Original Article

Cellular Effects of Wound Fluid (Seroma) from Tumor Bed on Human Breast Cancer Cell Lines

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Abstract

Introduction: Post-lumpectomy wound fluid (seroma) contains many proteins from tumor bed due to physiologic answer to operation and wound healing process. Some cellular tests had been performed on different types of breast cancer (BC) cell lines and normal cell line while treated with seroma.

Materials and Methods: The wound fluid samples were collected from BC patients. The human BC cell lines included MCF-7, MDA-MB-231 as well as normal non-tumorigenic epithelial cell line (MCF-10).

Results: Seroma could inhibit various cancer cells proliferation pattern in comparison with the normal cell. Concerning the cell death, aggressive MDA-MB-231 cells were put into the apoptosis process. Besides, seroma could decrease colony count and size and changed the clone morphology from holoclone to paraclone. Regarding the invasion assay, seroma significantly inhibited cell motility.

Conclusion: By remaining in tumor bed, seroma can induce inhibitory pattern of proliferation, and change the morphology of cancer cell colony and cell motility, consequently leading to positive impact on patients who suffer from cancer.

Keywords: Post-lumpectomy wound fluid, apoptosis, colony

1. Introduction

Breast cancer (BC) is a public cancer, identified in females universally and is reported to be the fifth reason of cancer-related deaths among this population [1, 2]. Breast cancer is the multipart sickness and has heterogeneity regarding histopathology, genomics and proteomics variation, treatment reaction and patient result [3]. Surgical treatment contains both mastectomy and breast-conserving surgery (BCS). Nowadays BCS, including surgical tumor elimination plus irradiation is the typical care for the BC controlling. Tumor bed or margin is the maximum risk region for local recurrence due to the possibility of tumor cells remaining in that area [4, 5].Tumor microenvironment (TME) shows a vital part in next cell fate result, in order to select surviving and proliferation or death/senescence [6]. Intercellular junctions or TME as cell-cell signaling clarify this role. Post-lumpectomy wound fluid (seroma) contains many growth inflammatory factors due to physiologic answer to operation and wound healing process [7]. Cancer cells have particular features such as cells with environmental independence for growth, cells with evasion of apoptosis, cells with limitless proliferative potential, cells with angiogenesis and cells with invasion as well as metastasis in different regions [8]. While many researchers showed that seroma has to

be extracted by surgeon from margin, some studies has approved the effectiveness of seroma in view of protection role, and they are against of vacuumming it at operating room. According to our knowledge and previous studies, it has been decided to design this study for better defining of seroma effectiveness through investigation the cellular effects and some molecular changes related to them and finally, providing answer to these questions; whether seroma has to be removed or remained in tumor bed? Is there a difference in obtained results' significance?

2. Materials and Methods

2.1. Patients and Sampling

The wound fluid (seroma) samples were collected from BC patients. Seroma was collected from the drain after 24 hours (h) of surgery. In this method, seroma was gathered directly from the wound healing surgery site. The seroma was allowed to gradually be collected in the drain during 24h and then was all gathered. All samples were obtained from different patients with different molecular and pathological signature who underwent BCS in the Shohada hospitals (Table 1). The gained seroma was collected in protease inhibitor solutions and was transferred under icepack to the laboratory. Seroma was centrifuged (300g in 5min), sterile filtered (0.22 and 0.45 µm) and stored at -80^{0}C.

Table 1. Patient information by study individual

		Pathologic data									
Pt Number	Age	Tumor history	Tumor size (cm)	Node status	Tumor grade	ER/PR status	HER-2 Expression	Ki67	P53	Tumor Necrosis	LVI
25	40-50 y	IDC	1	N0	1	+	Negative	30%	+	Negative	Not seen

2.2. Cell culture and proliferation assay

In this study, the human BC cell lines (MCF-7, MDA-MB-231) pulse control non-tumorigenic epithelial cell line (MCF10) were selected, all obtained from Iranian Biological Research Centre. All cell lines were developed in Dulbecco's Modified Eagle's Medium (DMEM). Media were added with 1% penicillin/streptomycin, 10% fetal bovine serum and 2 mM L-glutamine. The MCF10 cell line received more supplementary materials (DMEM/F12 medium with EGF, hydrocortisone, cholera toxin, and insulin). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO2. To examine cell viability in cure with different concentrations of seroma (5% and 10% of seroma) in medium condition without FBS, these concentrations were carefully chosen based on MTT assay. Two controls were controlled for all cell lines with 10% FBS and without FBS. Moreover, 96-well cultured plates with a mass of 1×104 cells were used to seed the cells. Then, all individual samples at different concentrations were used to treat these cells. By applying an ELIZA reader, the cell viability was investigated at absorbance range of 570 nm. The absorbance values were considered based on the control percentages, yielding percentage cell viability after 24, 48, and 72h of seroma treatment.

