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Research Project for Ph.D:

Exploring the Dynamic Nexus: Sphingolipids, Exosomes, and Post-Radiotherapy Interplay in Cancer Response, with Emphasis on Angiogenesis and Resistance

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ABSTRACT

Exosomes are extracellular vesicles released by cells, capable of carrying several signals such as proteins, nucleic acids, and metabolites, to other sites, having an essential role in intercellular communication. Their composition comprises several proteins and lipids, such as proteins in the tetraspanin family, heat shock (HSP), cholesterol, ceramides, and sphingomyelins. Different works have shown exosomes' role in cancer development, contributing to metastasis, angiogenesis, and the microenvironment. Angiogenesis is an essential mechanism for cancer development, and modulation of the microenvironment is paramount for cancerous cells to adapt and activate dormant endothelial cells to begin the angiogenic process. Radiotherapy is one of the most common therapies for cancer, being capable of affecting endothelial cell dysfunction and leading to apoptosis. Besides, pro-survival cytokines are released into the tumoral microenvironment, weakening radiotherapy's effects. After radiotherapy, it is unknown how sphingolipids can modulate and participate in exosome production and signaling by tumoral cells during radiotherapy, furthermore, how these exosomes impact angiogenesis and resistance. This research will include diverse experimental approaches such as irradiation assays followed by exosome isolation, exosome lipidomic profiling through mass spectrometry, flow orientation assays, and chick chorioallantoic membrane assays. Our work will bring knowledge on the profile and composition of exosomes post-radiotherapy, focusing on potential response markers and strategies to overcome radiotherapy resistance. Ultimately, it can impact in health and care of cancer patients.

Keywords: Sphingolipids; exosomes; endothelial cells; angiogenesis; cancer

1. INTRODUCTION

1.1 EXOSOMES AND CANCER

Exosomes are one of several extracellular vesicles (EVs) released by cells, carrying proteins, nucleic acids, and metabolites to other sites, playing an important role in intercellular communication (1,2). They are involved in processes such as immune responses (3), central nervous system communication (2), and pathological processes such as cancer (4).

Being membrane bound carriers, the exosomes originate from the endosome, formed by an encapsulation of cell membrane proteins through endocytosis, also aggregating intracellular proteins and nucleic acids. In addition to lipids forming the double-layer membrane, they deliver their contents to recipient cells such as growth factors, receptors, transcription factors, DNA, RNA, mRNAs, and non-coding RNAs. This early endosome maturates into a late endosome, forming multiple intraluminal membrane vesicles (ILVs) carrying those different proteins and nucleic acids, leading the late endosome to be called a multivesicular body (MVB). The MVB then secretes the ILVs into the extracellular space as the MVB fuses with the cellular membrane, and these secreted ILVs are exosomes (5).

Some of the proteins that make up the exosome are proteins such as members of the tetraspanin family (associated with exosome biogenesis and cargo selection) (6,7), endosomal sorting complex required for transport (ESCRT) proteins (8), heat shock proteins (HSPs) (9,10), proteins associated with transport and fusion such as GTPases and annexins (11,12), as well as different lipids, such as cholesterol, ceramides, and sphingomyelins (13–15).

Several works have shown how exosomes are important for cancer development, being capable of contributing to cancer metastasis, angiogenesis, and influencing on the cellular microenvironment (16). Reports have shown how tumoral cells secrete more exosomes than healthy cells (17,18), and these tumor-derived exosomes (TDEs) are also

capable of mediating the immune response against the tumors at many levels, thus promoting tumor progression (19).

The tumor microenvironment is a complex system that surrounds and maintains a tumor, with many non-cancerous cells being present to fulfill that role. It was reported how TDEs mediate the cross-talk between breast cancer cells and cancer-associated fibroblasts to enhance migration (20), with exosome transported miRNAs inducing vascular permeability and metastasis in distant organs (21); integrins and integrin ligands, carried by TDEs, have roles in the cancer cell colonization and formation of a pre-metastatic niche (22), with many TDEs inducing differentiation of tumor microenvironment cells to cancer-associated fibroblasts, which are some of the major cell presence in tumor microenvironment (23).

