Spring 5-5-2015

Effect of cmvIL-10 on Exosome Production by Human Breast Cancer Cells

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EFFECT OF CMVIL-10 ON EXOSOME PRODUCTION BY HUMAN BREAST CANCER CELLS

Thesis submitted for the Biology Honours Program
at the University of San Francisco

by

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May 2015

STATEMENT OF ORIGINALITY

I hereby declare that the work contained in this thesis is entirely my own, except where acknowledged, and has not previously been submitted for any purpose to this or any other University.

S. N. Basappa

ABSTRACT

Effect of cmvIL-10 on Exosome Production by Human Breast Cancer Cells

By

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Human cytomegalovirus (HCMV) is a ubiquitous virus that infects 70-90% of the general population, primarily the immunocompromised, but has been implicated in several forms of cancer, including breast cancer. Breast cancer is the second leading cause of cancer related deaths in women in North America, usually from metastasis. Exosomes are 30-100nm vesicles produced by most cells which carry protein and RNA to cells in their microenvironment. The aim of this study is to investigate the impact of HCMV-infection of a secreted viral cytokine, cmvIL-10, on exosome production by highly metastatic breast cancer cells.

MDA-MB-231 cells were cultured *in vitro*, and were treated with cmvIL-10. Exosomes were isolated from cell media via ultracentrifugation. A subsequent quantification colorimetric assay, quantitative polymerase chain reaction (qPCR), Western Blot and fluorescent tagging and reintroduction to untreated cells were used to determine number, content and localization of collected exosomes.

The results showed that there was a definite difference in the number of exosomes produced between MDA cells treated or not treated with cmvIL-10. There were significant quantities of exosomes produced by MDA cells treated for 72hrs, but not at earlier time points, nor in HEK293 cells at any time point, that could be measured. There was a statistically significant fold change in the amount of total RNA isolated from exosomes of MDA cells treated with cmvIL-10 ($p=0.011$). These data were further confirmed during the introduction of fluorescently tagged exosomes to untreated MDA cells, which demonstrated a perceptibly greater fluorescence in cells that took up exosomes treated with cmvIL-10. More exosomes per sample will need to be isolated to investigate miRNA or specific protein difference between groups; optimization of the protocol is required prior to further miRNA profiling and Western blot.

This study demonstrated that there is a difference in the quantity and content of exosomes produced by MDA-MB-231 cells following treatment with cmvIL-10. Further research may show whether these exosomic profiles can be viable biomarkers to indicate cancer and HCMV status.

LIST OF ABBREVIATIONS

cDNA	Complementary DNA
cmvIL-10	Cytomagalovirus Interleukin 10
CXCR4	Chemokine receptor type 4
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
exoRNA	Exosome RNA
FM	Fluorescent microscopy
GFP	Green fluorescent protein
Hsp70	Heat shock protein 70
HCMV	Human Cytomegalovirus
hIL-10	Human Interleukin 10
IL-10	Interleukin 10
miRNA	Micro RNA
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PMBC	Peripheral blood mononuclear cells
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
C_T	Threshold cycle
TBS-T	Tris buffered saline with tween

ACKNOWLEDGEMENTS

I would like to acknowledge and thank the members of my lab and the university for their guidance, help and support.

Firstly, I would like to thank Dr Juliet V. Spencer for allowing me the opportunity to work in her lab, to start my own project, even though it was a pilot study, and for her guidance and direction during my final year at USF.

Secondly, I would like to thank Margarette Mariano and Carolyn Tu for helping to oversee my progress, and for being the primary people to whom I would turn when I needed to learn a new assay for my project.

Thirdly, I would like to thank Vivian Young, Kathryn Allaire and Jordan Boeck for all of their technical assistance around the lab.

Fourthly, I would like to thank Dr Ketil Pedersen of Thermofisher Scientific for his exosome Western Blot protocol and advice, which forms a good basis for a future optimized protocol in our research.

Finally, I would like to thank my family and friends for all of their support throughout the course of my project.