2.3 Staining assay

At this study, for better understanding of how seroma compounds make an influence on apoptotic process of tumor cell, MDA-MB231 cell line was selected under Annexin V as a staining assay. In this regard, 10% of seroma in DMEM cell culture medium was used for the treatment of 6*105cells for two days. Then, centrifuging technique was performed at 2000 rpm for 5 minutes. After removing the supernatant, 100 µL of binding buffer was used to wash the pellets. Next, the cells

were put for incubation for 15 minutes on ice in the dark mixed with 5 µL of PI and 5 µL of Annexin V. After loading 400 µL of binding buffer, flow cytometer was done for study. The negative controls were the untreated cells.

2.4. Clonal survival assay

The MCF-7 cell line were chosen as the better cell line for showing colony formation. After seroma treatments, the cell line was trypsinated and suspended in fresh medium quantified, then 24/well cell culture plates was used to keep 500 cells to 1000 cells in triple copies. In addition, paraformaldehyde was used to stable the colonies after a week; in addition, crystal violet was applied to stain the mixture. Phase contrast microscopy was to specify the number of colony and shape in each plate. All data are accessible as mean colony number ± SD comparative to untreated controls.

2.5. Scratch assay

To study invasion, the MDA-MB-231 cells (7x105 cells/well of 24 well plates) with 80% confluence were treated with the seroma in variable concentrations (0, 2.5 and 5% of individual samples and 0, 10, 20 and 100% of pooled samples) for 48h. The cells were incubated with mitomycin (0.5mg/ml) for 2h and then were injured by the tip of a sterile 200-µL micropipette. The well was washed 3 times by PBS in order to remove all separated cells. Images were taken by a microscope (Nikon, ECLIPSE, TE 2000-4, Japan) at 0 and 24h to discover the rate of migration. First, the edge of scratch was determined, and then the scratch line in 0 and 24h measured. Finally, all data were presented as mean scratch line ± SD relative to untreated controls.

2.6. Data analysis

In this research, GraphPad Prism was performed for examination. The t-test was run to specify the level of significance among all groups. The index of P value was set at the significant level of $P < 0.05$. All information were introduced by way of the mean ± standard deviation (SD) of at least two independent tests.

3. Results

To study the effects of seroma (two concentrations 5 and 10 %.) on cancer and normal cell viability, according to previous studies and the researcher's knowledge, some critical cellular tests were selected. For investigation of some cellular effects of seroma like cell proliferation, apoptosis, clonal survival as well as cell invasion, more aggressive BC cell lines; MDA-MB-231cells were chosen.

Cell proliferation

On the way to assess the effects of seroma on cell proliferation, MTT examine was employed which indirectly measures cell growth [9]. As it was presented in Fig1, there was a significant reduction in the proliferation process compared to the control group (MCF-10). Furthermore, this consequence was illuminated in 48 and 72h. In addition, there is no difference between two selected concentrating of seroma (5 and 10%)

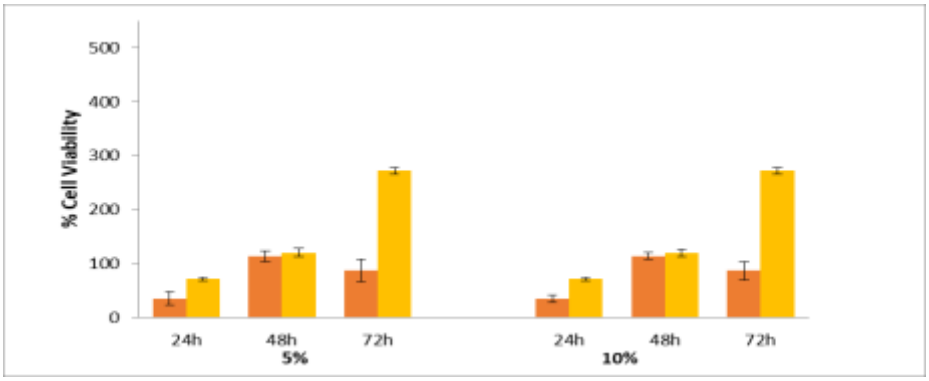


Figure 1. Two cell lines (MDA-MB-231and MCF10) incubated with seroma with 5 and 10% concentration whit the notion of three different times after treatment (24h, 48h, 72h). Data was presented as mean ± SD (n = 3). Data was analyzed by t-test.