With all this evidence, and much more still being explored, it becomes clear that tumor-derived exosomes have vital roles in cancer viability and progression. Questions on how these TDEs act and interact are still being posed, and there are many paths to be explored still.

1.2 ENDOTHELIAL CELLS AND THE TUMOR MICROENVIRONMENT

Blood vessels are created in embryogenesis by the differentiation of angioblasts transitioning into endothelial cells in a tightly regulated process mediated by transcription factors and signaling pathways (24). The blood vessels created by the proliferation of endothelial cells give rise to arteries and veins, with vascular endothelial growth factor (VEGF) and Notch signaling being responsible for artery differentiation whereas VEGF and COUP transcription factor 2 leading to vein differentiation (25) – with all of this process being called vasculogenesis, once it happens during embryogenesis.

Angiogenesis on the other hand, is the formation of blood vessels from already existing ones, and is regulated by VEGF and Notch signaling, though both of them act in an antagonistic way: whereas VEGF stimulates angiogenesis, Notch refrains it (26).

This angiogenesis process is incredibly important for tumor progression, once it supplies the nutrients and oxygen needed for the tumor to sustain itself (27). The blood vessels in these tumors consist of tumor endothelial cells (TECs), lining the interior of the blood vessels, and perivascular cells, surrounding the exterior of the blood vessels and being responsible for their contraction and relaxation (28). During cancer development, factors such as hypoxia stimulate angiogenesis by the activation of VEGF, granting it the capability of growing and metastasizing, with studies showing that the inhibition of VEGF has an antitumoral effect (29).

The modulation of the tumoral microenvironment is essential for tumoral cells to develop. By releasing cytokines, chemokines, and growth factors, the tumor cells are capable of adapting the microenvironment and activating the dormant endothelial cells to start the angiogenesis process (30). Through this, not only the tumor is capable of developing in the original tumoral space, but through use of exosomes is capable of spreading to other regions, since these exosomes are capable of carrying varying angiogenic factors (31). Endothelial cells internalize the TDEs (32), being affected by its contents and leading to the development of the blood vessels.

To deal with cancer progression, several therapies are employed, with one of the most common ones being radiotherapy (RT). Mainly used for treatment of localized cancers, RT has seen a development and improvement in its implementation, with high precision systems being created. Capable of killing tumoral cells, RT is also capable of affecting the tumoral microenvironment, such as blood vessels and immune system associated (33).

Because of the way endothelial cells proliferate in cancer, being more permeable than normal blood vessels, they are also more sensitive to irradiation than vessels surrounding healthy tissues. RT induces endothelial cell dysfunction, with increased permeability, detachment from the basement membrane, and apoptosis, which contributes to the development of radiation-induced inflammation and fibrosis (34). The irradiation induced apoptosis can lead to vascular destruction, which may lead to tumor

cell death. Despite this, pro-survival cytokines are also secreted into the microenvironment, inhibiting the apoptosis of endothelial cells and weakening the RT effects (35). Endothelial cells irradiation might actually trigger pro-survival processes such as the upregulation of angiogenesis mediated by vascular endothelial growth factor receptor 2 (VEGFR-2) (36). It was shown that in gastric cells, inhibition of VEGFR-2 hindered endothelial cell progression triggered by exosomes from irradiated gastric cancer cells (37), placing exosomes as vesicles of interest for cancer therapies, especially in association with RT.

JUSTIFICATION

Exosomes play important roles in cancer development and progression as well as resistance to therapy through cell-cell communication. Radiotherapy is a common modality of cancer treatment. However, it is not known how sphingolipids can modulate the exosome production by tumor cells exposed to radiotherapy and their potential impact in angiogenesis and resistance. Investigating the relationship between irradiated tumor cells and endothelial cells through exosomes is crucial. Furthermore, it is essential to study the profile and composition of exosomes post-radiotherapy, with a focus on identifying potential biomarkers or future therapies to overcome resistance.