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INTRODUCTION

1.1 Human Cytomegalovirus

Human cytomegalovirus (HCMV) is a ubiquitous virus that may affect between 70-90% of the general population (8, 15). The HCMV virion is an enveloped icosahedral capsid containing double-stranded DNA. HCMV is a member of the herpesviridae family, and is related to herpes simplex 1 and 2, varicella zoster and Epstein-Barr viruses (8, 17). HCMV is transmitted through bodily fluids, and can infect a large range of cells, most notably epithelial cells, endothelial cells, fibroblasts and smooth muscle cells, and can establish a life-long infection (12, 15, 16, 17). Although HCMV produces minimal symptoms or complications in the immunocompetant, it can devastate those persons who are immunosuppressed or otherwise immunoincompetant. Individuals with AIDS, transplant patients required to stay on immunosuppressive drugs to prevent organ rejection, and infants reliant on immunity acquired from their mothers' milk are all susceptible to illness resulting from HCMV infection, including pneumonitis, retinitis, and Blueberry Muffin Syndrome in infants (16, 17, 18). Patients undergoing treatments that are immunosuppressive, such as cancer patients, or whose immune systems are stressed are also at risk of developing HCMV-associated infections.

HCMV has many mechanisms for manipulation of host immune responses. Of particular note during HCMV infection is the production of a viral ortholog cytokine, cytomegalovirus interleukin-10 (cmvIL-10). As a mimic of human IL-10 (hIL-10), cmvIL-10 is capable of binding the host cellular IL-10 receptor (IL-10R), and does so with greater affinity than hIL-10 (4, 9, 12, 13, 16). Activation of the IL-10R Jak-Stat pathway results in immunosuppressive effects, such as inducing a decrease in proliferation of peripheral blood mononuclear cells (PBMC), production of inflammatory cytokines, and MHC expression in monocytes (13, 16, 17, 18). Endogenously, hIL-10 is utilized by the body to prevent long-

term, excessive immune response to pathogens; however, cmvIL-10 has high-jacked this mechanism to evade an immune response to the virus, inducing permissibility to HCMV and general immunosuppression (9, 13, 16). There is also some indication that late HCMV infection may predispose women to developing breast cancer independently (15).

1.2 Breast Cancer

Breast cancer is reportedly the second leading cause of cancer-related deaths in women in North America (1, 15, 16). Breast cancer, like all forms of cancer, is not a uniform disease; there are many cancer subtypes of different levels of danger. In cancerous tissues, mutations cause typically normal cell checkpoints to be disregarded, allowing cells to grow and divide out of control. If this unchecked growth and division occurs in a differentiated cell, the cell will behave like the surrounding tissue and may divide to produce a primary tumour that is unlikely to metastasize (the relocation of cells of a previously localized tumour to new tissue), and is not usually deadly. However, if a progenitor or stem cell is mutated similarly, the rapidly dividing cells are likely to have the ability to metastasize. If the stem cell is not killed by chemotherapy or radiation, it will continue to grow and spread throughout the body, leading to morbidity and death of the organism (19, 20).

Metastatic breast cancer composes an estimated 20-30% of all breast cancer cases (10), and is well known to be the most common cause of cancer related deaths. Metastasis will allow malignant cells to attach to tissue such as bone marrow tissue, or lung tissue, which can allow for tumour growth and stress on previously health tissue. In turn, resulting hypoxia of such tissues will allow cancer cells to thrive via the Warburg effect. Further, there can be permanent damage in the stressed organs, leading to apoptosis, necrosis and with extensive systemic damage, morbidity and death of the organism (10, 19, 20). Cancer cells, however, not only divide rapidly, but must signal to each other and to non-cancerous cells in their microenvironment in order for the immune system to remain permissive and to not destroy

these cells (16). One mechanism of cell signalling that may potentiate these effects is the production and release of exosomes.

1.3 Exosomes

Exosomes are small vesicles of approximately 30-100nm in size. Exosomes differ from microvesicles in that they are smaller and are derived from the endosomal complex, rather than by direct invaginations or pinching off of the cell membrane (7, 11). Of interest is that exosomes contain protein and RNA, and are able to carry their cargo between cells. Exosomes derived from different cell types under different conditions have distinct content (7, 11, 18). Current evidence suggests that protein, mRNA and miRNA specific to the exosome-producing cell can create a differential profile (11). If a profile specific to a diseased cell type under specific conditions was observed and recorded, then potentially same or similar exosome profiles can indicate a diseased state in that cell type. For example, according to Kruger et al, highly metastatic MDA-MB-231 cells and less metatstatic MCF7 cells are known to produce exosomes carrying only 27 proteins in common, with MDA-MB-231 cells producing 88 and MCF7 producing 59 proteins respectively (5). Hence, it is reasonable that exosomes from similar cancerous tissue may produce similar profiles.

