FONDAZIONE Buzzi Unicem MESOTELIOMA

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Pleural Microparticles and Malignant Mesothelioma

Introduction

Malignant mesothelioma (MM) is a devastating cancer mesothelial cells caused mainly by asbestos exposure. Limited knowledge regarding the detection of asbestos exposure and the early diagnosis of MM, as well as a lack of effective treatment options for this deadly cancer, underscores an immediate **need to understand the mechanisms of** MM development.

Much research is currently underway on the study of **nanovesicles** and their enormous potential to contain signature molecules representative of different diseases as well as to communicate with distant targets.

In this area of research, many scientists have been particularly interested the role of exosomes in the biology of MM.

In this **bibliography review**, we have summarized the most noteworthy topics from the published literature in the field.

We hope that the ongoing research in MM will help advance the field by revealing the mechanisms of MM development and survival so that we can develop new treatment strategies for this disease.

Microparticles

Extracellular vesicles are cell-derived membrane-bound vesicles in response to cell activation or apoptosis. They are very heterogeneous both in content and size, with a diameter ranging between 0.1 to 1 µm.

Microvesicles are miniature versions of the parent cell and can reflect it by expressing parental antigens.

"**Microvesiculation**" is a biological process and, as such, can occur in all types of cells and every fluid in our bodies.

There is very little data on microparticles and pleural fluid, although initial research has been published since the early 2000s.

The **pleural** fluid is theoretically an ideal biological fluid for studying microparticles: sampling is minimally invasive, there is abundant material available for analysis, there is little "background noise" because it usually has a low cell count and very little cellular debris.

Microparticles deriving from cancer cells are of great interest because, by definition, they reflect the cancer cell that they derive from and can therefore provide a lot of information about it.

They are membrane-bound heterogeneous sacs that are released from the surface of cancer cells into the extracellular environment.

Tumor cells can constitutively produce extracellular microvesicles apparently without the need for stimulation.

Although several features of these tumor microparticles have been described, they all show the great potential of tumor cells for the survival and growth of the cancer.

Exosomes

Exosomes are specific extracellular microparticles and are in fact nanospheres with a diameter of less than 150 nm.
Although they were first discovered in the 1980s, this research was not particularly appealing because at that time, exosomes were simply considered as cellular residues, or waste. This was probably because it was not possible to demonstrate the interactions ongoing between exosomes and nearby cells.
The **enormous potential** of these particles, whose surface is composed of a lipid membrane that can contain surface proteins, DNA, miRNA, RNA, lipids, etc., has only recently emerged.
Due to their supply of surface proteins, loading capacity and stability, exosomes are potential **extracellular messengers** that can reach very distant cells within our body. Exosomes are therefore essential for intercellular communication: these nanovesicles are in fact transported from producer cells to target cells is important to normal physiology as well as disease states such as cancer.
Exosomal communication is implicated in multiple biological systems such as immune function, tissue repair, nervous system signaling, cardiac health, etc.
The wave of studies in the field of exosomes has brought valuable information about **basic biology and diseases** into the scientific realm.
We now know that exosomes are more than simple waste receptacles used by cells to rid themselves of unwanted material, but are sophisticated molecular messaging systems that can act locally or distally from where vesicles are secreted.
This special ability opens up avenues of important research from **a diagnostic standpoint**: researchers are studying the association between the amount of exosomes in biological fluids and the presence of tumor cells, which show a strong "impetus to communicate", i.e., produce a high number of these messenger particles.
Exosomes are also potentially interesting **from a therapeutic standpoint**: cells could be targeted by targeted therapies based on specific exosomes, transformed into transporters of agents.
Because of the **pivotal role** exosomes play in **disease**, they could be used for biomarker identification for diagnostic and prognostic means and developing new therapeutic strategies.
Cancer is the most studied field where the roles of exosomes have been explored in various processes such as diagnosis, prognosis, metastasis and therapy.

