High-dose Irradiation Stimulated Breast Tumor Microenvironment to Enhance Tumor Cell Growth and Decrease Tumor Cell Motility

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ABSTRACT

Background: Surgery and radiotherapy are two main modalities of breast cancer treatment. However, surgery affects the tumor microenvironment negatively and promotes the growth of possible malignant cells remaining in the tumor bed.

Objective: The present study aimed to investigate the effects of intraoperative radiotherapy (IORT) on the tumor microenvironment. Therefore, the effect of surgical wound fluid (WF), collected from operated and irradiated patients on the growth and motility of a breast cancer cell line (MCF-7) was assessed.

Material and Methods: In this experimental study, preoperative blood serum (PS) and secreted WF from 18 patients who underwent breast-conserving surgery (IORT-) and 19 patients who received IORT following surgery (IORT+) were collected. The samples were purified and added to MCF-7 cultures. Two groups of the cells were treated with and without fetal bovine serum (FBS) and used as positive and negative controls. Applying 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and scratch wound healing assays, the growth and motility of MCF-7 cells were measured.

Results: Cell growth of the cells receiving WF from IORT+ patients (WF+) was statistically higher than the corresponding values of the cells received PS or WF from IORT- patients (WF-) (P<0.01). Both WF+ and WF- decreased the cells' migration ability compared to PS (P<0.02) and FBS (P<0.002), although WF+ caused a more significant reduction (P<0.02).

Conclusion: Wound fluid extracted from breast cancer patients who underwent both surgery and IORT increased the growth of breast tumor cells, but decreased their ability to migrate.

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Keywords

Breast Cancer; Intraoperative Radiotherapy; Neoplasms; Radiotherapy, Adjuvant; Tumor Microenvironment

Introduction

Tumor microenvironment comprises stromal cells, such as fibroblasts, immune cells, mesenchymal stem cells, and matrix molecules. Evidence suggests these nonmalignant components support initiation, promotion, and metastasis of tumors [1-3]. For instance, transforming growth factor beta (TGFB), secreted from fibroblasts and

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macrophages, prevents cell growth in normal tissues, while it has a positive impact on tumor cell survival through Mitogen-activated protein kinase (MAPK) and PI3K signaling pathways [4]. It is hypothesized that surgery causes changes in the tumor microenvironment in favor of tumor cell growth. The reason is the fact that wound healing occurs after surgery in order to recover the invaded tissue and neutralize acute inflammation which happens following surgery [5]. This event can facilitate the proliferation of the remaining cancer cells in the tumor bed. Radiotherapy may have a similar effect on the irradiated tissues because it causes inflammation and invades the irradiated tissues. Besides, radiation has an impact on tumor cell migration [6]. One reason, supporting this idea is the radiation contribution to tumor cell reprogramming which facilitates their conversion to mesenchymal and cancer stem cells [7-10]. Nevertheless, radiotherapy is applied after breast surgery to eliminate remaining cells from surgery and to prevent local recurrence. Different fractionation schedules and radiotherapy techniques, including conventional radiotherapy (2 Gy/fraction), whole breast hypofractionation, accelerated partial breast irradiation, intraoperative radiotherapy, and volumetric modulated arc therapy, have been used for breast cancer treatment to prevent tumor recurrence [11]. Some research indicates breast intraoperative radiotherapy (IORT) has a better outcome compared to conventional radiotherapy [12], while a clinical trial showed that tumor recurrence was 4.4% for intraoperative radiotherapy and 0.4% for external radiotherapy [13]. In another clinical trial, no difference was observed between these two techniques [14].

In the present study, in order to investigate the effects of intraoperative radiotherapy on tumor microenvironment, surgical wound fluid (WF), containing the components of the tumor microenvironment, was collected from breast cancer patients who underwent breast-conserving surgery with and without IORT (IORT+ and IORT- respectively) and added to the medium of MCF-7 cultures. Then using MTT and scratch wound healing assays, the effects of WF on the growth and motility of the cells were assessed.

Material and Methods

Before launching the experiments of this experimental study, human ethics approval was obtained from Mashhad University of Medical Sciences and then the following steps were performed.

Collection of patients' samples

Preoperative peripheral blood serum (two hours before surgery) and WF (18 hours after surgery) were collected from 37 breast cancer patients who underwent breast-conserving surgery at the Pastorno hospital (Mashhad, Iran). The patients were 40-80 years old, the stage of their cancer was 2, and their cancer grades were 2 or 3. Nineteen patients received IORT (20 Gy of 50 kV X-rays) immediately after the surgical excision (IORT+), while others were only operated (IORT-). Blood samples were centrifuged for 10 min (3000 rpm) and passed through a 0.22 μm filter to extract their serum. WFs underwent similar procedures, but they were passed through 0.22 μm filters several times. The serum extracts (PS), as well as WF from IORT+ (WF+) and IORT- (WF-), were stored at -80 ˚C.