In relation to HCMV infection, it is likely that cells affected by HCMV will produce exosomes with specific profiles, and that cancerous cells infected with HCMV or treated with cmvIL-10 will also have such specific profiles. Therefore, if these profiles can be characterized, exosomes may serve as biomarkers for cancer and virus infection status, easily collected from bodily fluids like blood or urine. The goal of this project was to determine the effect of either HCMV infection or exposure to cmvIL-10 on exosome production and content in exosomes derived from human breast cancer cells. Future research may indicate whether exosomes produced under these conditions may be profiled and whether they may prove as viable biomarkers indicative of health status.

MATERIALS AND METHODS

2.1 Model and Exosome Isolation

MDA-MB-231 human breast cancer cells (American Type Culture Collection, Manassas, VA) were cultured in L-15 Leibovitz's Medium (Corning, Manassas, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) or 10% exosome-free fetal bovine serum (System Biosciences, Mountain View, CA) and maintained at 37^{o}C with atmospheric CO_2 according to the suppliers instructions. Human embryonic kidney (HEK) 293 cells were grown in Eagle's minimal essential media (MEM) with 10% fetal bovine serum or 10% exosome-free fetal bovine serum in a humidified incubator at 37^{o}C and 5% CO2 atmosphere. Purified recombinant cmvIL-10 was purchased from R&D Systems (Minneapolis, MN).

Cells were grown to 80% confluence in 75cm^2 or 175cm^2 flasks, then were treated with exosome-free media and 100ng/mL cmvIL-10 for 24, 48 or 72 hours. Media was harvested for exosome isolation with Exo-Quick TC reagent (System Biosciences, Mountain View, CA) according to the manufacturer's instruction. Subsequent experiments resulted in revision of exosome isolation to ultracentrifugation adapted from Lasser et al for improved exosome yield (6). Briefly, harvested media was subjected to centrifugation 300 x g for 10 min at 4°C to pellet cells, then was transferred to ultracentrifuge tubes and subjected to ultracentrifugation 16,500 x g 20 min at 4°C to pellet cell debris. Supernatants were transferred to new tubes and subjected to ultracentrifugation 120,000 x g 70 min at 4°C to pellet exosomes, which were resuspended in assay-dependent buffer.

2.2 Exosome Quantification

Exosomes isolated by ultracentrifugation were resuspended in 150μL 1X phosphate buffered saline (PBS) and stored in -20°C until quantified. ExoCET assay kit (System Biosciences, Mountain View, CA) was used to quantify exosomes according to the

manufacturer's instructions. Briefly, 20µL exosomes were lysed with provided buffer and heated at 37°C for 5 min to liberate exosome proteins. Samples were centrifuged at 1,500 x g 5 min to remove debris. Serial dilutions of provided standard were prepared for a standard curve, and both sample and standards were mixed 1:1 with reaction buffer, incubated at room temperature for 20 min and read with a spectrophotometer at 405nm. Data were quantitated from the standard curve and statistical analysis was determined by unpaired t-test. One-way ANOVA was used for comparison between multiple groups.

2.3 RNA Quantification

Exosomes isolated by ultracentrifugation were resuspended in 150 µL exosome lysis buffer, incubated at room temperature for 5 min, and were stored at -20°C. SeraMir Exosome RNA Amplification Kit (System Biosciences, Mountain View, CA) was used to quantify exosome RNA (exoRNA) according to the manufacturer's instructions. Briefly, 5 µL of provided spike-in RNA control were added per sample. Then 200 µL 100% ethanol were added per sample and added to a spin column. Samples were spun down at 13,000 rpm for 1 min and flow through was discarded. Then 400 µL wash buffer was added per sample and centrifuged 13,000 rpm for 1 min three times, with flow-through discarded each time. Samples were dried by centrifuging 13,000 rpm for 2 min. The collection tube was replaced, 30 µL provided elution buffer was added to spin column membrane, sample was centrifuged 2,000 rpm for 2 min to load buffer, and then centrifuged 13,000 rpm for 1 min to elute exoRNA. Purity was checked with NanoDrop Spectrophotometer. RNA was used immediately, and remaining sample was stored in -80°C.