Malignant mesothelioma and exosomes

The earliest research on exosomes and MM was focused on identifying exosomes and their protein cargo from the pleural effusions of cancer patients.
Exosomes were isolated by sucrose-gradient ultracentrifugation from the pleural fluid of patients with MM, lung cancer, breast cancer or ovarian cancer. The matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis indicated large amounts of peptides originating from immunoglobulins and various complement factors, as well as previously undescribed exosomal proteins such as the sorting nexing protein (SNX25), B-cell translocation protein gene 1 (BTG1), and pigment epithelium-derived factor (PEDF). Both BTG1 and PEDF were in increased abundance in exosomes from malignant processes and likely involved in the tumor exosome biogenesis. Moreover, Western blot analysis verified the presence of the MHC class II molecule, HSP90, and immunoglobulin G and M.
Research has also been conducted on the **protein composition of exosomes** that are secreted from MM tumor cells. Knowledge about tumor antigens in MM is limited and so research in this area has been useful to delineate the proteomic load of MM exosomes. MM tumor cell lines were created; exosomes were isolated using ultracentrifugation and were characterized by TEM for their morphology and size. The exosomal proteins were subjected to MALDI-TOF analysis, and of these identified proteins, four were also confirmed by Western

blot analysis, namely: fascin, -tubulin, HSC70 and HSP90.
In addition, as reported in in vivo systems, these tumor exosomes were also enriched with MHC class I molecules, and the researchers also indicated high levels of annexins which may be involved in membrane-cytoskeleton dynamics. This report revealed several proteins that had not yet been indicated on tumor exosomes or in MM cell lines, thus providing novel information on MM and tumor exosomes as a whole that could advance our understanding of the disease.
In 2005, researchers published their work on **the immunological functions of exosomes** secreted by cancer cells (breast cancer and mesothelioma), and how these tumor exosomes altered the expression of the NKG2D receptor on target blood leukocytes. The exosomes secreted from these MM cancer cells were positive for their expression of NKG2D ligands, which was directly related to the ability of MM exosomes to decrease the capacity of effector T cells to kill target cells. This research demonstrated that the two MM cell lines used had a high expression of NKG2D ligands and appeared to correlate with the aptitude of MM exosomes to more effectively suppress NKG2D expression on target cells. Overall, this report indicates a role of MM exosomes in phenotypically altering immune cells that can aide cancer cells in immune evasion through the presence of exosome ligands to NKG2D.
A promising field of therapeutic cancer research is focused on the use of tumor-associated antigens (TAAs) present on tumor exosomes as a mode of **dendritic cell-based immunotherapy** immunotherapy. The concept being that tumor exosomes bearing TAAs, mostly secreted from immunogenic cancers, are adept at inducing antitumor responses in mouse cancer models through the activation of dendritic cells. This is an exciting advance in this field because MM is regarded as a non-immunogenic cancer with very few TAAs known. This research investigated whether MM exosomes were potential antigen sources for dendritic cell-based immunotherapy. Initially, a lethal dose of MM tumor cells was injected into BALB/c mice. After seven days of tumor formation in the mice, a single bolus dose of dendritic cells was injected for immunotherapy. These dendritic cells, however, had been loaded with either MM exosomes or MM cell lysate to test whether exosomes had an immunogenic priming capacity on the dendritic cells. The median overall survival of the tumor-bearing mice was significantly increased in the dendritic cell immunotherapy loaded with MM tumor exosomes compared to the cell lysate, suggesting that the exosomes could be a potential immunotherapeutic approach for MM as well as other non-immunogenic tumors.
Subsequent research on exosomes in MM has focused on the **formation of tunneling nanotubes** (TnT), which are actin-based cell extensions involved in intercellular cargo transport. The relationship between TnT formation and their communicatory effects with MM tumorigenesis is unknown, which led to research on exosomes as possible mediators for TnT formation in MM. MM exosomes were purified and added to MM cells cultured separately, showing that in these conditions, MM cancer cells produced significantly more TnTs than the cells cultured without the addition of exogenous exosomes. The researchers suggested that the added tumor exosomes were enriched with TnT, a process that could be involved in how exosomes interact with target cells. It has also been shown that exosomes can localize and "surf" on filipodia (cellular protrusions similar to actin filaments) before internalization. The uptake of MM exosomes by MM cells apparently facilitated more TnT connections between the tumor cells, and the connected cells had nearly twice as many lipopods. Overall, MM exosomes may act as an induction agent of TnT formation between MM tumor cells and perhaps this connection could be an important conduit of cellular information vital for MM progression.
To increase our **understanding of the MM secretome**, researchers published a study on MM-derived exosomal proteomic cargo. Using quantitative proteomics, they delineated the protein composition of exosomes from four human MM cell lines and identified a total of 2178 proteins from all cells, with 631 common exosomal proteins between the groups. Of these MM exosome proteins, the researchers delineated candidate biomarkers based on clinical relevance, including tubulin isotypes TUBB4A, Q8IWP6 and B3KPS3;

