PROGETTO DI RICERCA – DOTTORATO IN ONCOLOGIA E CHIRURGIA SPERIMENTALI (XXXIV CICLO);
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Tumor-derived exosomes as factors that promote metastatic niche formation: evaluation of the effects induced by colon cancer derived exosomes on functional activities and structural features of Hepatocytes

Background

Many laboratories are focusing their efforts to find the mechanism by which the primary tumor prepares the soil suitable for colonizing the tissue in advance in another organ. We do not know how a "metastasis" develop, how and why a cell can detach and nest in one particular organ rather than another, and from there initiate a widespread tumor process. Exosomes could provide a warning that a cancer is about to metastasize. This is a little known area of a clear link between exosomes and metastases.

There is emerging evidence that exosomes play a key role in many cancers. Exosomes have unique features that could be used as biomarkers to facilitate improved detection and treatment of the cancer. One basic way of cell communication is by releasing extracellular molecules such as nucleotides, lipids and proteins into the environment, where these molecules will then bind to receptors on surrounding cells, leading to intracellular signalling and modification of the recipient cell (1). Exosomes have been described to contain messenger RNA (mRNA), microRNAs (miRNAs), double-stranded DNA (dsDNA) and proteins that could serve as diagnostic, predictive and prognostic biomarkers for the different tumors. Exosomes are formed by inward budding of endosomal compartments called multivesicular bodies (MVBs). The discovery that exosomes can deliver mRNA and microRNA has sparked interest even more (2), with evidence being presented that mRNA contained in exosomes can be translated into functional protein, indicating that exosomes can directly transfer genetic information that may modify recipient cell behaviour. Accumulating evidence indicates that exosomes are also “mediators of metastasis” (3,4,5). Many different tumor cells may secrete exosomes including breast, colon/rectum, brain, ovary, prostate, lung, and bladder cancer (6). It has recently been demonstrated that during metastatic cascade, tumour-derived exosomes (TDEs) are able to model the microenvironment of secondary sites such as the liver, where non-parenchymal cells (NPCs) are reported to be the main targets cells.

It is observed that colon/rectum cancer (CRC) has a tendency to "extend" to the liver. The liver is one of the most vascular organs in the body and its main cellular component, the hepatocyte, plays a central physiological role metabolizing nutrients, potentially harmful endogenous substances, and xenobiotics (7,8). Moreover, data collected during the years shows us the multiple roles played also NPCs namely to regulate both pre-metastatic niche formation and the liver tumour cell colonisation. But, while the bibliography speaks a lot about the role of non-parenchymal cells (liver sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells and liver-associated lymphocytes) in the regulation of premetastatic niches / metastases, little is known about the role of hepatocytes in this phases. Hepatocyte-driven liver regeneration is the default pathway in response to mild-to-moderate acute liver damage. Hepatocytes are also the main detoxifying cell type of the body for drugs, alcohol, and other chemicals, and thus they are broadly used as a cellular model for toxicology screening and drug development.

To date, some scientific papers explored the impact of hepatocytes on colon cancer. While there are evidence that the colorectal cancer is known to preferentially metastasize to the liver and prove interactions between colon cancer cells and hepatocytes in relation to metastasis (9,10), no data are

available on the role of Heps during the pre-metastatic niche formation and the role played by tumor-derived exosomes (TDEs) in affecting structural and functional features of Heps.

At the light of these considerations, the purpose of the present research proposal will analyse the effects induced by metastatic colon cancer derived exosomes (M/CCDEs) on functional activities and structural features of Heps, and then to assess how M/CCDE-preconditioned Heps may moderate the behaviour of colon cancer cells. In order to do this I shall be using colon cancer as a model for our analysis.

Moreover, immunological function will be evaluated to see different approaches in the hypothetic care to cancer for molecular characteristics. Many studies have shown that dendritic cells and macrophages are critical tumorigenic regulators of the microenvironment in hematologic malignancies. Normally the mutated cells that can give rise to a tumor are attacked by lymphocyte activation, but binding of PD-1 to its ligands PD-L1 and PD-L2 reduces T-cell activity (11,12). However, the cancer cells can escape this fate thanks to the production of a particular protein PD-L1 that interacts with PD-1 generate adaptive resistance unimpeded and enhances tumor rejections.