Exosome cDNA was prepared according to the manufacturer's instructions. Briefly, 5 µL exoRNA was added to PCR tubes with provided 2µL 5x poly A buffer, 1 µL $MnCl_2$ (25 mM), 1.5 µL ATP (5 mM), 0.5 µL poly A polymerase and incubated 37°C 30 min. Provided 0.5 µL SeraMir 3' Adaptor Oligo were added and samples were incubated 60°C 5 min, at room temp

2 min, then was placed on ice. Then 10 µL exoRNA, 4 µL 5X RT Master Mix, 1 µL 5' Sera Mira Switch Oligo, 1 µL reverse transcriptase and 4 µL RNAase free water were incubated at 42°C for 30 min, 95°C for 10 min, and maintained at 15°C.

To test total exoRNA, qPCR was performed according to the manufacturer's instructions. Briefly, 0.5 µL exo cDNA, 15 µL 2x SYBR mastermix, 1 µL 5' SeraMir spike-in assay primer, 0.5 µL SeraMir 3' reverse qPCR primer and 13 µL RNAase free water were added to a 96 well PCR plate. Then, qPCR was performed: 50°C 2 min, 95°C 10 min, 95°C 15 sec, 60°C 1 min, with 40 cycles of 95°C 15 sec, 60°C 1 min, and data read at 60°C 1 min. Differences in threshold cycle (C_T) values indicated fold changes, which were normalized as ΔC_T. Statistical analysis was determined by unpaired student's t-test.

To test specific miRNA qPCR was performed according to the manufacturer's instructions using specific primers. Briefly, a provided 384 well microRNA primer plate was reconstituted with 22 µL RNAase free water, then 1 µL of one primer was added to each well in a 96 well PCR plate (primers 1-48 per sample for two samples) with 5 µL of Master mix (143.8 µL2x SYBR mastermix, 4.88 µL SeraMir 3' reverse primer, 0.625 µL exo cDNA and 136.3 µL RNAase free water) per well. Then, qPCR was performed: 50°C 2 min, 95°C 10 min, 95°C 15 sec, 60°C 1 min, with 40 cycles of 95°C 15 sec, 60°C 1 min, and data read at 60°C 1 min. As C_T values were extremely high (between 30-40 cycles per miRNA), optimization of miRNA extraction per sample condition was required before difference between treatment groups could be quantified.

2.4 Protein Quantification

Exosomes isolated by ultracentrifugation were resuspended in 7.5 µL PBS and stored in -20°C. Exosomes were lysed with 1.5 µL of 5X RIPA buffer with protease inhibitor and sonicated 10 s at 20% duty cycle at 4°C. Lysed exosomes were incubated for 15 min at 4°C

to liberate exosome proteins. Exosome lysate was boiled 70°C 10 min after 9.3 μL 2xSDS (non-reducing) and 1 μL non-reducing loading buffer were added. Exosomes were subjected to SDS-PAGE and subsequent Western Blot onto nitrocellulose or PVDF membranes, blocked with 5% milk in 1X TBS-T, and probed with the following 1^{o} antibodies (Ab): PCNA, EGF, EGFR, IL-10, IL-10R (Santa Cruz, Dallas, TX), and Hsp70, CD63 and CXCR4 (VWR, Radnor, PA) at 1:1000 dilution in blocking solution. After washing, the membranes were incubated with a 1:2000 dilution AP-conjugated 2^{o} antibody and bands were detected using Western Blue stabilized AP substrate (Promega, Madison, WI).