galectin-3-binding and LGB3P; alpha enolase, annexin 1 and G6PD. Furthermore, it was also shown that exosomes contained mesothelin, calreticulin, vimentin and superoxide dismutase, all highly expressed in MM. This research also uncovered the presence of 26 immunoregulatory components in MM exosomes (such as oncostatin-M oncostatin receptor (OSMR), the drug resistance-associated protein 1 (ABCC1), and the SUMO-1 activating receptor SUMO-1 (SAE1)), as well as 16 tumor-derived antigens, including glycan-1, which has been identified in many tumor-derived exosomes and considered a potentially valuable biomarker for pancreatic cancer. Importantly, this study also provided valuable information showing that MM exosomes regulate the cells in the tumor microenvironment by increasing the migratory capacity of fibroblasts and endothelial cells in vitro. Overall, this research suggests that MM exosomes contain many proteins relevant to cancer, angiogenesis, metastasis, migration and immune regulation.

The known complexity of the MM secretome was also elucidated using iTRAQ proteomic analysis. Using six MM cell lines in comparison with three primary mesothelial cell cultures, it was seen that MM cell secretomes contained higher abundances of exosomal proteins.

Another study analyzed a small number of patient samples demonstrated the potential utility of extracellular vesicles (including exosomes, microvesicles and apoptotic bodies) in diagnosing benign or malignant MM. The ratios of mesothelin, galectin-1, osteopontin, and VEGF were higher in MM samples compared with benign effusion, whereas exosomal angiopoietin-1 was higher in MPM samples. These findings are encouraging and need to be validated with larger sample populations.

Although more emphasis has been placed on the exosomal proteomic signature, a study suggests that a specific exosomal microRNA signature can discriminate malignant pleural mesothelioma (MPM) from past asbestos exposure (PAE) subjects. This study was conducted in a small number of subjects and needs to be verified in larger cohorts.

Later, researchers using the MM tumor stromal pattern demonstrated that endothelial cell-derived exosomes enriched in miR-126 were differentially distributed within the stroma. These findings suggest an important role regarding the exosomal transfer of miR-126 in its antitumor response in MM. The same researchers further demonstrated that MPM-derived spheroids, when treated with the miR-126-enriched exosome, showed antitumor effect initially. However, this effect later vanished due to the loss of miR-126 from the cells that could be restored by inhibition of exosome secretion.

Researchers investigated the **proteomic cargo and gene modulatory effects of** exosomes from asbestos-exposed cells. First, epithelial lung cells (BEAS2B) or macrophages (THP1) (the first known cells to encounter asbestos during inhalation) were cultured with asbestos and their exosomes isolated. These asbestos exosomes were subjected to tandem mass spectrometry for protein identification. 145 proteins were identified in the epithelial cell exosomes, of which 55 were significantly different in abundance in the asbestos-exposed group, including plasminogen activator inhibitor 1, vimentin, thrombospondin and glycan-1, and glycan-1. Exosomes from asbestos-exposed epithelial cells were also found to lead to genetic changes in the target primary pleural human mesothelial cells (HPM3), which were reminiscent of epithelial to mesenchymal (EMT) transition: down-regulation of E-cadherin, desmoplakin and the IL1 receptor antagonist. 785 proteins were identified upon proteomic analysis of the macrophage exosomes, of which 32 had significantly different abundances between the exosomes in the asbestos-exposed group and the control. Fifteen of these exosomal proteins were in greater abundance in the asbestos group compared with the control, and interestingly, vimentin and SOD were among those that showed an increase in exosomes from the macrophages after asbestos exposure. In response to exposure of asbestos exosomes from macrophages to target primary mesothelial cells, it was shown that significant genetic alterations occurred in the mesothelial cells: 498 genetic changes in total, with 241 up-regulated and 257 down-regulated. As a positive control, the group used asbestos fibers on mesothelial cells, and found that 206 genes were mutually altered in the asbestos-exposed