HYPOTHESIS

From previous results, we known that there is alteration in sphingolipids metabolism in oral cancer cells and is associated with stemness and cell transformation. However, a correlation between sphingolipids and exosomes production affecting angiogenesis and cell resistance during radiotherapy in OSCC cells.

2. OBJECTIVES

The aim of the research is to study the effects of extracellular vesicles, specifically exosomes, that are produced by OSCC cells exposed to radiotherapy, on human vascular endothelial cells and angiogenic process.

3. WORK TIMELINE

Activities	2024	2025	2026	2027
Cell culture	■	■	■	■
Cell irradiation assays	■	■	■	■
Exosome isolation	■	■	■	■
Plasmatic extracellular vesicles isolation			■	■
Protein analysis	■	■		
Exosome lipid composition		■	■	■
Lipidomic and sphingolipidomic assays		■	■	■
Exosome size analysis	■	■	■	
Viability/proliferation assays	■	■	■	
Invasion assays	■	■	■	
HUVEC tubule formation and proliferation			■	■
Flow-induced orientation analysis			■	
Chick chorioallantoic membrane (CAM) assays (in vivo)			■	
Results analysis, manuscript, and Ph.D. thesis writing				■

This project will be developed in collaboration with Dr. Julie Gavard, CNRS Research Director Team leader, UMR_S 1307 Centre de Recherche en Cancérologie et Immunologie Intégrée Nantes Angers, France. Ana Julia plans to apply for an international “sandwich” with Dr. Gavard as her co-advisor to work on aspects and approaches related to angiogenesis.

Cell culture, irradiation assays, exosome isolation, and exosome size analysis will be done both in Brazil and France. Plasmatic extracellular vesicules isolation, lipidomic assays, flow-induced orientation analysis, and chick chorioallantoic membrane (CAM) assays will be done under Dr. Julie Gavard’s supervision in France. Protein analysis,

exosome lipid composition, sphingolipidomic assays, viability/proliferation assays, invasion assays, and HUVEC tubule formation and proliferation will be done under Prof. Andreia M. Leopoldino's supervision in Brazil.

4. METHODOLOGY

Cell culture

Head and neck squamous carcinoma cell lineages (Cal-27, HN13), non-tumoral oral keratinocyte over expressing or knocked-down for sphingosine kinase 2 (NOK-SK2), and human umbilical vein endothelial cells (HUVEC; ATCC) will be cultivated in DMEM medium (Sigma-Aldrich) supplemented with 10% bovine fetal serum (Gibco), antibiotics and antimycotics (Sigma-Aldrich) at an incubator with 5% CO_2, 37°C.

Cell irradiation

Cells will be seeded in culture dish to receive the same irradiation per area unit. They will be irradiated with 2, 4, and 6 Gy doses in the Radiotherapy Service of Hospital das Clinicas of School of Medicine of Ribeirão Preto, University of São Paulo. It will be used the biological research irradiation equipment RS 2000 (Rad Source Technologies, Inc., Suwanee, GA), with 160 kVp and 25 mA, with a standard Cu filter of 0.3 mm. X-rays or gamma rays created under such conditions have a 95 kV to 160 kV energy spectrum, with half of the beam value being 0.62 mm of Cu. X-ray dosage gradient in tissue is 10% for 0.5 cm deep. Quality control will be done through Nanodot dosimeters (Landauer, Inc., Glenwood, IL), with dosage readings on top of the wells used to calculate treatment time beam-on.

Exosome isolation

Exosome extraction will be done following a differential ultracentrifugation method (38,39), seeking to obtain only exosomes from irradiated and control cells. Samples will be successively centrifuged at 300 x g for 5 minutes, 2,000 x g for 10 minutes, 10,000 x g for 30 minutes, and 100,000 x g for 2 hours. After the centrifugation at 300 x g and 2,000 x g, supernatants will be centrifuged at 16,500 x g for 30 minutes, to obtain a pellet of microvesicles. The remaining supernatant will be filtered at 0.22 µm, and centrifuged at 120,000 x g for 2 hours, and washed with filtered PBS. All of the centrifugations will be done at 4°C.