2.5 Exosome Localization

MDA-MB-231 cells were seeded into 6-well plates at a density of 3×10^{5} cells per well and incubated for 24 hrs at 37°C. Exosomes previously harvested via ultracentrifugation in PBS were thawed, and 20 μL of thawed exosomes suspended in PBS were diluted in 480 μL PBS, were treated with either 50 μL 10X Exo-Red or Exo-Green and were incubated 37°C 10 min to label exosomes. 100 μL provided Exo-Quick TC was added to stop labelling reaction, and exosomes were incubated on ice 30 min. Samples were centrifuged 14,000 rpm 3 min to pellet exosomes. Supernatent with excess label was removed from exosome pellet, which was resuspended in 500 μL PBS. Cells were treated with labelled exosomes for 2-3 hours, then exosome localization was visualized with the Zeiss AxioObserver fluorescent microscope using Zen Black software.

RESULTS:

3.1 Exosome Quantity

In order to determine the effect of cmvIL-10 on exosome production by MDA-MB-231 breast cancer cells, exosomes were isolated and the quantities produced per condition were measured. System Biosciences Exo-CET exosome quantification kit was used to detect the numbers of exosomes released into media of cmvIL-10 treated cells at different time points. From the standard curve used, a minimum detection limit of 2.0 x 10^8 exosomes was required to identify a significant quantity of exosomes per sample condition.

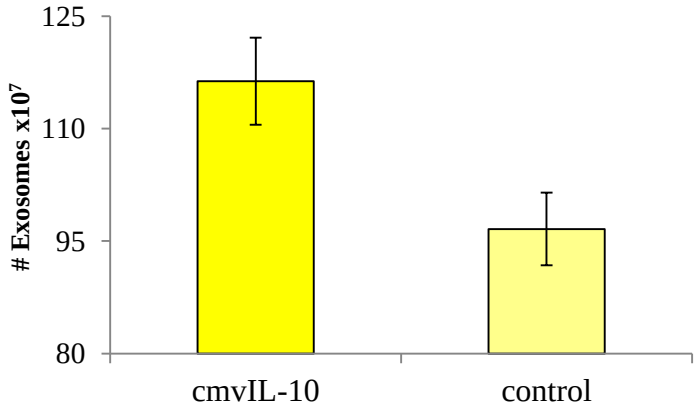

Figure 1. Exosome Quantity Post Treatment: MDA-MB-231 cells were cultured in the presence or absence of 100ng/mL cmvIL-10 for 72 hrs, and exosome numbers were measured via exoCET ELISA.

After 72hrs exposure to cmvIL-10, MDA-MB-231 cells produced more exosomes than the control cells. HEK293 cell controls and MDA-MB-231 cells treated for 24 or 48 hour post-treatment time points did not produce sufficient quantities of exosomes to be detected through the absorption assay. These other samples may have produced appreciable quantities of exosomes; however, none produced any within the 10^7 standard curve range required for this assay. While not statistically significant, there was an increase in the total number of exosomes produced by cmvIL-10 treated cells as compared to the controls ($p>0.05$) (Figure 1). The use of additional replicates may result in statistical significance between these groups. These results suggest that cmvIL-10 stimulates exosome production in MDA-MB-231 breast cancer cells.

3.2 Total RNA Quantity

In order to determine the effect of cmvIL-10 on exoRNA packaging, total RNA was measured in isolated exosomes produced per condition. System Biosciences SeraMir RNA quantification kit was used to acquire RNA samples, produce cDNA from the RNA, and prepare for subsequent qPCR to quantify amounts of total cDNA, which reflects the total RNA per exosome sample. Samples with more RNA were amplified at earlier cycles than those with less RNA. Both treatment groups were compared to a negative control (water instead of exo cDNA), which had late threshold cycles compared to either sample group, indicating the lack of DNA in the negative control (data not shown).

