Original Article:

The probability of influence of the abscopal effect on reduction of similar far away 4T1 cell line tumors by irradiation of main tumor

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ABSTRACT

The most important problem with radiotherapy is the limitation of whole body irradiation of a metastatic patient. There are evidence showing that similar effect will occur in non-irradiated tumors similar to the irradiated ones. This effect is called abscopal effect. In the present study, the abscopal effect on local induced mice breast cancers has been investigated. One million of 4T1 mice breast cancer cell line was injected to balb/c mice subcutaneously while being under anesthesia. After the growth of tumors till becoming palpable, one of two induced tumors were exposed to total 28 Gy, with gamma rays emitted from a cobalt -60 tele-therapy machine in 14 fractions with 2 Gy daily doses. Tumor volumes were measured, using the caliper. The data was analyzed by the use of non-parametrical and ANOVA tests. Similar growth in non-irradiated control tumors was seen. After 10 or 11 fractions of one- side irradiation and total dose of 20 to 22 Gy, however, non-irradiated tumors, similar to irradiated ones, showed similar effect, reduction of size and volume different from control groups ($P<0.05$).The reduction of non-irradiated tumors relative to irradiation of another tumor in the same mouse is the emblem of occurrence of the abscopal effect. The mechanism of the abscopal effect is unknown but it could be related to the release of cytokines from irradiated tumors with their receptors existing on the surface of non-irradiated tumors. By induction of this effect, the remission probability of small metastases after local irradiation could be possible.

Keywords: breast cell line; tumor volume; irradiation; abscopal effect

INTRODUCTION

Nowadays cancer is one of the main reasons of death in developing and developed countries [1]. Presently, three main modalities exist for management of cancer. Surgery is the first strategy of cure in low risk tumors and tumors with high chance of succeeding [2]. The goal of radiotherapy is to deliver the higher dose to tumor and lower to normal surrounding tissues [3]. To investigate the effect of each daily fraction of dose in radiotherapy, the dose-response and iso-effect curves have been proposed [4]. According to the target idea and its effect on these curves, it had been considered that damage to cells merely occurs for cells in irradiation field. In other words, radiation should directly hit the cells [5]. This theory was obsolesced after Dr. Mole's finding in 1953 that neighbor cells also show the similar effect, similar to genomic instability and chromosomal damages [6]. After this invention, the new era of radiobiology is emerging and researches have inclined toward finding of its mechanism. These effects had been called off-

target effects including bystander and abscopal effect [7]. The bystander effect is damages in near or close cells to irradiation field and the abscopal effect is the existence of similar effect in faraway cells [8]. Induction of off-target effects has been reported within both in-vitro cell culture and in-vivo animals [8, 9]. There are several case reports that irradiation of main tumor causes remission of metastases [10-12]. Some studies done with high dose per fraction and / or fractions of irradiation proved to be successful [13]. Other studies were done over the combination of drugs, such as anti-CTLA with irradiation [14]. Similarly, they are interested in induction of the abscopal effect in bracy-therapy and along with radical and / or palliative goal [15]. Occurrence of abscopal effect is related to releasing cytokines, inflammatory factors or tumor necrosis factors [16-22].

According to these studies, induction of the abscopal effect alone, without the use of any drug are rare. So, if one could induce the abscopal effect without any drug, it could be a new regulatory arm in treatments. For the purpose of achieving that goal, a study similar to human conventional fractionation irradiation is needed. The aim of this study is to investigate the reciprocal influence of irradiation on a tumor and its effect on the non-irradiated one.

MATERIALS AND METHODS

The population of this experimental study were tumor bearing Balb/c mice. The sample size was determined by using the following formula:

$$N = \frac{(Z\upsilon + Z1 - ß)}{¼\{log\ (1 + r/1 - r)} + 3$$

According to this formula, the sample size, with a 95 % of confidence and significance value of $p<0.05$ was 10 mice but as the deaths of mice was a probability, 15 mice were chosen. During the experiment 5 mice died. This study was carried out with regard to the guidelines for animal experiments at the University of Tokyo and Iran Ministry of health. All the scarified mice by inhalation of CO_2 gas and the dead mice were burnt with discards of hospital.