Apoptosis assay

To investigate the apoptotic effect of seroma, MDA-MB-231 cells were selected. As it is shown in Fig2, MDA-MB-231cells were treated with seroma (5% in DMEM for 48 h), and were stained, using Annexin V-FITC/PI and analyzed using flow cytometry. The effect of seroma on MDA-MB-231 cells with the control group (MDA-MB-231 cells without seroma) was detected. Regarding the apoptosis test in figure 2, apoptosis process occurred in comparison to control group.

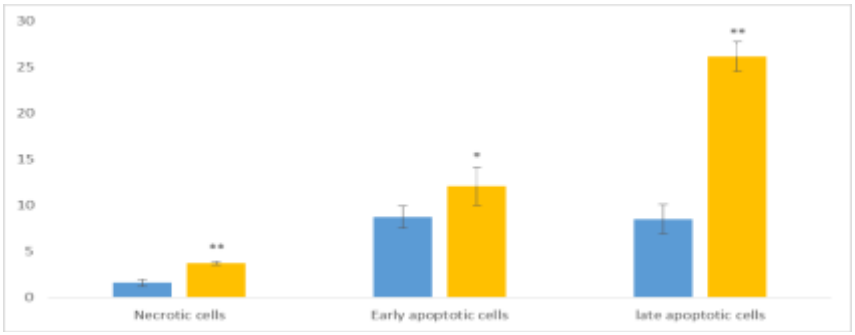


Figure 2. Annexin V-FITC and PI staining were used to evaluate apoptosis in MDA-MB-231 cells following seroma and MDA-MB-231cells without seroma as control group.

Clonal survival

Clonal survival assays were carried out on MCF-7 cell line, which was treated with seroma. As it is represented in figure 3 (crystal violet stained colony plates) it seems like an increasing paraclone was occurred. Colony shape changes were observed in cell line.

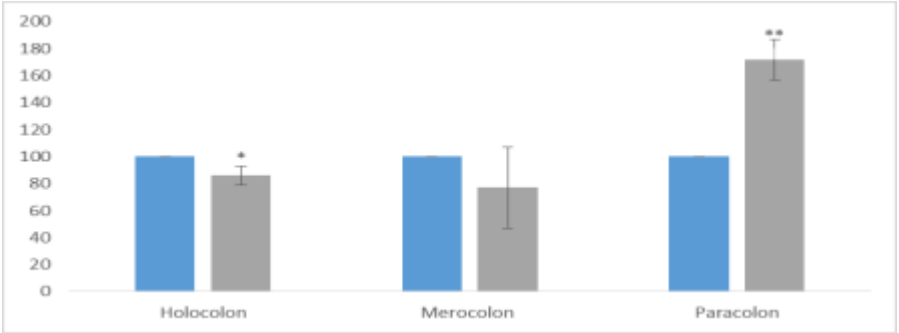
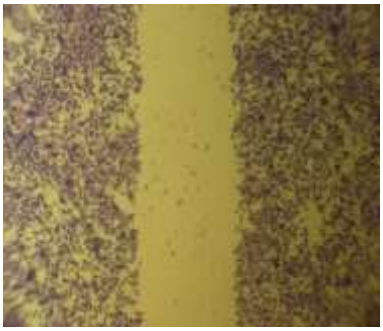


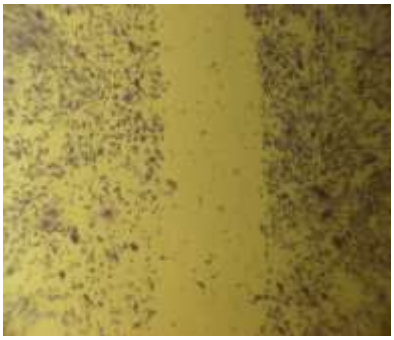
Figure 3. Clonogenicity of MCF-7 cells following the treatment with and without seroma were assessed by colony formation assay.