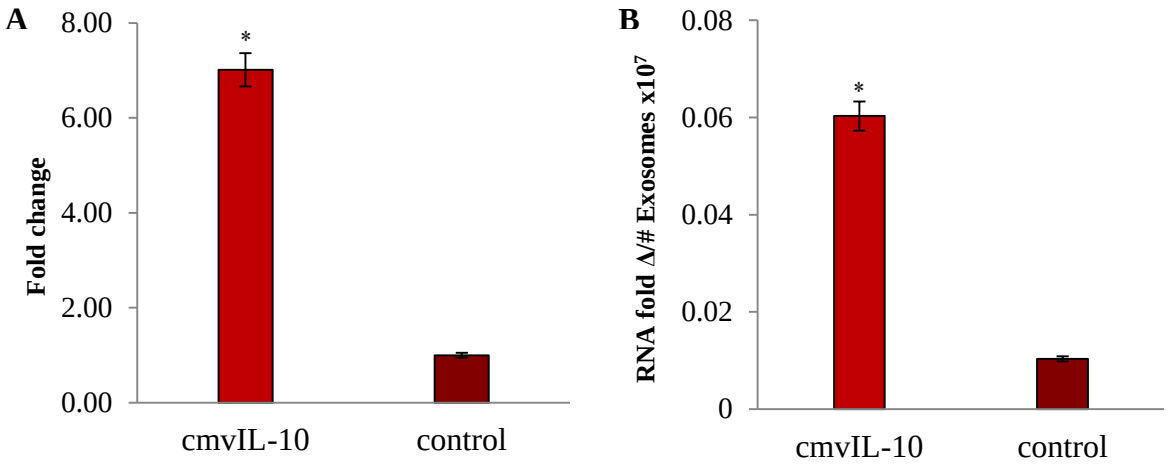

Figure 2. RNA Quantity Post Treatment: MDA-MB-231 cells were treated with 100ng/mL cmvIL-10 or were not treated, as controls, for 72hrs. Fold change was quantified (a) and normalized to exosome quantity on average (b). It was determined that $p = 0.011$ with a one-way ANOVA.

Cycle threshold (C_T) values of the test group, in absence of a specific housekeeping gene, were normalized to the controls, and fold changes per replicate were averaged. It was determined that cells treated with cmvIL-10 produced more exosomal RNA than control cells ($p<0.05$) (Figure 2a). These values were further normalized to average exosome quantity previously determined via Exo-CET analysis, and despite the difference in exosome quantity per condition, statistical significance persisted ($p<0.05$) (Figure 2b). These results indicate that exosomes produced in the presence of cmvIL-10 contain more RNA.

3.3 miRNA Content

In addition to examining the total amount of RNA per condition, and per exosome, an attempt to determine the levels of specific miRNAs was undertaken. Quantification of miRNA was attempted with System Biosciences' SeraMir kit, which may also be used for this purpose. With the same cDNA samples produced and used for total RNA quantification, further qPCR with sequence specific primers was undertaken. High C_T values (between 30 and 40) resulted for all cDNA per sample condition (data not shown). Low C_T values are preferable because they indicate high quantities of the desired cDNA in the sample, and therefore high original miRNA in the exosomes.

For the quantity of exosomes and RNA available, there were still low quantities of miRNA, including the high fold change of the cmvIL-10 treated samples. These data suggest that the majority of RNA change in the exosomes was primarily due to the presence of substantially increased mRNA or other RNAs, not significant changes in the levels of miRNA. Further optimization of exosome isolation and recovery is required to have significant and useful quantities of miRNA that allow differentiation between up or down regulation in each sample. miRNA profiling may allow for the potential development of a profile specific to exosomes produced by cmvIL-10 treated cells. Therefore, these data further suggest that more exosomes per condition need to be isolated in order to have sufficient miRNA to determine changes in exosomes produced in the presence of cmvIL-10.

3.4 Protein Content

To determine the effect of cmvIL-10 on exosome protein packaging, an attempt to measure the amounts of specific proteins in isolated exosomes produced per condition was also undertaken. It was expected that cmvIL-10 had the potential to change the protein concentration and types of protein carried by breast cancer cell derived exosomes. Western Blotting was used to attempt to determine the presence and quantity of the following proteins: PCNA, Hsp70, CD63, EGF, EGFR, IL-10, IL-10R, and CXCR4.

PCNA was initially thought to be a potential exosome-specific protein for MDA-MB-231 cells that would indicate the presence of exosomes and to be of low quantity in whole cell lysate; however, PCNA was detectable in whole cell lysate but not in the exosome lysate (data not shown). Subsequently, Hsp70 and CD63 were identified in the literature as general exosome marker proteins (System Biosciences), and CXCR4 was identified as a potential co-receptor for viral entry (2), which might be an indicator of permissibility of cells exposed to cmvIL-10 for HCMV virions. IL-10, cmvIL-10 and IL-10R were all identified as proteins associated with the immunomodulatory IL-10 pathway, while EGF and EGFR were identified as markers of chemotaxis status, as given by Valle-Oseguera and Spencer (16).