Cell culture

The MCF-7 cell line was purchased from the Pasteur Institute, Tehran, Iran. The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All materials were bought from Gibco Company (Germany). The cultures were incubated at 37 ˚C in a humidified air containing 5% CO_2. When the cultures were 80% confluent, the cells were removed from the bottom of the dishes, centrifuged, and cultured in 96 and 6-well plates for MTT and scratch wound healing as-

says, respectively.

MTT assay

5×10^3 cells /well were seeded in 96-well plates and incubated for 24h. Afterward, the PS and WF of each patient were added to the wells at a concentration of 2%. Six wells were devoted to each patient, three wells for PS and three wells for WF. In addition, mixtures of PS (M_{PS}), WF+ (M_{WF+}), or WF- (M_{WF-}) collected from all patients were separately prepared and added to six wells in a 2% concentration. As the control groups, six wells received 2% FBS (positive control group) and six wells received nothing (negative control). After 48 h of incubation, the media were removed from the wells, a mixture of 20 µl MTT (Sigma, St. Louis, MO) and 200 µl fresh medium was added to individual wells and then they were incubated for 4 h. In order to resolve formazan crystals, the media of the wells were replaced with 200 µl dimethyl sulfoxide and shacked for 10 min. Finally, using a multi-well scanning spectrophotometer (ELISA reader Epoch, USA), the light absorbance of the wells was measured at 570 and 630 nm.

Scratch wound healing assay

The cells were seeded in 6-well plates (5×10^5 cells/well) and incubated for 24 h. The confluent layers of the cells were scratched with a 100 µl sampler tip, and the wells were washed with PBS. Then 3 ml culture medium mixed with 2% PS, WF+, WF- or FBS was added to each well. Using an inverted microscope (Nikon, Japan), the wells were assessed 12, 36, and 48 h later. Images of the wells were analyzed and the area of the scratches was measured by an image software (Leica LAS software from http://www.Leica-Microsystems.com).

Statistical analysis

SPSS version 24 was used to perform statistical analysis. On the basis of the Kolmogorov–Smirnov test, when the data distribution was normal, one-way ANOVA, Tukey's multiple comparison and the students' T tests were performed to compare the groups at $P<0.05$. When the distribution was not normal, Kruskal-Wallis and Mann-Whitney tests were performed. The light absorbance of the wells treated with individual patients' samples was presented as the mean of at least three wells, while the gap-area of M_{PS}, M_{WF+}, M_{WF-}, and the control group were presented as the mean of at least six wells.

Results

Figure 1 shows the light absorbance of the cells which received samples from each patient (a: Ps from all patients, b: WF+ from IORT+ patients, and c: WF- from IORT- patients). M_{PS}, M_{WF+}, and M_{WF-} represent the wells which received the mixture of PS, WF+, and WF-, respectively. The mean values (AV) of light absorbance for each group have been illustrated in the graphs as well. Statistical analysis showed that there were no significant differences between the mean values, AV_{PS}, AV_{WF+}, and AV_{WF-}, and corresponding mixtures, M_{PS}, M_{WF+}, and M_{WF-} ($P>0.05$ for AV_{PS} and M_{PS}, AV_{WF+} and M_{WF+}, AV_{WF-} and M_{WF-}). Therefore, the mixtures were used instead of the patients' samples for scratch wound healing assay.

Figure 2 reveals the cell viability percentage of the groups. The cell viability percentage represents the ratio of the light absorbance of each group to the light absorbance of the positive control. WF+ group had higher cell viability compared to the negative control ($P<0.004$); however, there was no difference between this group and the positive control ($P>0.05$). WF- and PS were not statistically different from the negative control ($P>0.05$), but the difference between these groups and the positive control was significant ($P<0.05$). In order to assess the impact of surgery and IORT on cell viability, WF-, WF+, and PS were compared to one another. No difference was observed between WF- and PS ($P>0.05$), while the cell viability of WF+ was statistically higher than PS ($P<0.001$) and WF- ($P<0.003$).

