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Evaluating the combined effects of platelet inhibition and natural killer cell enhancement on cancer cell survival

Overview

One of the greatest challenges in treating cancer is its ability to spread to distant parts of the body, away from the primary site of disease. This process is known as metastasis. Metastasis is the leading cause of treatment failure in cancer, and overall cancer-related deaths. [1] There is currently no effective treatment option available for individuals with this advanced stage of the disease, and there is therefore an urgent need for the development of therapeutic intervention specifically targeted to the prevention of metastasis. [2] The major mechanism through which tumour cells spread to other parts of the body is through the bloodstream, and therefore most prevention methods target this area exclusively. [3] Current drugs mainly target primary tumour development and the processes that lead to metastasis, such as epithelial-mesenchymal transitions (EMTs), cell migration, and tumour invasion/intravasation into blood vessels. [2] However, studies have shown that while thousands of cells detach everyday from primary tumour sites and enter the bloodstream, [4] only a small percentage of these cells are able to exit the bloodstream and form secondary tumours (a process known as extravasation). [3] Therefore, the most efficient way of preventing metastasis appears to be through targeting the limited number of cells that manage to survive the migratory process.

Most research in the area has focused on increasing the effectiveness of the anti-tumour activity of the innate immune system in the bloodstream through cytotoxic natural killer (NK) cells. NK anti-tumour activity is generally effective in eliminating malignant cells from the bloodstream; however, since this process requires direct contact between NK cells and tumour cells, physical shielding of tumour cells can prevent NK attacks and increase the chances of metastasis. In this way, tumour cell-activated platelets have been shown to provide protection to tumour cells by coating them and providing a physical barrier from NK cells and their cytotoxic effects. [5][6]

Research has shown that the enhancement of NK cell activity is beneficial for metastasis prevention. [7][8] This has been largely achieved through the use of the cytokine interleukin-2 (IL-2), which has been shown to enhance and activate NK cell responses in vivo and in vitro. [9] Meanwhile, the deactivation of platelets in the blood has been shown to decrease the probability of tumour cell survival and to prevent metastases from forming. Most research in this area has focused on anti-platelet drugs or

anticoagulants such as Aspirin or heparin, which have consistently shown positive results on tumour cell survival and cancer prevention. [10][11][12][13]

Despite these results, there have been no published studies investigating the combined effect of natural killer cell enhancement and platelet inhibition. This is surprising, given the high potential for positive outcomes. This research project proposes to investigate the combined effect of NK enhancement and platelet inhibition in a co-culture environment with NK cells, platelets and cancer cells. Specifically, it will make use of CD56+ (NK) and MDA-MB-468 (breast cancer) cell lines, platelet concentrate, IL-2 (NK enhancement) and Aspirin (anti-platelet activity). The effects of combined IL-2 and Aspirin treatment will be compared to IL-2 and Aspirin alone, and outcomes will be recorded as cancer cell survival levels over a predetermined time period.

Significance

The results of this study will determine whether combining the effects of platelet inhibition and natural killer cell enhancement can have a synergistic effect on killing tumour cells and preventing metastasis through the bloodstream. This is an important question to consider, as the effects of IL-2 and Aspirin in the same environment have not previously been investigated, and there is no indication that their combined result will be the sum of their individual effects. There is also the possibility that IL-2 and Aspirin may combine to produce a negative effect on metastasis prevention, or that they simply are not compatible with each other in the same environment. Preventing metastasis is one of the major goals of modern cancer research, since it allows oncologists to focus treatment on the site of the primary tumour, where patient outcomes are much more optimistic. Therefore, this is a significant study for the future of cancer treatment strategies, as successful completion of the project will provide researchers with data on which to base the development of new drugs aimed at the prevention of metastasis, and allow for further research in the field of metastasis prevention through the targeting of extravasation.

Hypothesis

Given that inhibition of platelet activation and enhancement of natural killer cell cytotoxicity have been shown to negatively affect cancer cell growth and metastases, it is predicted that inducing both of these effects will result in a greater tumour cell response than either strategy alone. Therefore, it is hypothesized that the addition of interleukin-2 and Aspirin to a culture of CD56+ NK cells, MDA-MB-468 breast cancer cells, and platelets, will result in decreased MDA-MB-468 cell survival over a predetermined period of time, when compared to the effects of interleukin-2 or Aspirin alone.

The specific aims of the study are:

- determine a method for culturing platelets, cancer cells and natural killer cells in a culture medium that prevents activation of platelets and allows for cell migration
- determine the length of time over which the effects of interleukin-2 and Aspirin occur after being added to the cell culture, specifically with respect to the activity of NK cells against cancer cells
- determine the mechanism by which cancer cell survival is primarily affected (whether it is due to platelet inhibition, NK activity, culture environment changes, etc.) through comparison to different control cultures

Methodology and Project Timeline

The goal of this experiment is to record data regarding the viability of cancer cells in co-culture with platelets and NK cells in the presence of factors influencing the activity of these cells. Human interleukin-2 will be used to promote NK cell activity, while acetylsalicylic acid (Aspirin) will be used to inhibit platelet aggregation around cancer cells. The cancer cell line that will be used is MDA-MB-468, a human breast adenocarcinoma. If NK cells cannot be obtained from a donor, they will be replaced with Jurkat cells, a T lymphocyte leukemia cell line, and hydrogen peroxide, to induce cancer cell apoptosis. [14]

The project relies on the ability to stain the cancer cells with an appropriate dye that will last through the lifetime of the cells and act as an indicator of cell viability. The stain selected for these purposes is Calcein AM [15], a metabolic fluorescent dye that fluoresces green until deactivated upon cell death. The stain will be combined with the cancer cell line prior to co-culture, allowing for viability measurements. The initial dye concentration must be sufficient to allow for visualization after multiple cycles of cell division.

