each tube.

Proliferation assay

Cells were seeded in 96-well culture plates at a density of 10,000 cells/well in 100 μl of 10% FBS supplemented media. Following 24 h of serum starvation, cells were incubated with or without OSCC-derived EVs for another 24 h. Proliferation rates were determined by immunosorbent assay for the detection of Ki-67 (clone MIB-1, DAKO, Denmark) expression levels, as described previously (Klein et al, 2000).
Migration assay

Cell migration assay was conducted in 6.5 mm transwell insert containing 8 µm pores (Corning, USA). Serum starved HUVEC cells (8x10^4 cells/well) were plated to the top chamber in 200 µl of serum-free media with or without OSCC-produced EVs. The bottom chamber was completed with 500 µl of 10% FBS supplemented media. Following 24 h of incubation, a cotton swab was used to carry out the gentle removal of nonmigratory cells in the top compartment and migratory cells that stayed on the lower surface of the membrane were subjected to fixation and staining with a 1% solution of toluidine blue/borax. Measuring absorbance was measured at 650 nm.

Apoptosis analysis

Cells were seeded in 6-well culture plates at a density of 2x10^5 cells/well in 5 ml of 10% FBS supplemented media. Following 24 h of incubation with or without EVs derived from OSCC cells, the cellular apoptotic index was analyzed in a FACScalibur flow cytometer (BD Biosciences, USA) by quantifying annexin V-PE positive and 7-AAD negative cells as described previously (Bufalino et al, 2015).

Statistical analysis

The results are reported as mean ± standard derivation (SD). Statistical analysis was performed using Mann-Whitney’s U test or ANOVA, followed by Tukey’s multiple comparisons test, and significance level was set at 5% (p≤0.05). All experiments were carried out, at least, in triplicates.
RESULTS

Characterization of the OSCC-derived EVs

Morphological characterization revealed that OSCC-derived EVs exhibited an integral and rounded surface without any apparent intervesicular fusion or aggregation (Fig. 1A). As shown in Figure 1B, both OSCC-derived EVs were enriched with CD63 and Annexin II, but a higher expression was detected in EVs derived from HSC3 cells. Moreover, the average particle concentration and size distribution of the EVs from SCC15 (3.37 ± 0.45 x 10¹⁰ and 142.06 ± 24.24 nm, respectively; Fig. 1C) and HSC3 (2.79 ± 0.55 x 10¹⁰ and 166.06 ± 7.60 nm, respectively; Fig. 1D) did not show significant variation.

SCC15-derived EVs induce angiogenesis

To evaluate the effects of SCC15-derived EVs in the processes related to angiogenesis, HUVEC cells were cultured with increasing EV concentrations and assessed in proliferation, migration, apoptosis and tube formation assays. Even at highest concentration, SCC15-derived EVs did not alter significantly the proliferation of HUVEC cells (Fig. 2A). At 5x10⁷ EVs, proliferation was reduced in ~32% (mean 31.7% ± 16.7) (p=0.36), and the treatment with 5x10⁸ EVs showed great variability, with a mean of induction of ~12% (mean 12.9% ± 64.2) (p=0.95). However, the treatment with 5x10⁸ (p=0.03) SCC15-derived EVs promoted a significant increase in the number of apoptotic cells in comparison with the control (Fig. 2B). Additionally, increased cell motility was detected after treatment with 5x10⁷ and 5x10⁸ particles (p=0.02 and p=0.03, respectively, Fig. 2C). Significant tube formation was identified when HUVEC cells were incubated with SCC15-derived EVs (Fig. 2D-E). In fact, a significantly higher number of tubes was observed as the concentrations of EVs increased (p=0.02 for 5x10⁷ EVs and p=0.0001 for 5x10⁸ EVs). However, no expressive alterations were identified in the length of vascular tubes (Fig. 2F).
**HSC3-derived EVs suppress angiogenesis**