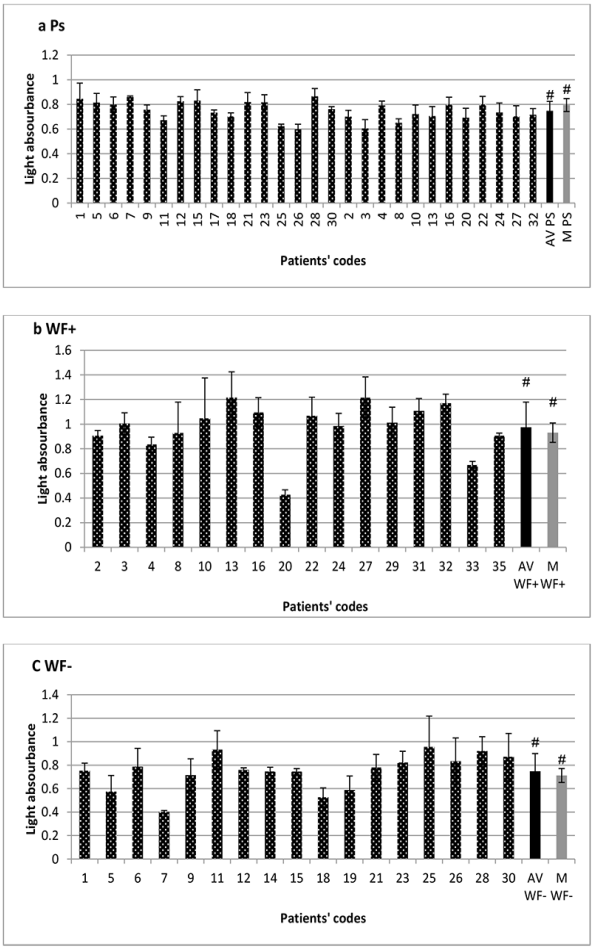

Figure 1: Light absorbance of the cells which received individual patients' samples or their mixtures, (a) preoperative blood serum (PS) from each patient or a mixture of PS (M_{PS}), (b) wound fluid from each irradiated patient (WF+) or a mixture of WF+ (M_{WF+}), (c) wound fluid from each operated patient (WF-) or a mixture of WF- (M_{WF-}). The mean values of the groups have been illustrated by AV (AV PS, AV WF+, AV WF-) and M indicates the mean values of the wells receiving the mixture of PS or WF (M PS, M WF+, M WF-). The error bars indicate ± standard deviation of three wells devoted to each patient and six wells which received the mixtures. Standard deviations of the mean values were obtained based on the light absorbance variation of the groups. # indicates no significant difference between AV and M

Figures 3 and 4 reveal the results of the scratch wound healing assay. The shape and the size of the scratches at the time of creation (0) and 48 h later have been illustrated in Figure 3. Figure 4 shows the percentage of the remaining gap at different time points. The scratch of the control group (treated with FBS) was covered faster than of the other groups (P<0.001 compared to M_{WF+} and M_{WF-}, P<0.01 compared to M_{PS}). At all time-points, except time 0, the remaining scratches in M_{WF+} and M_{WF-} wells were larger than in M_{PS} wells (P<0.02). Statistical analysis also showed that the remaining scratches in M_{WF+} wells were larger than in M_{WF-} wells (P<0.03).

Discussion

The present study aimed to investigate the effects of IORT on the tumor microenvironment; therefore, WF, containing the components of the tumor microenvironment, was collected from breast cancer patients who underwent breast-conserving surgery with and without IORT and added to the medium of MCF-7 cultures. Then using MTT and the scratch wound healing assays, the effects of WF on the growth and motility of the cells were assessed.

The results showed that the light absorbance of the cells receiving WF+ was more than those received PS. It indicates that the tumor microenvironment extracted from the patients who underwent IORT supported the tumor cell growth and proliferation. WF- had no impact on cell growth, but both WF+ and WF- decreased cell motility.

Enhanced cell growth as a result of IORT is not unexpected. It has been accepted for a long time that radiotherapy accelerates cell proliferation in tumors. It is a reaction of tumors to cell loss, which occurs following irradiation [15]. Many researchers have tried to find and apply new radiotherapy techniques and fractionation schedules to manage cell proliferation following radiotherapy [11]. In addition to accelerated proliferation, radiation creates