Samples underwent Western Blotting for all proteins of interest, but the lack of any significant bands in any exosome lane indicated that there was not enough protein in the exosome samples for Western Blotting detection. An exosome specific protocol was obtained, but further modification of this protocol is required for optimization of exosome Western Blotting under the required conditions. The treatment of new cells with exosomes harvested from cmvIL-10 treated cells, with subsequent Western Blotting for the whole cell lysate, may also indicate differences in protein levels. These protein levels are being studied further. These data suggest that more exosomes per condition need to be isolated in order to have sufficient protein to detect changes in exosomes produced in the presence of cmvIL-10.

3.5 Exosome Localization and Total RNA Confirmation

In order to determine the effect of cmvIL-10 on exosome localization and uptake, treated exosomes were introduced to untreated cells. Fluorescent labelling of exosomes was undertaken with System Biosciences Exo-Glow kit, using provided Exo-Green to label protein and Exo-Red to label RNA. Fluorescent microscopy (FM) was used to detect the labelled proteins and RNA of the introduced exosomes, and indicated that exosomes did localize to cells and were taken up by them (Figure 3). This uptake was especially apparent in the younger, newly divided cells compared to older cells. Cells were not treated with dye as a control, as excess label was removed from exosomes prior to treatment in the test groups.

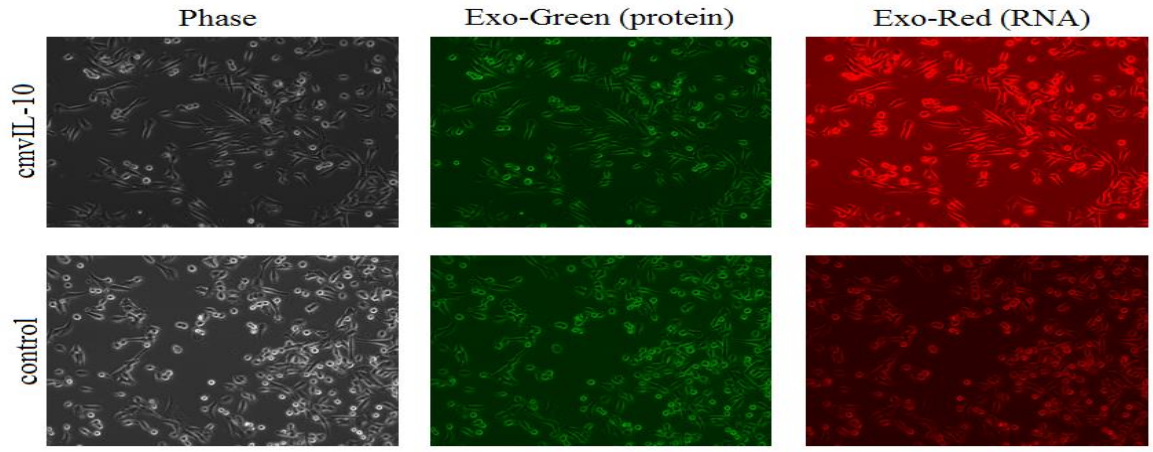

Figure 3. Exosome Localization in Untreated Cells: MDA-MB-231 cells were treated with 100ng/mL cmvIL-10 or were not treated, as controls, for 72hrs. Exosomes were harvested and introduced to wells seeded with $3x10^5$ MDA-MB-231 cells. Treated cells were observed after 3 hours with fluorescent microscopy.

Qualitatively, greater fluorescence for the Exo-Red dye in the sample from cmvIL-10 treated cells reflected the larger quantity of RNA in that group, which further confirmed the greater presence of RNA in cmvIL-10 treated cells (Figure 3). Exo-Green fluorescence was roughly the same in both treated and untreated cells, indicating that the overall amount of protein per sample had not changed in cmvIL-10 treated exosomes (Figure 3). However, while the total protein concentration may not have changed, the amount of any given protein may have changed. A change in proteins that increase metastatic potential, for example,

might have occurred, thereby validating the need for an optimized exosome Western Blot protocol. These data indicate further that cmvIL-10 treated exosomes do contain more RNA than untreated cells, and that exosomes produced under both treatments are taken up by untreated cells.

