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Effect of Myeloma-Derived Microparticles on in Vitro Proliferation and Viability of Alloimmune Human Peripheral Blood Mononuclear Cells

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Abstract

Background: Microparticles (MPs) are small vesicles released from the cell membrane. Accordingly, these contain active molecules to mediate biological processes, including cell proliferation and cell cycle progression. The fusion of myeloma cell lines with immunized B lymphocytes is a critical step of hybridoma technology. MPs modulate lymphoproliferation, thereby facilitating B cell expansion and successful immortalization. Human alloimmune B cells are considered valuable sources of monoclonal antibodies, and peripheral blood is a pool of B lymphocytes. The present study aimed to investigate the role of myeloma-derived MPs in the proliferation of alloimmune peripheral blood mononuclear cells (PBMCs).

Materilas and Methods: In the current experimental study, ultracentrifugation isolated MPs from three different myeloma cell lines, including U266, P3x63Ag8, and SP2/0. PBMCs were extracted from the whole blood of a patient with thalassemia alloimmune exposed to MPs. Thereafter, both the proliferation and cell viability were evaluated using inverted microscopy and the trypan blue staining method.

Results: MPs derived from the P3X63Ag8 myeloma cell line were shown to have the most effects on cell proliferation and viability (p=0.0005). MPs of the SP2/0 cell line initially increased the proliferation of PBMCs, but viable cells were drastically reduced in the following weeks. As well, U266 cell-derived MPs increased the proliferation of PBMCs (p=0.0001), but it was half of the group receiving P3x63Ag8 derived MPs. However, the viability of treated cells remained almost constant for 5-weeks.

Conclusion: Altogether, the obtained data indicated that P3X63Ag8 and U266 derived MPs could increase the viability of PBMCs. If the complimentary examination is confirmed, these MPs could also be used as the potential agents in B lymphocyte proliferation, thereby helping in immortalization and antibody production.

Keywords: Alloimmune, Cell-Derived Microparticles, Cell Proliferation, Myeloma, Peripheral Blood

Introduction

Microparticles (MPs) are a heterogeneous population of small vesicles (ranging from 100 to 1000 nm) that originated from the plasma membrane of different cells (1). Accordingly, these contain different molecules, including proteins, phospholipids, RNAs, and DNA, which can transmit among cells (2).

MPs contribute to the different physiology and pathophysiology processes, including blood coagulation, inflammation, intercellular signaling, cell proliferation, and cancer development (3).

MPs can be isolated either from biological fluids (e.g., blood, synovial fluid) or cell cultures using centrifugation techniques (4). Several studies have previously shown that MPs increase in both solid and hematological malignancies like myeloproliferative neoplasms (5-7).

It was indicated that in Multiple Myeloma (MM), as a neoplastic plasma cell disorder, uncontrolled proliferation of malignant plasma cells occurs in the bone marrow (8). In 1975, Milstein and Kohler discovered a method based on the hybridoma technology for the immortalization of B cells of interest by fusing them with cancer cells (myeloma) to produce immortal hybridomas (9). Alternative methods proposed for immortalizing mammalian cells also use viruses expressing HPV-16 E6/7, EBV, and

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SV40 T antigens; hTERT, siRNAs of p53; and RB and ras & myc oncogenes (10).

Human hybridoma technology is an important approach used for monoclonal antibodies production. The fusion of two human lymphocyte lines and the fusion of spleen lymphoid cells from patients with Hodgkin's lymphoma with U266 human myeloma cell line were the first two successful processes related to the production of human monoclonal antibodies (11). Moreover, the fusion of mouse myelomas cell lines such as P3X63Ag8.653, Sp2/0-Ag14, NS1, and NS0 with primary human B cells is another way to produce human hybridoma cells (12). A major limitation of hybridoma technology is its low efficiency. Various cytokines and feeder cells can help expand an antigen-specific single B cell to clone (13). Thereafter, the expanded antigen-specific cells can be immortalized by fusion with myeloma in order to form a hybridoma (11).

MPs modulate lymphoproliferation and may indirectly affect the immunoglobulin production (14).

Additionally, MPs derived from tumor cells can regulate immune cells as well as changing the immune response (2).