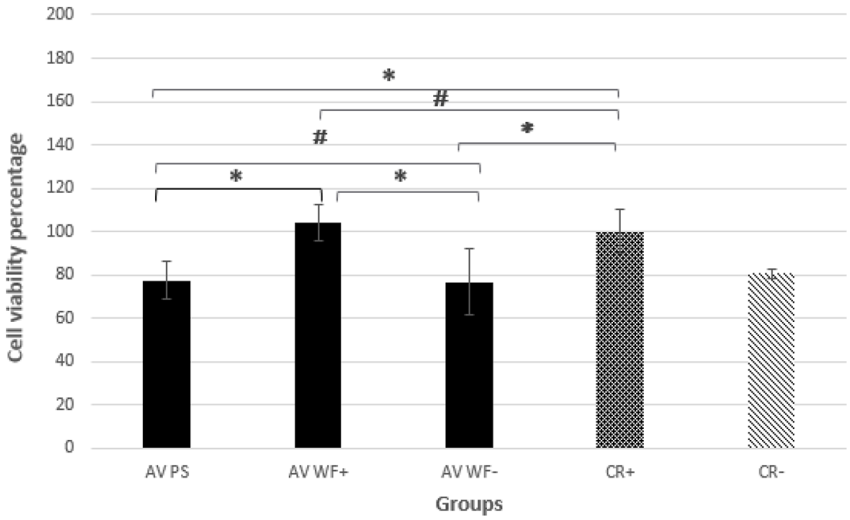

Figure 2: Cell viability percentage of the groups. AV PS, AV WF+, and AV WF- denote the mean viability percentage of PS, WF+, and WF- groups, respectively. CR+ represents the positive control group and CR- denotes the negative control group. * Represents $P<0.05$ and # represents $P>0.05$ PS: preoperative blood serum, WF+: wound fluid from irradiated patients, WF-: wound fluid from operated patients. Error bars indicate±standard deviation

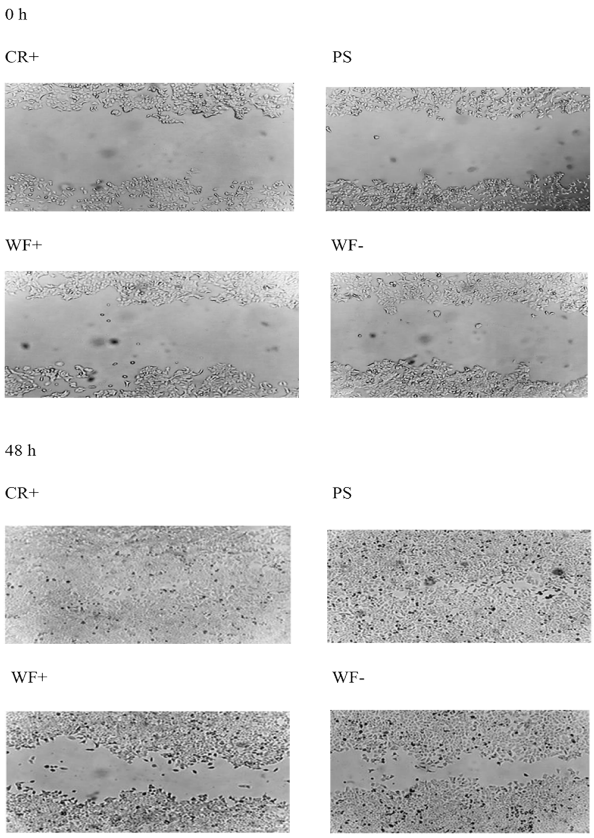

Figure 3: Images of the scratches at 0 and 48 h later.

an inflamed microenvironment supporting tumor cells. What we observed in WF+ group may represent the accelerated repopulation or inflamed microenvironment induced by WF+. In addition, Vilalta et al. demonstrated that irradiation of breast tumors, despite its beneficial effects, can attract tumor cells circulating in the blood vessels and increase local tumor recurrence [16].

Unlike our results, Belletti et al. observed that both WF+ and WF- increased short-term cell growth and when they let the cells grow for 15 days and create colonies, they found that the impact of WF+ on cell growth was lower than WF- [17]. Weldwijk et al. also treated tumor cells with WF+ and WF- and observed none of them affected the tumor cell growth [18]. The discrepancy may be explained as a result of diverse experimental conditions used in these studies. For instance, we collected the wound fluid 18 h after surgery, while in the aforementioned studies, it was collected after 24 h. It is likely the composition of wound fluid alters at different times after surgery and consequently

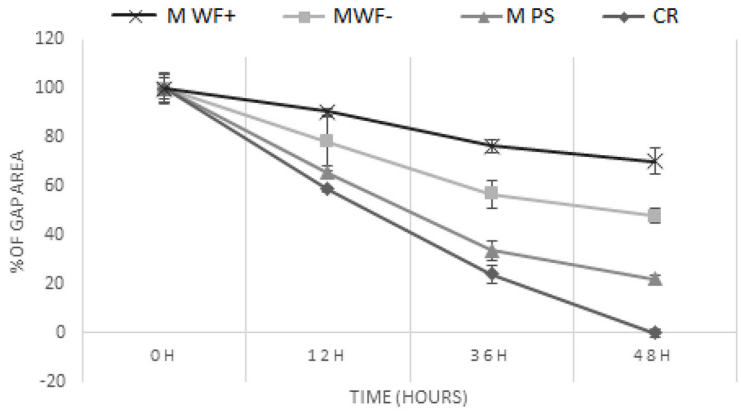

Figure 4: Percentage of the gap areas that remained at different duration after creating the scratches. Error bars indicate±standard error. CR, M WF+, M WF-, and M PS represent the groups that received fetal bovine serum (FBS), a mixture of wound fluid from irradiated patients, a mixture of wound fluid from operated patients, and a mixture of preoperative blood serum, respectively.