DISCUSSION

Human cytomegalovirus is a significant hazard to human health. While the immunocompetant normally do not suffer disease, HCMV is capable of remaining latent in the human body and reactivating in events of immunosuppression (13, 18). The development of cancer, and subsequent use of immunosuppressive chemotherapies and radiation therapy, is one possible trigger for reactivation. When an individual is susceptible to HCMV reactivation, it is necessary to be aware of the associated potential illnesses that may arise, and to act according to the situation. For situations wherein an individual has both cancer and HCMV, it is therefore also necessary to know the serostatus beforehand in order to treat that person with greater precautions, and with antivirals, in order to prevent or at least mitigate the potential complications that arise with concomitant HCMV infection, like retinitis and pneumonitis. The use of exosomes as biomarkers is a promising field, and may be used as early determining indicators of both HCMV serostatus, and of cancer prognosis. Therefore, a future goal of this research is to develop reliable exosome profiles of HCMV infection as a prognostic indicator for highly metastatic, triple negative (ER^-, PR^-, and $HER2^-$) breast cancer (3, 14).

The immediate goal of this project, however, has been to confirm that HCMV infection impacts the production and content of exosomes, such that the relevant profiles can later be produced. Previous research has indicated that MDA-MB-231 cells are more likely to survive and metastasize with treatment of the immunosuppressive viral cytokine, cmvIL-10, and therefore we used cmvIL-10 to treat MDA-MB-231 cells, after which exosomes were

harvested and characterized (16). It was found that treatment with cmvIL-10 induced greater exosome production in MDA-MB-231 cells at 72 hours as compared to all controls (Figure 1). While this trend is not statistically significant, it does indicate that, should these exosomes contain cmvIL-10 protein, or should it contain protein or RNA produced in response to treatment, they could be taken up by surrounding cells, thereby propagating the effects of the immunosuppressive ortholog. Furthermore, as infection of these cells has not yet been undertaken, it is expected that treatment with live virus may in fact cause these cells to produce even more exosomes with different content due to other viral means of infection, replication and host immunotolerance of the virus.

Furthermore, it was observed that treatment of MDA-MB-231 cells has also induced a statistically significant upregulation of the total RNA contained in the exosomes, even when normalizing for the increase in total exosome number with the treatment condition (Figure 2). The high C_T counts with the miRNA isolated from exosomes prevented their characterization. Nevertheless, as indicated by both their low quantity and by their rough similarity in C_T number per specific miRNA, one possibility is that the majority of the RNA upregulated in the exosomes was due to mRNA, or potentially other, unconfirmed RNA content.

The total protein content of exosomes did not seem to increase, as shown by similar levels of tagged protein fluorescence as compared to the greatly increased RNA fluorescence in the treatment group (Figure 3). However, because the RNA content in the exosomes harvested changed so drastically, even when the total protein amount remained constant, it is likely that there was a change in the specific proteins packaged. Further characterization of specific proteins of interest potentially affected by treatment of cmvIL-10 is necessary. To that end, until an exosome protocol under the necessary conditions can be developed, treatment of new cells with previously harvested exosomes may indicate changes in the amount of protein produced under these conditions. The treatment with cmvIL-10 produced

or normally produced exosomes will indicate the modifications induced to specific proteins or RNA contained therein.

The final goal of this project was to prove that the immunosuppressive HCMV cytokine, cmvIL-10, has a significant effect on cellular production of exosomes, such that a similar effect of HCMV infection can also be similarly characterized. Secondly, once these effects are confirmed, it will be possible to produce condition-specific exosome biomarkers of HCMV serostatus and breast cancer prognosis. Further studies to characterize exosomes of the less metastatic, less resistant MCF-7 breast cancer cell line will also be possible. Triple negative breast cancer, while the most difficult to treat, comprises only 15-20% of all breast cancers, while ER^+ lines like MCF-7 that are more responsive to chemotherapy and hormone treatment are the most common, estimated to be about 40% of all breast cancers, and are therefore also of interest (3, 14). In these ways, it is hoped that we may offer a simple, reliable method of the use of exosomes, easily harvested from blood, saliva or urine, as a medical diagnostic tool.

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