The effects of the EVs released by the highly invasive HSC3 cells were also analyzed in HUVEC cells. HUVEC cells incubated with $5 \times 10^7$ and $5 \times 10^8$ EVs reduced the Ki67-labeling index by approximately 35% (mean $35.1\% \pm 25.8$) and 40% (mean $40.1\% \pm 12.9$) respectively, in comparison with the control without EVs. However, a significant reduction in the proliferation of HUVEC cells was observed only after treatment with $5 \times 10^8$ EVs ($p=0.05$) (Fig. 3A). No significant effects on apoptosis or migration rate were associated with HSC3-derived EVs (Fig. 3B, C). On the other hand, a progressive suppression of tubulogenesis, characterized by a significantly lower number of tubes, was observed at concentration of $5 \times 10^8$ EVs ($p=0.04$, Fig. 3D, E). No significant differences in the total tube length were observed (Fig. 3F).

**DISCUSSION**

The dynamic and reciprocal interactions established between cancer cells and the tumor microenvironment orchestrate critical aspects for the development of primary and metastatic tumors, as well as for maintaining a permissive environment at the tumor-stromal interface. One way by which cancer cells can establish communication and modifications in the surrounding tissues is through the release of EVs (Atay and Godwin, 2014). Within this context, the present study aimed to characterize OSCC-derived EVs and to evaluate their effects on angiogenesis in vitro.

Under electron microscopy, EVs derived from both cell lines exhibited the classical spherical aspect and expressed the transmembrane protein CD63 and the cytosolic protein Annexin II, which are supposed to be enriched in EVs (Lötvall et al, 2014). However, despite secreting similar amounts and size distribution of vesicles, HSC3-derived EVs presented higher amounts of those markers compared to SCC15-derived EVs, a fact that can reflect differences in the content, source or release process of the two cell lines (Willms et al,
Increased production of EVs by tumor cells occurs to facilitate the intercellular communication and to support the tumor growth in different ways (Zlotogorski-Hurvitz et al., 2016). In accordance with this and comparing to previous studies using the same method of quantification, we obtained higher EV yields by the polymer-based precipitation method compared to sequential gel-filtration (Heinemann et al., 2014) and ultracentrifugation (Zlotogorski-Hurvitz et al., 2016). In addition to the fact that different tumor cells are able to produce higher amounts of EVs in comparison with non-neoplastic cells (Taylor and Gercel-Taylor, 2008), these data also reflect the efficiency of the methodology used in this study. Moreover, the diagnostic applicability of EV recovery with same method was confirmed in earlier publications (Shin et al., 2015; Yap et al., 2017). This methodology also enabled us to obtain EVs of similar size from both OSCC cells, in contrast to wide range of nanoparticle size distribution previously reported (Zlotogorski-Hurvitz et al., 2016). However, despite their seemingly homogenous characteristics, our results showed that EVs derived from different OSCC lines mediated distinct effects on the angiogenic properties of recipient endothelial cells.

SCC15-derived EVs induced significantly HUVEC migration and tube formation, and EVs derived from HSC3 inhibited significantly the proliferation and tube formation. Like non-neoplastic tissues, cancers need stromal support and O$_2$, as well as an alternative pathway to eliminate CO$_2$ and metabolic residual products (Hanahan and Weinberg, 2011). Therefore, the pro-angiogenic phenotype induced by SCC15-derived EVs can be explained by the fact that the angiogenic process is fundamental for tumor progression and metastatic spread. Previous studies have shown that EV uptake can induce upregulation of angiogenesis-related genes, promoting endothelial cell proliferation, migration, and sprouting (Nazarenko et al., 2010). Regarding the apoptotic effects triggered by SCC15-derived EVs on HUVEC cells, our results showed an increased number of apoptotic cells. In addition to its involvement in the remodeling, homeostasis and development of blood vessels, this
elevated apoptotic index can equally contribute to the angiogenic process and to during the phase of tubular morphogenesis (Affara et al, 2007).