Plasmatic extracellular vesicles (EVs) isolation

Mice plasma will be collected and isolated following André-Gregoire et al. (40) protocol. The mice will be prepared adequately, using ketamine and xylazine to anesthetize it, followed by a buttonhole incision on the upper part of the thoracic cage so an intracardiac puncture can be done. Following blood extraction, the mice will be euthanized immediately with approved procedures. Afterwards, blood and plasma will be separated by centrifugation at 1,000 x g for 15 minutes at 4°C, transferring the supernatant to appropriate tubes. Plasma can then be stores until further analyzes are done, or the protocol can be continued immediately. Extracellular vesicles will be obtained by size exclusion chromatography (SEC), using resin columns (gEVoriginal/70 nm Gen 2) associated with an automatic fraction collector (AFC, Izon Science), according to the manufacturer's protocols. Plasma samples will be centrifuged at 10,000 x g for 30 minutes at 4°C, before loading them on SEC columns to eluate particles. The extracellular vesicles can be concentraded by ultracentrifugation at 100,000 x g for 2 hours at 4°C. The final pellet will be resuspended in particle-free PBS.

Protein analysis

Proteins associated with exosome such as integrins, CD81, CD63, heat shock proteins (Hsp90 or HSP70) will be assessed through western blot, following laboratory's protocols already established. Other proteins can also be assessed in cells, such as ATM,

p53, HDAC6, gammaH2AX (DNA damage response), VEGF and VEGFR-2 by Western blot and/or ELISA.

Exosome lipid composition

Following exosome isolation, their composition will be analyzed through mass spectrometry (LC-MS/MS). 100 µl of each sample will be used, with the addition of 100 µl of methanol:chloroform solvent (1:1). They will be vortexed for 5 minutes and put on ultra-sonication bath for 2 minutes. Afterward, the samples will be centrifuged at 21,000 x g for 5 minutes at 4°C, collecting the supernatant and vaporizing the solvent in the supernatant in liquid nitrogen. Following that, the samples will be diluted in 100 µl of a MeOH:$CHCl_3$ solution (1:1), with 5 µl of this solution being applied in the LC-QqQ-MS/MS and LC-ESI-MS/MS chromatographer, for quantitative and qualitative analyses with the same sample, in triplicate. The results obtained will be compared with previously described patient profiles obtained by Andreia M. Leopoldino´s research group.

Lipidomic and sphingolipidomic assays

For lipidomic and sphingolipidomic analysis, OptiPrep top to bottom density gradient 5% to 40% was performed after size exclusion chromatography on polyallamor tubes. Fractions will be collected from top to bottom and washed in filtered PBS for 3 hour at 100,000 x g at 4°C, and pellets will be resuspended in an ammonium bicarbonate 50 mM solution. Next, lipids will be extracted by the Bligh and Dyer method for mass spectrometry analysis.

Exosome size analysis

Zetasizer Nano ZS will be used to determine and compare exosome size and number produced by the cell lines under control and irradiation condition, after irradiation, 48 hours after irradiation, and a week after irradiation. Transmission electron microscopy will be performed to identify the exosomes. Concentration of exosomes will also be measured by single particle tracking (Interferometry Light Microscopy, VideoDrop,

Myriade), measuring particle concentration in a real-time nanometer-scale optical method.

Alongside this, cryo-electron microscropy will be performed. Exosomes will be separated and concentrated, being deposited on glow-discharged electron microscope grids, followed by vitrification and quick freezing into liquid ethane using an automatic plunge freezer (EM GP, Leica). Exosomes will be analyzed by cryo-transmission electron microscropy afterwards at the Microscopy Rennes Imaging Center (MRic, Université de Rennes, France) using a 200 kV Tecnai G2T20 Sphera microscrope (Thermo Fisher).