Cell line

The 4T1 cell line (ATCC number: CRL- 2539), (Triple-negative breast cancer adenocarcinoma) was bought from Shahid Beheshti University, Tehran, Iran. These cells were cultured in RPMI-1640 culture medium (Gibco, Germany) including 10% fetal bovine serum (Gibco, Germany), penicillin 100unit/ml and streptomycin 100 mg/ml and stored in 5% co_2incubator[26]. The subculture was done after reaching 80% confluence by the use of trypsin- EDTA 0.25% (Merck, Germany).

Animals

Male 4-6 week-old balb/c mice were provided from animal lab of Iranian Pasteur institute. Mice were caged in groups of 3-5, and fed with animal standard mouse pellet and water ad libitum [26, 27]. Their backs and hind limbs were shaved. All of the mice were anesthetized in isoflurane chamber before all procedures (cell injection and irradiation) and were observed until fully recovered. Scarificing was done with lethal inhalation of carbon monoxide two days after the irradiation had finished. The tumor volume was calculated using the formula: tumor volume = (major axis) × $(\text{minor axis})^2$ × 0.5236[26, 27], and after that, by dividing the irradiated tumor volume by non-irradiated ones, ratios of different volumes achieved. All animal experiments were carried out with respect to the guidelines for animal experiments at the University of Tokyo.

Tumor measurement

In the flank region of mice, 4T1 cell line were injected (1×10^6 cells) [27]. When tumors became palpable, the mice were randomized and irradiated on one side of the tumor while the other whole side of the body was shielded with lead. Randomization was performed at the day of implantation to eliminate any potential observer bias caused by randomization at a later time point. Both treatment and control groups had 5 mice. Both tumors in flank region were 4T1. Then, by means of a caliper which accuracy was 1mm, size of tumors were measured [26]. Irradiation was started in day 16 (needed days to grow the tumors to be palpable) and 28 Gy delivered in 14 fractions (14×2Gy), (5 fractions per week), similar to conventional human radiotherapy course. The control groups received sham treatment by immobilization of mice through anesthetization on coach of cobalt-60.

Tumor irradiation

Irradiation was done with gamma rays generated from Co^{60} teletherapy machine (Theratron 760-c, AECl Canada). The field size was 5×5 cm, in 80 cm and SSD technique, each fraction 2 Gy and 5 fractions per week for 14 fractions, with dose rate of 54 cGy / min [24]. During irradiation, all of the mice were anesthetized with isoflurane to avoid movement.

Irradiation dose Calculation

Tumor bearing mice were held in prone position and radiation delivered to the targeted tumor-side. The reference point for the prescription of dose was set as 0.5 cm depth (d_m) from the skin. The irradiated field was determined using computer tomography-based simulation set to spare hematopoietic and critical organs. The absorbed dose into the dorsal spine was calculated to below 10% [6].

Statistics

SPSS 16.0 software was used for data analysis. The non-parametrical Mann- Whitney test was used to determine the significance of differences between variables. Mean and standard Errors were used to report the values of measured variables, with a 95 % of confidence (significance value of $p<0.05$ was considered).

RESULTS

After 14±2 days, those mice with visible bulks of tumor were categorized randomly. Then, irradiation after day 16 was started according to 2 Gy in 5 fractions at week.

Table 1. Data of tumor volume measurement in some different days (the days have been added up with initial 16 days needed for being palpable)

Fraction of treatment	After injection`s days measurement	Mean ± SE	
		Irradiated site	Shielded site
1st	17th	6.778 ± 0.1982	7.068 ± 0.1403
7th	25th	1.074 ± 0.2524	1.045 ± 0.1351
14th	33th	1.072 ± 0.3030	1.072 ± 0.1313

All mice in the control groups, as seen in the figure 1, had continuous fluctuation in tumor volume ratios (p=0.002). The two stages of fluctuations were seen, before 8th fraction with its unpredictable rate and after 8th fraction with its continuous decline with fractionation (figure 1).