In contrast, the antiangiogenic properties of EVs produced by HSC3 cells highlight the complexity and heterogeneity in which these structures perform their function in the tumor microenvironment (Meehan and Vella, 2016). Anti-angiogenic effects of EVs generated in vitro from various cell types or isolated from plasma have been associated with the activation of the CD36-dependent signaling pathway, which inhibits microvascular cell migration and tube formation. Furthermore, HSC3 cells are highly aggressive and hypoxia-responsive (Teppo et al, 2013) and exogenous hypoxia is an important factor with a strong impact on EV secretion, load and, consequently, on angiogenic properties (Webber et al, 2015). More potent angiogenesis has been associated with EVs obtained from glioblastoma and multiple myeloma cells during hypoxic exposures (Kucharzewska et al, 2013; Umezu et al, 2014). Moreover, leukemia cells under hypoxia are significantly enriched with miR-210 that promotes the EV angiogenic activity (Tadokoro et al, 2013). Thus, further studies should be conducted to assess the content and the effects produced by HSC3-derived EVs obtained under hypoxic conditions.

CONCLUSIONS

In closing, our findings reflect the complexity of the coordinated events and interactions related to tumor angiogenesis mediated by EVs. While SCC15 cell-derived EVs promoted a pronounced angiogenic response as well as high apoptosis and cellular migration of endothelial cells, EVs secreted by the more invasive HSC3 cell line showed opposite effects. These results not only increase our understanding of the interactions between tumor-derived EVs and endothelial cells but may also contribute to future investigations on EVs and to the evolution of therapies that use EVs as target.
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REFERENCES


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FIGURE LEGENDS

**Figure 1.** Morphological characterization of OSCC-derived EVs. (A) Transmission electron microscopy of OSCC-derived EVs revealed structures with an integral and rounded surface (Scale bar = 20 nm). (B) Detection of CD63 and Annexin II expressions in OSCC-derived EVs by dot-blot. Average concentration and size distribution of EVs isolated from SCC15 (C) and HSC3 (D) cells in three independent experiments by nanoparticle tracking analysis.

**Figure 2.** SCC15-derived EVs promote angiogenesis. (A) The proliferation assay based on detection of the Ki-67 protein did not show a significant induction of HUVEC cell proliferation. (B) Apoptosis of HUVEC cells was significantly induced after the treatment with $5 \times 10^8$ (p=0.03) SCC15-derived EVs. (C) Migration of HUVEC cells was significantly induced at concentrations of $5 \times 10^7$ and $5 \times 10^8$ particles (p=0.02 and p=0.03, respectively). (D) Representative photomicrographs showing a significant tube formation after the treatment with SCC15-derived EVs for 24 h (original magnifications of 40x). (E) All concentrations of SCC15-derived EVs produced a higher number of vascular tubes (p=0.02 for $5 \times 10^7$ particles and p=0.0001 for $5 \times 10^8$ particles). (F) No significant alterations were observed on the length of vascular tubes. Bars represent the means ± SD of three independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001.

**Figure 3.** HSC3-derived EVs block endothelial-like tube formation. (A) The proliferation of HUVEC cells was significantly inhibited at concentration of $5 \times 10^8$ HSC3-derived EVs (p=0.05). No significant effects on apoptosis (B) or migration (C) were associated with HSC3-derived EVs. (D) Representative photomicrographs showing a significant reduction of tube formation after the treatment with HSC3-derived EVs for 24 h (original magnifications of 40x). (E) All concentrations of HSC3-derived EVs produced a progressive suppression in tubulogenesis and a significant lower number of vascular tubes was observed at
concentration of $5 \times 10^8$ HSC3-derived EVs ($p=0.04$). (F) No significant alterations were observed on the length of vascular tubes. Bars represent the means ± SD of three independent experiments. *$p \leq 0.05$. 