causes different results. Watt-Boolsen analyzed the leukocytes in the surgical wound fluids of operated breast cancer patients at different duration after surgery. They concluded that wound fluid is not a collection of blood components, instead, it consists of inflammation-related secretions [19]. Chow et al. studied the composition of surgical wound fluid at different periods following the surgery and found IL-6 increased at first, but decreased after a while, whilst TNF-α increased steadily. They found IL-6 as a cause of inflammation in surgical tissues [20]. In another study carried out by Baker et al. wound fluids collected from 73 patients were analyzed. Researchers observed that the concentration of growth factors such as EGF, PDGF, bFGF, TGF-beta1, and VEGF had decreased by 60% of the patients [21]. Changes in the concentration of IL-6, IL-1β, IL-1α, and TNF-α through time have also been observed [22].

Although supporting tumor cells through either accelerated repopulation or inflammation is a deleterious impact, the benefits of IORT for breast cancer patients cannot be denied. In addition to tumoricidal effects, IORT may have effects similar to other hypofractionated radiotherapy techniques (e.g. stereotactic ablative radiotherapy). High-dose irradiation creates a tumor surveillance ability through the immune system, which in addition to removing in situ tumor cells, preventing tumor metastasis [23, 24] and is expected to occur in IORT+ patients as well.

Performing invasion assay, Belletti et al. observed that WF- increased tumor cell mobility, while WF+ had no effects. As WF+ was collected from the patients who firstly underwent an operation and then received IORT, they concluded that IORT abrogates the cell migration and invasion induced by operation [17]. Kulcenty et al. came to a similar conclusion in a recent study [25]. Our result regarding the cell motility of WF+ group supports the aforementioned observations; however, it is not consistent with the radiation-induced epithelial to mesenchymal transition (EMT) hypothesis. This hypothesis indicates that radiation alters tumor cells and their microenvironment and promotes EMT, invasion, migration, angiogenesis, and metastasis [26, 27]. Kawamoto et al. irradiated tumor cells with 5 Gy of X-rays and observed enhanced cell migration and invasion. They also examined the molecular changes which were consistent with EMT [26]. In a study carried out by Sundahl et al.

the results of several studies investigating the effect of radiation on tumor cell invasion were reviewed. All reviewed studies revealed that radiation promoted tumor cell invasion [27]. As our results and those obtained by Belletti et al. and Kulcenty et al. are all related to IORT, it is likely that radiation dose, which is very high in IORT, explains the discrepancy between our results and radiation-induced EMT. However, we have yet to determine whether this suggestion is correct or wrong.

Conclusion

The wound fluid collected from the patients who received IORT increased the growth of tumor cells. This observation can be explained by radiation-induced accelerated repopulation and inflammation, which support tumor cell growth. Nevertheless, this result is not consistent with the results of previous research. The apparent discrepancy may be related to the time at which the wound fluids have been collected. Since in the present study WF was collected 18 h following irradiation, while in previous studies, it was gathered after a more prolonged duration. Therefore, it is proposed to investigate the impact of duration between irradiation and collecting the wound fluids on tumor cell growth in future studies. Furthermore, the present study, consistent with previous research, revealed that WF collected from the patients who received IORT decreased the motility of the tumor cells. It can be considered as a beneficial impact of IORT on tumor cells.

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Authors' Contribution

S. Torabinejad did literature, conceived the idea, designed the experiment, and prepared the manuscript draft. Sh. soleymanifard managed the project, approved the data, and modified the manuscript. S. Sayah did literature, performed the experiments, analyzed the data, and prepared the manuscript's figures and images. F. Behnam Rasouli consulted the experiment design and modified the manuscript. All authors read and approved the final version of the manuscript.

Ethical Approval

The Mashhad University of Medical Sciences Ethics Committee approved the study's protocol (Ethic code: IR.MUMS.fm.REC.1396.279).

Informed Consent

The informed consent letter was prepared, confirmed by "The Mashhad University of Medical Sciences Ethics Committee" and signed by the contributed patients.

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Conflict of Interest

None

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