Viability/proliferation assays

Cell viability and proliferation will be done through the MTT assay, as previously described by Duarte et al. (41), seeking to evaluate the cell's metabolic activity. Irradtiation will be done 12 hours after plating, and the cells will be incubated with MTT (5 mg/ml) 24 hours, and a 7 days after radiotherapy irradiation, with absorbance being read afterwards.

Invasion assay

Invasion assays will be performed as previously described by Sobral et al. (42), seeking to observe the cells capacity to migrate after radiotherapy. Cells will be seeded in 24-well plates using a modified Boyden chamber inserts with filter membranes containing 8-µm pores. Matrigel will be diluted with serum-free medium and used to coat the filter membranes, with cells being seeded onto the upper compartments of the transwell chamber, with DMEM with 10% FBS on the lower chamber for stimulation. After 24 hours and 48 hours incubation, the medium in the upper chamber will be removed and the filters will be fixed with 10% formalin for 15 minutes. The cells in the lower surface will be stained with DAPI, and photographs will be taken using a Zeiss Axiovert 40 inverted microscope.

Clonogenic assay

Clonogenic assays to measure cell capacity to form colonies *in vitro* after radiation will be done following Brix et al. (43), on expanded methods by Franken et al. (44). Cell cultures will be maintained for 48 hours, 72 hours and one week after irradiation, and will be fixed in neutral buffered formalin and stained with 0.5% crystal violet solution (Sigma-Aldrich). Counting will be done in ImageJ software.

HUVEC tubule formation and proliferation

HUVEC cells will be cultured with conditioned medium obtained from control and irradiated OSCC cells cultured for 24 and 48h after irradiation. HUVEC cells will be trypsinized and inactivated with Reagent Pack Subculture Reagents kit (#CC-5034; Lonza Bioscience) and seeded 2-3 x 10^4 cells/mL. Seeding will be done in a 96-well plate, with 50 µl Matrigel (BD Biosciences) in each well. The following day, the cells will be washed with Hank's solution followed by addition of calcein (1:1000) for 30 minutes at 37°C. The plate will be photographed in Leica MZ16F microscope, with Leica DFC7000T camera.

Flow-induced orientation assay

To analyze the orientation of epithelial cells, flow-induced orientation analysis will be done as described by Vion et al. (45). HUVEC cells will be cultivated on 0.2% gelatin-coated slides, with unidirectional laminar shear stress being applied by perilstatic pumps connected to a glass reservoir and the chamber containing the slide. To analyze the orientation of the cells, the angle formed between the vector of the flow direction and the orientation vector (given by the orientation of the main axe of each cell) will be calculated.

Chick chorioallantoic membrane (CAM) assays

Chick chorioallantoic membrane assays will follow Ribatti et al. (46). Fertilized eggs will be incubated at 37°C and 60% humidity for 48 hours. Afterwards, 2-3 ml of albumin will be aspirated from the egg on the third day of incubation, with a square window being cut into the shell to reveal the embryo and CAM vessels. On the eighth day of incubation,

the window will be opened under sterile conditions and a gelatin sponge will be implanted onto the CAM. Following the gelatin placement, exosomes can be delivered onto it. On the twelveth day, the embryos will be fixed with their membranes *in ovo* by pipetting a Bouin's fluid solution onto the CAM surface, allowing the embryos to be fixed for 3 hours at room temperature. Following the fixation, the sponges and underlying CAM portions will be cut and transferred onto a culture tube, being dehydrated in ethanol solutions, followed by clearing them in toluene and then immersing them in paraffin for 2 hours. With a microtome, the samples will be cut into serial sections, parallel to the surface of the membrane and stained with a 0.5% aqueous solution of toluidine blue for 1 minute at room temperature. Macroscopic evaluation will be done by analyzing the convergence of blood vessels towards the graft, the variations of distribution and density of CAM vessels next to the site of grafting or analyzing the branching of vessels.

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