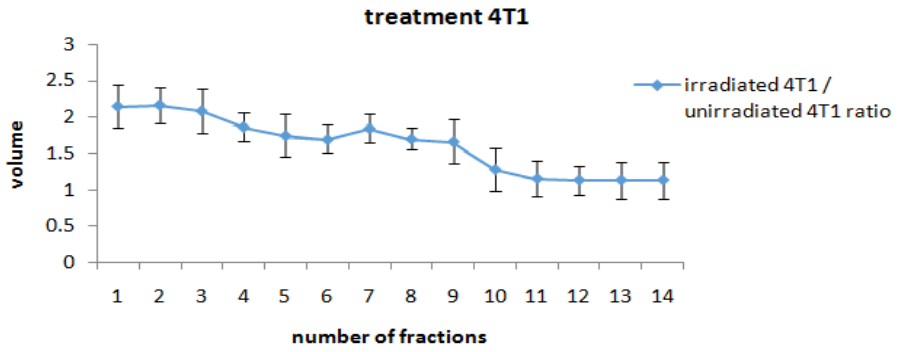

Figure 1.Unstable and unpredictable trend of tumor growth in control group

The rate of tumor volumes variation in the control groups were faster than that of the treatment ones (p=0.000).The tumor ratios were different in treatment group compared with the sham group. Also the behavior of curves between treatment and control groups were different. In the treatment groups, curves became flat after induction of abscopal effect (p=0.001). The dose level that was required for induction of abscopal effect is shown in figure 2. Based on our data analysis, 20 or 22Gy is needed for the abscopal effect to be observed (P = 0.000). Non-irradiated tumors in treatment groups showed similar variation to the exposed ones (figures 1 and 2).

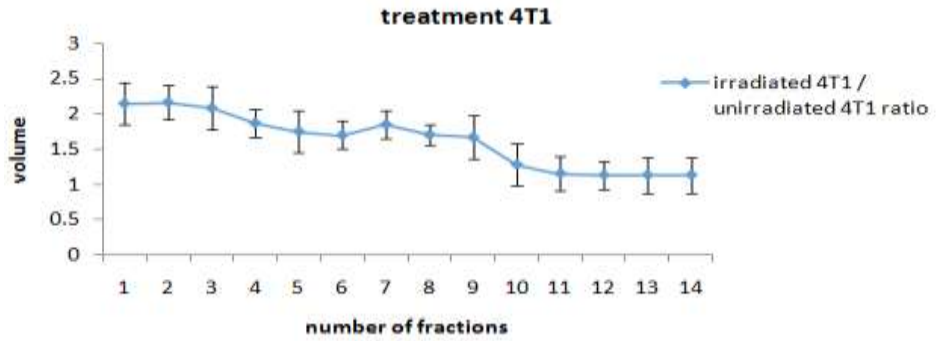

Figure 2. Illustrating the irradiated / non-irradiated ratio during 14 fractions of irradiation in treatment group

Different sizes of tumors were used where the trend of growth of all the groups were similar (p = 0.000).

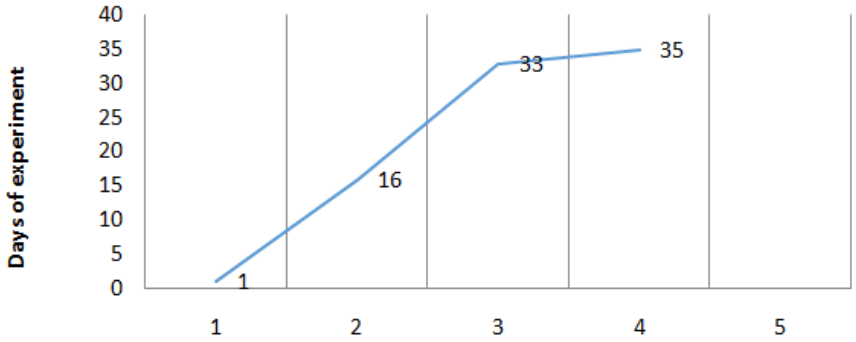

Figure 3. Time table of all processes ofthe study from beginning of study till scarification.