PD-L1 is unquestionably a negative predictive marker, and it is often overexpressed in different tumors including lymphoma, melanoma, colon cancer, lung cancer and other types of cancer. Tumor cells, resting dendritic cells, and macrophages in the tumor microenvironment express PD-L1 and proinflammatory cytokines like IFN-γ and IL 6 upregulate PD-L1 on tumor cells. As a result, tumor cells attenuate T-cell signaling to evade immune surveillance. Blocking PD-1/PD-L1 interaction has been shown to restore T-cell activation and antitumor response, providing the rationale for therapeutic intervention PD-1/PD-L1 as target (13,14). But, the mechanisms of PD-L1 upregulation in macrofages and in the non-tumoral cells (Heps) mediated by M/CCDEs remain unknown. Aim of the project will be to evaluate the role of M/CCDEs, through the profile of Interleukin-6 (IL-6) expression in PD-L1 in healthy cells (Heps) and in the immune system checkpoint. Aim of this project will be also to investigate the modulation of PDL-1 at Heps (liver microenvironment) and evaluating the role of IL-6 in PD-1/ PDL-1 axis modulation.

RESEARCH PROPOSAL

For the purposes of this research I will focus on three specific tasks:

the first OBJECTIVE to determinate the significance of circulating exosomes levels in colon cancer, I prospectively will isolate and characterize exosomes from SW480 and SW620 cell lines. The isogenic colon cancer cell lines, SW480 (ATCC CCL-228: colorectal adenocarcinoma; Dukes'type B) and SW620 (ATCC CCL-227: colorectal adenocarcinoma from metastatic site; lymphnode; Dukes'type C derived from the same patient), will be used in this study as tumor model. Caco-2 cells (ATCC HTB-37) and THLE2 cell line will be used as control, since even if originally derived from a colon carcinoma they are widely used as a model of normal enterocytes.

The second OBJECTIVE I shall do analysis of the effects induced by M/CCDEs on functional activities and structural features of Heps. I will perform a first set of experiments by using the cell culture system in which Heps will be treated at different time points and with several doses of exosomes released by SW620 cells, SW480 cells and Caco-2 cells. This set of experiments will be useful to evaluate the role of the M/CCDEs in inducing the modulation of PD-L1 in cells through IL-6 thereby reducing metastatic niche formation in liver.

The third OBJECTIVE is to focus on analysis of effects induced by M/CCDEs-preconditioned Heps on colon cancer cell behaviour. I will evaluate two different aspects: (i) the attractive capability that M/CCDE-preconditioned Heps can exert on metastatic colon cancer cells, promoting their liver homing (pre-metastatic phase) (ii) if when preconditioned with M/CCDE, Heps form a weaker barrier that tumor cells

can more easily across, thus creating a microenvironment that facilitate metastatic colonization of liver parenchyma (phase of metastatic expansion).

EXPERIMENTAL PHASE OF THE PROJECT.

The experimental activities and functional assays that will be performed within each objective of this project are briefly described below:

AIM 1:

- western blot analysis
- dynamic light scattering analysis
- SWATH-based quantitative proteomic analysis

AIM 2:

- Selected time point of treatment as well as effective dose of exosomes will be used for subsequent experiments on LiOrgs and for final validation of results by using exosomes isolated from plasma of CRC patients (*in vivo* exosomes).
- To determine whether exosomes are taken up efficiently by Heps, we will add PHK26 labeled exosomes to cultured Heps, the treatment will be done at several time points and with several doses of exosomes
- To assess the influence M/CCDEs on the proliferative activity of Heps, we will perform viability and apoptotic assay, as well as analysis of cell cycle on Heps treated for several time points and with several doses of exosomes
- Albumin secretion, ammonia metabolism and markers of detoxification capability, such as cytochrome P-450 enzyme activities, will be evaluated to determine Hep functions.
- To evaluate modulation of several cytokines, chemokines and signaling pathways I will use multiplex immunological assays using Luminex ® Technology.

For gene and protein analysis I will use:

- confocal microscopy
- real time PCR
- western blot analysis
- Flow cytometry
- ELISA

AIM 3:

- Tumor cell growth will be followed via measurements of fluorescence by FACS or by observations at confocal microscope.
- Cell cycle will be analyzed by flow cytometry, following standard procedures.
- Evaluation of the ability of M/CCDE-preconditioned Heps to affect the adhesion of metastatic colon cancer cells will be performed.

Conclusions

Exosomes derived from pancreatic cancer cells educate some liver cells to secrete molecules that create the fibrous environment conducive to the growth of cancer cells through a process called “pre-metastatic niche formation”(A). The treatment of metastatic disease in primary tumors and metastases is needed to

fight cancer as a life-threating disease and, the efficacy of targeted therapies in the metastatic setting might be exceeded by the use of exosomes.

Understanding how the parenchymal component operates in moderating liver metastic cascade in the pre-metastatic phase, will provide a clearer picture on how the liver prepares to support the growth of the metastases. This research sheds a different light on the role Heps has in modulating liver metastatic cascade already in pre-metastatic niche formation. In the long term, this project could be propose a mechanism that controls metastatic progression through the crosstalk between tumor-derived exosomes and Heps.

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