Messer et al. have demonstrated that in an in vitro system, MPs could contribute to the B-cell activation through the production of soluble mediators (15).

In a previous study conducted on acute leukemia, which is a disease defined as the uncontrolled extension of hematopoietic progenitor cells (16), in vitro leukemic and non-leukemic B cell-proliferation were observed after exposure to leukemia-derived small Extracellular Vehicles (EVs). In this regard, further studies have also revealed that these EVs contain some factors affecting cell proliferation and survival (17).

In the allogeneic blood transfusion, as a system of temporary transplantation, different foreign antigens are present in the recipient, which can consequently lead to an immune response (18) as well as some severe consequences. Human alloimmune B cells can be used as a valuable source of monoclonal antibodies against allogeneic antigens. The produced antibodies can also be used to screen antibodies to find compatible blood for alloimmune patients.

As mentioned earlier, the expansion of B cells is known as an important factor for their successful immortalization and subsequent antibody production. Accordingly, Peripheral Blood Mononuclear Cells (PBMCs) are recognized as the important pools of B lymphocyte cells.

In the present study, the effects of myeloma-derived MPs on the proliferation of alloimmune PBMCs were investigated using both microscopic evaluation and dye exclusion staining methods.

Materials and Methods

Cell lines and growth conditions

At this stage, three myeloma cell lines, including U266 as a human, and P3x63Ag8 and SP2/0 as mouse sources, were evaluated. All cell lines were obtained from Pasteur Cell Bank and then routinely propagated in RPMI 1640 (Bio-Idea, Iran) and supplemented with 10% FBS, 100 U penicillin/mL, and 100 mg/mL streptomycin. The cultures were incubated in a humidified atmosphere at 37˚C containing 5% CO2 and passaged following the logarithmic growth phase (approximately once every three days).

In vitro generation and isolation of MPs

In this phase, myeloma cell lines supernatants were centrifuged at 1500 rpm for 5 minutes, leaving the cell pellet behind. Afterward, the cell-free supernatants were collected and then centrifuged at 16,000 rpm for 15 minutes at 4 °C to remove any cell debris. The supernatant was discarded and the pellets were washed two times with PBS and suspended in 200 µL of RPMI medium. Isolated MPs were filtered through a 0.22-µm filter.

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Determination of MPs concentration by Bradford assay

The protein concentration of platelet-derived MPs was determined using Bradford assay via a spectrophotometer (TECAN, Australia). At this stage, briefly, varying dilutions of bovine serum albumin (BSA) were prepared (including 312.5 µg/mL, 625 µg/mL, 1250 µg/mL, 2500 µg/mL, 5000 µg/mL, and 10000 µg/mL). Afterward, BSA (10 µL) or MPs samples were mixed with Bradford reagent (200 µL), and then the absorption rate was measured at 595 nm. Finally, a standard curve was drawn using the BSA samples, which was then used to determine the concentration of MPs.

The isolation of PBMCs with Ficoll density gradient

In this experimental study, PBMCs were obtained from the whole blood belonging to a thalassemia patient who was alloimmunized against RhE antigen using centrifugation. Accordingly, the PBMCs layer was isolated after Ficoll-Hypaque (Baharafshan, Iran) density gradient centrifugation at 1800 rpm for 20min, and then centrifuged again at 2000 rpm for 12 min. Finally, the supernatants were removed, and the cell pellet was washed three times with PBS at 1500 rpm for 5min.

Exposure of PBMCs to myeloma-MPs

PBMCs were cultured at a density of 2×10^4 cells per /well in 96-well plates and then incubated with 5 µl of MPs in the RPMI culture medium, which was previously supplemented with 10% fetal bovine serum, 10,000 IU/ml penicillin, 10,000 ug/ml streptomycin, and 2 mmol/l L-glutamine. Afterward, these were incubated for a 5-week duration at 37 °C and 5% CO2. The total concentrations of P3x63Ag8, SP2/0, and U266 MPs were obtained as 4200 µg/ml, 3000 µg/ml, and 3600 µg/ml, respectively. Of note, PBMCs without any MP were used as a control group.