In order to observe interactions between the three cell types, an appropriate medium for co-culture must be established. Most media commonly used for NK and MDA-MB-468 cultures contain calcium, which activates platelets and causes aggregation. This is not suitable for the purposes of this experiment, since all platelet activity should be derived from interactions with the cancer cells themselves. Therefore, cells will be cultured in a modified Tyrode's solution (calcium-free) with added HEPES buffer. The components of the medium are as follows: 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.2 mM Na_2HPO_4, 12 mM $NaHCO_3$, 5.5 mM D-glucose, 25 mM HEPES. [16][17]

Once the cells have been trypsinized, washed with PBS, and cultured in the medium, the experiment will be carried out. The experiment features two negative controls, a positive control and three test cases. One negative control for the experiment will feature MDA-MB-468 cancer cells cultured in the prepared medium, and will act as a baseline assessment for all experiments since there will be no influence from NK cells or platelets. A second negative control will feature a co-culture of all three cell types in the prepared medium, which indicates the baseline level of interaction between platelets and NK cells, and the effect that they have on the viability of the cancer cells. The positive control for the experiment will be NK cells co-cultured with cancer cells (in prepared medium) without platelets, which is expected to result in low cancer cell viability due to the cytotoxic effects of the NK cells. Once controls have been established and the culture medium is deemed appropriate for the survival of the cells, the final step is to investigate the test cases and measure the results. Aspirin is indicated to have an effect in vitro at concentrations of 325 mg/L [18], while the concentration of interleukins has been shown to have activating effects at ratios of 20:1 to NK cells. [19] The affectors will be added to samples of the culture individually (one IL-2, one Aspirin), and a third test case will feature both IL-2 and Aspirin. This will provide indications as to the individual effects of IL-2 and Aspirin, as well as their combined effect.

Each of the six experiments will be executed in triplicate (three separate cultures for each experiment). Cancer cell viability will be measured through the use of two techniques: fluorescence intensity readings on a microscope, and a manual viability cell count using a hemocytometer. Three separate measurements will be taken using both methods at each specified timepoint. Each culture will be measured at 1 hr, 48 hrs and 1 week after culturing (which takes place Tuesday morning) and will be incubated in the CO_2 incubator at 37 °C in between measurements. Cancer cell viability data will be analyzed to determine the effectiveness of IL-2 and Aspirin in combination, as well as to measure the effectiveness of the treatment over time. The proposed project timeline is summarized below:

Week 1: Culture MDA-MB-468 cells in standard RPMI-1640 medium, stain with Calcein AM. Measure cancer cell viability to ensure quantification can be performed as outlined in protocol using fluorescence assays and hemocytometer cell counting. Culture Jurkat cells in RPMI-1640 medium and combine with MDA-MB-468 culture. Repeat viability measurements, place culture in incubator. Contact research labs to request NK cell donations, gather materials needed to create modified Tyrode medium.
Week 2: Assemble modified Tyrode medium with HEPES buffer. Assess viability of triple co-culture with MDA-MB-468, NK Cells and platelets. Record final viability measurements of week 1 culture. If NK cells are not yet obtained, attempt to request from other possible suppliers/labs.

Week 3: If NK cells are still unavailable, switch to contingency plan of Jurkat cells and hydrogen peroxide. Culture control experiments (two negative controls, positive control) in prepared Tyrode medium, and record viability measurements over the week. Store in incubator.
Week 4: Record final viability measurements of control experiments. Culture test cases (IL-2, Aspirin, and IL-2 – Aspirin combination) in prepared Tyrode medium, and record viability measurements over the week. If control experiments fail, re-culture this week. Store all experiments in incubator.
Week 5: Record final viability measurements of test cases. If any experiment fails, re-culture this week.
Week 6: Buffer week for experimental delays or protocol changes. Data analysis and reporting.

Budget

The following table summarizes the items needed for the research project, with supplier, amount and cost information. The total cost of $273.01 is under the prescribed budget of $300 CAD, with some room for flexibility over the length of the project. Currency conversions were calculated using the Bank of Canada's official exchange rates for February 10, 2020. [20]

Item	Supplier	Product #	Size/amount	Cost (CAD)	Notes
Primary CD56+ NK Cells	Donor (Information: ATCC)	ATCC PCS-800-019	1.0 mL	$0	Not yet obtained. Info: [21]
MDA-MB-468 Cells	IBBME Teaching Lab (Information: ATCC)	ATCC HTB-132	1.0 mL	$0	Info: [22]
Platelet Concentrate	zenbio	SER-PCEX	60 mL	$178.47	[23]
Interleukin-2	GenScript	Z00368	10 μg	$86.57	[24]
Aspirin	Wal-Mart Canada	4008121	3.025 g	$7.97	[25]
Calcein AM	IBBME Teaching Lab (Information: ThermoFisher)	C1430	1 mg	$0	Ordered by another research group, made available. Info: [26]

Trypsin, HEPES, PBS, NaCl, KCl, $MgCl_2$ Na_2HPO_4, $NaHCO_3$, D-glucose, Jurkat cells and hydrogen peroxide (if necessary) are available from the IBBME Teaching Lab, according to inventory as of January 24, 2020. [14]

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