1- Injection of cell line into mice
2- Onset of irradiation
3- End of irradiation course
4- Scarification Day

DISCUSSION

In this study we showed that with merely local radiotherapy, the induction of abscopal effect in mice bearing 4T1 cell line is achievable. Prevention of tumor growth which was observed with diverse mouse tumors [8], including LLC, Meth Afibro sarcoma, and Colon adenocarcinoma suggests that the induction of abs copal effect was irrelevant to a specific tumor type [29]. After 14 fractions of 2Gy irradiation in 3 weeks, volume of tumors rather than increasing led to steady plateau phase (figures 2 and 3), which may suggest enough production of effective agent(s) to prevent more growth and /or less production to achieve remission. After 10 fractions of irradiation, the dose threshold to induction of steady level was seen which would be the indicator of dose dependency. When the same tumor, located in different site were irradiated, the similar trend of tumor growth in non-irradiated one became significant. Different and similar trend in control and non-irradiated groups were seen respectively, proving that abscopal effect in treatment group has occurred. Furthermore, when different bulks of tumors were irradiated, in order to investigate whether this effect depends on tumor size, all fluctuations in the treatment groups were resembled to previous exposed ones. To make the data more quantifying and feasible for comparison, this study's datais indicated in terms of irradiated /non- irradiated ratio. This ratio obtained by the measured volumes for each tumor in irradiated site was divided by non-irradiated ones (figure2).

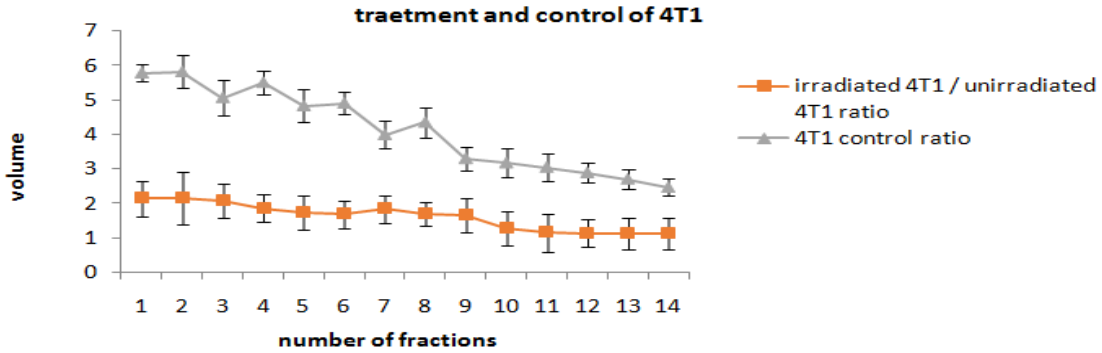

Figure 4. Comparison of trends of volume growth in treatment and control groups. There is Plateau phase in treatment group while in control group the volume variations are more intensive.

Although several different efforts in terms of inducing and enhancing of abscopal effect are in hand [23,24 and 28,29], induction of this effect without adding up the immune stimulatory factors [23, 28] or gene engineering [24] has not been previously investigated. The usage of clinical abscopal effect should include the induction probability and its magnitude in conventional irradiation scheme. Our data is the first document which serves as citable paper to future non-consecutively irradiation studies. Existing data in fractionation style are methodically different with human clinical regimens, indicating that those data are far from being used in clinics [24, 28-30]. Employing the Patched1 mice model, Mancuso et al have shown that a single dose of 1, 2, 3 or 10 Gy of x-rays in mice proposed a dose level for induction of abscopal effect [7]. The present data suggest that the effect of fractionation dose led to growth inhibition in both tumors of treatment group. The mechanisms responsible for our findings compared with that single dose irradiation difference in their illustrated end points necessitates further investigation.

CONCLUSION

All in all, based on similar volume variation in un-irradiated tumors in treatment group, this study shows that systemic effect of irradiation (OFF-TARGET effects) is achievable. Also, appearance of this effect with different sizes of tumor bulk is a good indicator of its irrelevancy. Regression of both tumors in irradiated group after 20-22 Gy indicates the existence of a threshold dose. Lesser growing speed of volume changes in treatment group, in comparison with sham one implies the effectiveness of abscopal influence on the reduction of tumor growth. Taken together, the results of this study pertains to our investigation of in vitro and in vivo procedures, all of which needed to declare that induction of abscopal effect is reproducible.

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CONFLICTS OF INTEREST

The authors whose names are listed in this paper certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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"The authors declare no conflict of interest"

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