Evaluation of lymphocytes proliferation and viability

Inverted light microscopy evaluated cell proliferation. In addition, a trypan blue (TB) dye exclusion assay assessed cell viability. At this stage, in brief, the cells exposed to MPs were firstly collected and then stained with 0.4% trypan blue solution on the 7th day of co-culturing, which continued once a week for five weeks. Correspondingly, the used method was based on the fact that intact cell membranes of live cells exclude some certain dyes like trypan blue, whereas dead cells do not perform in this way. The enumeration of viable cells was also achieved using a hemocytometer under a phase-contrast microscope.

Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was performed using the Student t-test. All the analyses were performed using Prism 8 software. P-value < 0.05 was considered as statistically significant.

Ethical consideration

The blood sample used in the present study was obtained from an alloimmune thalassemia patient who was referred from Adult Thalassemia General Clinic. Informed consent was obtained from all patients referred to this clinic. The human ethics protocol in this study was approved by the Iranian Blood Transfusion Research Center Human Ethics Committee (Approval # IR.TMI.REC.1399.016).

Results

Inverted Microscope Observations

The evaluation of cells under the inverted microscope showed a considerable proliferation in the test groups, especially among PBMCs exposed to mouse myeloma-derived MPs, P3X63Ag8, compared to the control group without any exposure to MPs. Figure 1 shows the accumulation of multiplied cells after the treatment with different myeloma-derived MPs. However, the cell proliferation rate in the group that received human myeloma MPs, namely U266, was lower than that of the group that received P3X63Ag8 MPs. Notably, no considerable change was observed in the proliferation rate after the

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cells treatment with mouse myeloma derived-MPs, namely SP20.

Cell viability measurement

At this stage, trypan blue solution stained lymphocyte cells for counting live cells with a hemocytometer. Table I shows the number of viable cells and their mean±SD after receiving MPs once a week. The mean of cell viability in the test groups was obtained to be higher than that of the control group. The highest number of viable cells was observed in the group treated with P3x63Ag8 mouse myeloma cell MPs (p=0.0005).

Although the cell viability increased in the group that received SP2/0 mouse myeloma cell MPs at the first week, it started a decreasing trend in the next weeks. A significant increment was also found in cell viability in the group treated with U266 human myeloma cell MPs (p=0.0001). However, it was lower than that of the group treated with mouse myeloma cell MPs. A bar chart of viable cells in each of the MPs treated groups compared to the control group during five weeks is shown in Figure 2.

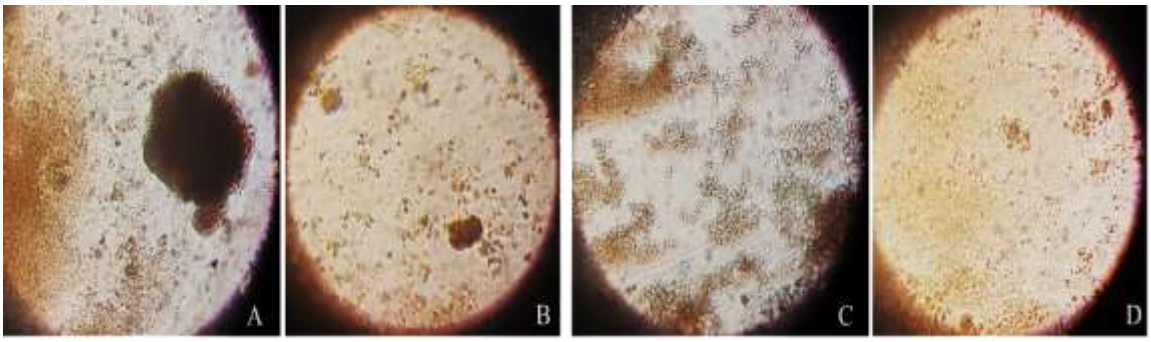

Figure 1. Microscopic image of the cells exposed to MPs. A and B: the cells exposed to MPs derived from mouse myeloma cell lines P3X63Ag8 and SP2/0, respectively, C: the cells that received MPs from human myeloma U266 cell line, and D is the control group. The most proliferation and cell accumulation were seen in the groups treated with P3X63Ag8 and U266 MPs (A and C). No considerable changes were observed in cell proliferation of the group treated with SP20 MPs (B).