Migration ability

In this study, Scratch test was used to assess the effects of seroma on MDA-MB-231cell migration. As it is shown in figure 4, the scratch test indicated that seroma significantly inhibited the migration of MDA-MB-231 cells in comparison with control (MDA-MB 231 without seroma).



(With seroma)



(Control)

Figure 4. Regarding scratch test in fig 4, Scratch test was used to investigate the migration ability of MDA-MB-231 cells comparing with control (MDA-MB 231without seroma).

4. Discussion

Physiological response to operative trauma and wound healing process due to potential inflammatory effects contribute to microenvironment promoting motility and invasion of residual tumor cell [10, 11]. Local recurrence of breast cancer is related to the residual tumor cell or cancer stem cell re-activation in the tumor microenvironment region [12]. Stimulatory effects of post-lumpectomy seroma on cell proliferation *via* activation of stat-3 was reported [10]. In addition, a research reported pro-oncogenic cytokines expression in post-surgical wound fluid [13].

It is not clear if seroma remained about a certain time after surgery or if it can have positive impact on changing the margin composition; consequently, a reducing local recurrence rate would take place.

The present study results show that seroma have beneficial effects on tumor control. There were some studies about the effectiveness of seroma on the inhibition and diverting of cancer cell potential abilities [12, 14]. IORT-treated seroma has a lower stimulatory effect on stem cell phenotype in comparison with not treated WF and abrogation of expression of MIR-21. MIR-155 and MIR-221 followed by IORT were observed and reported [15].

This study was designed according to six famous cancer hallmarks. Some important cellular process such as cell proliferation, apoptosis, clonogenicity and cell migration were studied and analyzed. The rate of proliferation was different between sample and control cell line. As it is shown in Figure 1, cancer cells had less proliferation rate in comparison with the normal cell. In other words, cancer cells with aggressive behavior such as MDA-MB-231 showed less proliferation response compared with MCF-10. Our finding showed that proliferation process is not dependent on seroma concentration but over time, different cells showed different proliferation response.

The Annexin V/ PI procedure is a normally used method for recognizing apoptotic cells [16]. According to Figure 2, there was much more apoptosis in cells under seroma in compared to control group.

Cell clonogenicity potential and ability to create sphere is related to stem cell. There were three types of clones according to morphology; holoclone, meroclone and paraclone which are related to stem cell. The holoclone and meroclone types have a much more reproduction ability and self-renewal to create the sphere, and can produce tumor and overexpress stem cell markers [17]. According to Figure3,

obtained results from the colony formation assay revealed that seroma could decrease colony count and size and changed the clone morphology from holoclone to paraclone.

Cancer cells have the ability to leave the original tumor, migrate, and invade the surrounding tissues. Tumor microenvironment interacts with cancer cells and can facilitate migration and invasion. Cancer cells can express some enzymes such as matrix metalloproteinase to lysis ECM glycoproteins, so they can migrate and invade the surrounding tissues. Scratch assay test is the easy, cost-benefit and developing technique which measures in vitro cell migration [18]. The study of cell migration in cancer research is of particular interest as the main cause of death in cancer patients is related to metastatic progression [19]. As can be seen in figure 4, regarding the invasion assay, the cells under seroma significantly inhibited cell motility.

By reviewing all the above results, it can be concluded that by timing, seroma can have positive impact on margin cells protection. Hence, based on the results suggested by clamping seroma immediately after surgery, it could be seen that the tumor bed is modified in favor of suppressing cancer cells, which is left over from surgery.

5. Conclusion

By reviewing and comparison of obtained results of this study, it can be concluded that there is an inhibitory profile of proliferation of breast cell lines, treated with seroma. In addition, the ability of seroma for inducing residual tumor cell death and modification of tumor bed and confirming the inhibitory pattern of proliferation is stated. Since one of the post-surgical recurrence problems is residual stem cell at the margin, in this study, seroma proved to have the ability of inhibiting cancer stem cell reproduction via inducing changes in the morphology of cancer cell colony and converting holoclone to paraclone. Seroma significantly changes the ability of cell motility and migration, leading to inhibition of cancer cell migration and decreasing metastasis risk that finally increases the overall survival of breast cancer patients. It is suggested that more studies be done from cell line toward primary human tumor cell culture and personalized medicine be considered.

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Conflict of interest

The authors declare no conflict of interest.

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