exosomes or asbestos-exposed group of mesothelial cells. This exciting discovery suggests that exosomes from asbestos-exposed cells are able to modify the genetics of mesothelial cells in similar ways to how asbestos fibers would change on their own.
As an initial step towards an **in vivo study**, researchers began to define the proteomic signature of mouse serum exosomes in an asbestos-exposure model. C57/Bl6 mice were exposed to asbestos via oropharyngeal aspiration, and 56 days later, the serum exosomes were isolated for proteomic analysis. Tandem mass spectrometry for protein identification again showed that there were 376 quantifiable proteins present in the mouse serum exosomes, with the majority of proteins being more abundant in the asbestos-exposed group. Of these more abundant proteins in the asbestos-exposed group, three were validated by Western blot analysis, all of which were acute-phase proteins: haptoglobin; ceruloplasmin, the copper carrying glycoprotein previously seen to be increased in the blood of MM patients and asbestos-exposed individuals, and fibulin-1, which is implicated in asbestos exposure and MM. The findings on the secreted exosomes of mesothelioma cells compared with healthy mesothelial cells showed that the tumor cells secreted significantly different patterns of miRNA compared with their healthy counterparts. In particular, it was shown that miR-16-5p expression was significantly increased within the exosomes released by the cancer cells. The hypothesis was that the mesothelioma cells developed a preferential secretion mechanism to rid themselves of miR-16-5p due to its well-established tumor suppressor functions. Many studies have indicated the functionality of this secretion and the possibility of targeting this pro-tumor phenotype.
Another study looked at a number of **different human cancers** by analyzing the vesicles and extracellular particles (EVPs) via a comprehensive proteomic analysis. This research demonstrated that EVP proteins can be used for cancer-type detection. Focusing on the mesothelioma data, the paper showed that immunoglobulins were the main family of proteins found in EVPs at a high frequency in mesothelioma. The study suggested that plasma-derived EVP protein signatures could be beneficial for cancer-type detection in patients. While encouraging, these findings need to be further validated and tested in a larger cohort of patients to confirm the results.
Besides the above-mentioned published studies, numerous studies have been performed with human mesothelioma cells, plasma from **asbestos-exposed** samples and mesothelioma patient samples. Studies have also been performed on plasma exosomes isolated from healthy volunteers, from the asbestos-exposed non-cancer group and asbestos-exposed mesothelioma group. Although the number of exosomes per ml of plasma did not differ in the various groups, there was a greater quantity of exosomal proteins in the various disease groups compared with the controls. The proteomic analysis performed on these samples showed the presence of coagulation-related proteins in the exosomes from the disease groups (mesothelioma and asbestos-exposed) compared with the controls. The control group plasma exosomes presented a signature comprising immunoglobulins, lipoproteins, and platelet-bound proteins. These data indicate an altered immune surveillance in MM samples concomitant with the increase of coagulation factors.

Conclusions

The studies reviewed above provide an initial framework for understanding potential biomarkers and the underlying biology of MM and asbestos exposure. Based on these findings, researchers can try to further identify means for early detection of asbestos exposure or the development of asbestos-related diseases, as well as discovering much-needed therapeutic targets.
Understanding the mechanism of how MM develops and progresses is an important area that can be used for the treatment of MM patients.
Ultimately, we hope that exosome research in MM will continue along this path and that more significant discoveries will be made toward understanding how asbestos causes cancer and finding ways to identify

dangerous exposure to asbestos and the early detection of cancer before a fatal diagnosis.
Although the field of exosome research is very prolific and has offered many opportunities for advancement in the medical field, it is not without challenges and limitations. Areas for improvement include methods for isolating exosomes, understanding the mechanisms of biogenesis, and the characterization of exosome cargo. We have achieved remarkable progress in this area, which gives us hope for potential breakthrough treatments and / or technologies.

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