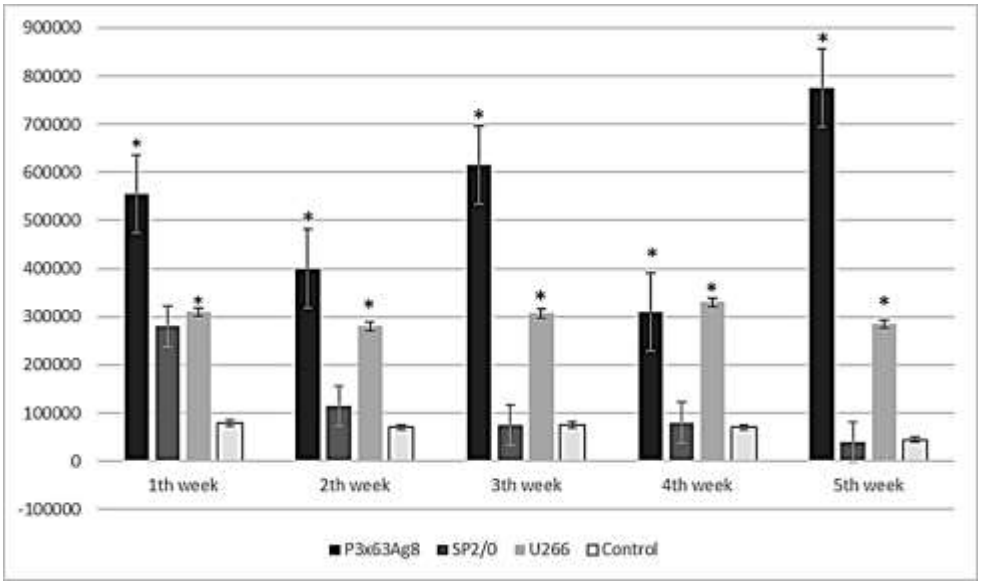

Figure 2. The chart of viable cells after receiving different mouse and human myeloma-derived MPs compared to the control group.

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Table I: Statistical findings of studied groups after exposure to MPs

	Control	Mean± Standard deviation	P3x63Ag8	Mean± Standard deviation	P Value	SP2/0	Mean± Standard deviation	P Value	U266	Mean± Standard deviation	P Value
1st week	80000	68000± 13509.2	555000	531000±182462.3	0.0005	280000	118000±94379.5	0.2	310000	302500±20310	0.0001
2nd week	70000		400000			115000			280000		
3rd week	75000		615000			75000			307500		
4th week	70000		310000			80000			330000		
5th week	450000		775000			40000			285000		

Discussion

Mammalian blood cells can generate different MPs under different conditions. Additionally, the effective role of MPs overexpression under different physiological and pathophysiological conditions has been confirmed earlier (19, 20). Both in vitro cell lines and in vivo animal models revealed the importance of MPs as key mediators in some processes, including cancer's growth, proliferation, apoptosis, and metastasis (21). Moreover, it was found that MPs can initiate coagulation by thrombin generation and clot propagation (22, 23). In addition, MPs affect the enzymatic systems that control oxidative stress generation (24). Furthermore, the other roles of MPs include their direct contribution to the inflammatory response (25) and regulation of angiogenesis by increasing post-ischemic capillary density and proliferation (26). MPs contain pro-survival factors, including FGF2 and VEGF mitogens (27), which can transfer biological messages between cells and act as intermediates in the homeostasis of multicellular organisms by maintaining a balance among cells stimulation, proliferation, and death (28). Although approximately 80% of circulating MPs are derived from platelets (29), they can be generated from other cellular origins like cancer cells (30, 31). Compared to normal cells, cancer cells shed considerably larger amounts of MPs, which can cause more invasion and disease progression (21). Cancer cells-derived MPs predispose the way for normal cells to enter the tumorigenic stage as well as enabling them for both phenotypical transformation and tumor growth by promoting proliferative signaling. Although the mechanisms of MPs in cell proliferation and cancer are not completely known yet, the similarity of tumor MPs to the tumor cells in such a way that both of them contain tumor-specific antigens has been previously confirmed in several studies (32, 33). In this regard, in the Bernimoulin's study, the expression of tumor-origin antigens has been reported in plasma derived-MPs of patients with cancer (i.e., Mucin-1 or MUC-1) (34). MPs can also lead to aggressive phenotypes being transferred by facilitating the intercellular spread of oncogenes. As well, MPs are capable of transitioning normal cells in the tumor microenvironment (TME) into anaplastic cells (21). Remarkably, this process could have important overlaps with other microenvironmental stimuli like B-cell receptor stimulation. In another study, EVs derived from mesenchymal stem cells of leukemia patients were indicated to prevent the leukemic cells' entrance to the

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apoptosis phase, which consequently resulted in greater movement and stronger gene alteration (35). Tumor cells can shed microvesicles (MVs) in an increased, dysregulated fashion, resulting in the presence of circulating MVs in patients with different cancers, including lung, breast, melanoma, and CLL (32). Although numerous publications in recent years have demonstrated MV shedding from tumor cells, this area of investigation is still considered a relatively novel area, especially in the field of MM.

The current study investigated the effect of MPs derived from human and mouse myeloma cell lines on the alloimmune lymphocyte cells proliferation. The effects of a 5-week exposure with MPs were investigated using PBMCs prepared from the whole blood belonging to an alloimmune patient in terms of both cells viability and proliferation. Moreover, TB dye exclusion assay was used in this study to evaluate cell viability because it is considered a standard cell viability measurement method and is still the most commonly used method in this field. Additionally, the combination usage of TB and hemocytometer is known as the standard approach for the estimation of living cells number in the culture (36). The present study revealed that following the treatment with mouse myeloma cell -P3X63Ag8- and human myeloma cell-U266, both proliferation and the number of viable PBMCs increased. Of note, there were some increments in the proliferation of cells treated with mouse myeloma cell SP2/0 MPs in the first week, but a decreasing trend started in the next weeks.

Based on previous surveys in this field, this is the first study investigating the effects of myeloma-derived MPs on PBMCs as one of the important sources of B lymphocyte cells. It was shown that myeloma derived-MPs could induce proliferation of PBMCs. Previous studies showed that MPs, released by multiple myeloma cells, play a role in stimulating MM cell proliferation (22). In another study by Gargiulo, it was shown that small extracellular vesicles released by Burkitt's lymphoma cell lines could stimulate B cell proliferation and antibody production (37). Patel et al. have reported that in acute leukemia patients, EVs shedding from leukemic clones enhanced proliferation and survival of the low density growing cells (38). In addition, lymphoma-derived small EVs increased the release of IL-10, thereby causing persistent Breg activation and expansion (37). Moreover, MPs are known as major sources of microRNAs (miRNAs) and transfection of immune cells with platelet miRNAs was found to result in cancer promotion indirectly (39). On the other hand, MPs derived from platelets were observed to be able to induce B lymphocytes for antibody production (40). Other biologically active molecules such as DNA, RNA, nuclear proteins, cell adhesion molecules, growth factors, and cytokines, which are present in MPs, may also enable them as inducers of cell proliferation (21, 35, 41-43). In acute leukemia, the shedding of MPs containing miR-118 and miR-116 promoted cell proliferation and prevented cells from apoptosis (44). Finally, discovering the mechanism of MPs in the mediation process of intercellular communication could be an important component in laboratory research, especially in finding novel strategies for cell proliferation, immortalization, and even for antibody production.

Conclusion

In the present study, myeloma-derived MPs were found to have the ability to induce both proliferation and viability of PBMCs. By considering B-lymphocytes as one of the cell populations present in PBMCs, myeloma-derived MPs can act as effective agents in the proliferation of alloimmune B cells. Besides, they can enhance their chance for the subsequent antibody production process. However, our study population was small and included only a patient's peripheral blood cells, so in order to confirm the obtained results, analysis of

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further samples is needed. Moreover, additional studies, including serological and molecular tests, are required to evaluate better the effective role of myeloma-derived MPs in human monoclonal antibody production.

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Conflict of intrests

The authors declare no conflict of interest.

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