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Effect of Radiotherapy on Antitumor Immune Response in Breast Cancer Patients

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Abstract

Background: Radiotherapy induces tumor cell death through DNA damage. Direct relationship between radiation therapy and the immune system is responsible for many of the antitumor effects of radiotherapy. The effects of radiation therapy extend beyond direct cytotoxicity on tumor cells to additional systemic antitumor effects. The objective of this study was to evaluate the effect of radiotherapy on the circulating levels of tumor necrosis factor-α (TNF-α), Interferon-gamma (IFN-γ), CD8+ cytotoxic T lymphocytes and CD56+ natural killer cells in breast cancer patients.

Methods: This study included 90 women divided into two main groups: Group I: 45 patients with stage II-III invasive breast carcinoma, group II: 45 healthy women as a control group. All studied patients received adjuvant radiotherapy after surgery. Enzyme-linked immunosorbent assay provided the measurement of TNF-α and INF-γ. Flow cytometry assessed the levels of CD8+ cytotoxic T lymphocytes and CD56+ natural killer cells.

Results: A significant reduction occurred in the percent of CD8+ and CD56+ cells in breast cancer patients after radiotherapy. There was a significant increase in both TNF-α and INF-γ in the plasma of breast cancer patients after radiotherapy.

Conclusions: TNF-α and INF-γ cytokines significantly increased while the percent of CD56+ and CD8+ cells significantly decreased following exposure to radiotherapy. Such immune modulatory potential and increase in the knowledge of radiation induced out of field and systemic effects, foresee a rapid progress in the development and clinical application of new combined radiotherapeutic and immunotherapeutic approaches.

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Introduction

Scientists used to believe that radiotherapy controlled cancer predominantly by inducing tumor cell death through DNA damage. However, new emerging data strongly suggest a direct relationship between radiation and the immune system. This, in turn, mediates many of the antitumor effects of radiotherapy.[1]

During radiation therapy, the irradiated tumor cells survive for certain periods of time. During these periods, they remain metabolically active and release different immune-stimulatory molecules involved in cell death, producing a phenomenon called immunogenic cell death.[2] The actions of these molecules are called damage-associated molecular patterns. These patterns are both local, initiating the recruitment of antigen presenting cells in the tumor and systemic, eliciting a general adaptive antitumor immune response through secretion of soluble cytokines and chemokines.[3]

Traditionally, radiotherapy has a significant effect on the immune system modulation through activating cytokine cascades by both tumor cells and tumor-infiltrating lymphocytes. These agents may be immune-stimulating or immune-suppressive. Also, they can possess both stimulatory and inhibitory effects depending on their concentration, the microenvironment, and the type of cells that produce them. These molecules also act at a systemic level outside the tumor burden.[4] Moreover, the tissue damage induced by radiotherapy itself causes the release of several cytokines and chemokines that mediate response to injury through recruiting cellular subtypes important in immune response.[5,6]

Proinflammatory cytokines may impact the effectiveness of radiotherapy, either by inducing or suppressing a radiation-mediated immune response. Tissue-resident macrophages mainly produce cytokines such as tumor necrosis factor-α (TNF-α). This cytokine has antitumorigenic effects at high concentrations; however, it promotes angiogenesis, metastasis, and cell survival at lower concentrations.[7,8] Once the immune cells migrate to tumor microenvironment, they mediate several cytotoxic effects by releasing various interferons. Type 2 interferon (IFN-γ) is an inflammatory cytokine that is up-regulated following radiation and plays an important role in antitumor immune responses.[9]

Several in vitro and in vivo experiments confirmed that radiotherapy was able to increase the subpopulations of CD4+T-cells and enhance their response.[10,11] Similarly, enhanced response of CD8+ cytotoxic T cells (CTLs) was reported following radiotherapy.[12] Tumor infiltrating lymphocytes (TILS) are found in different types of tumors and result from the immune response against cancer antigens. Tertiary lymphoid structure (TLS) might also have a prognostic impact on patients treated with chemo-radiotherapy.[13]

The employed radiation fraction size and delivery methods may modify the radiation effects on the tumor microenvironment and immune system. The direct and indirect effects of radiation are mainly based on the conventional standard dose fractionation of two Gy per fraction.[14] However, a variety of hypotheses as to the specific impact of different fractionation regimens on the antitumor response are under investigation. In preclinical studies, the use of hypofractionated high doses rather than high single-dose schedules showed the best results with regards to the pro-immunogenic effect of radiation.[15-17]

In addition, larger doses had more pro-immunogenic effects.[18] However, in vivo studies suggested the presence of more complex relationship between the immune response and both dose and fractionation.[16,19] So far, the published preclinical data have not detected the optimal dose schedule to stimulate the immune system.

The aim of this study was to evaluate the effect of radiotherapy on the circulating levels of TNF-α, Interferon-gamma (IFN-γ) and markers of enhanced immune function, CD8+ cytotoxic T lymphocytes and CD56+ natural killer cells, in breast cancer patients.

Subjects and Methods

This case-control study included 90 women divided into two main groups:

Group I: 45 patients with stage II-III primary invasive breast carcinoma.

Group II: 45 healthy women of matched age and menopausal status with the patients as a control group.

Patients were recruited from Cancer Management and Research Department, Medical Research Institute (MRI), Alexandria University. All contributors provided informed consent according to the declaration of Helsinki and the study was approved by the Ethics Committee of Medical Research Institute.

The exclusion criteria were the clinical manifestation of infection and receiving immunomodulatory agents or blood transfusions over the past three weeks.

We performed full history recording and thorough clinical examination on all studied patients. Clinical evaluation included physical examination, blood tests, chest X-ray, abdominal ultrasound, breast mammography, and ultrasound. In patients with locally-advanced tumors, we added bone scintigraphy and body computed tomography to the staging workup. Immunohistochemistry assessed pretreatment estrogen, progesterone receptors and HER-2 status. We further performed either modified radical mastectomy or breast-conserving surgery with or without reconstruction on the patients. Chemotherapy included anthracyclines containing regimens with or without taxanes. Patients whose tumors overexpressed HER-2, received trastuzumab concomitantly with taxanes. Patients with positive hormone receptors received hormone therapy.

We further applied adjuvant radiotherapy to patients treated with breast-preserving surgery and those undergoing mastectomy with any of the following criteria: primary tumor >5 cm, pre- or post-chemotherapy T4 and/or N2-3, premenopausal status with pN+, or positive resection margin. To delineate the target volume, all cases received CT-based treatment plan. We conducted radiotherapy at a dose of 44 Gy over a period of 16 days in a daily fraction of 2.75 Gy delivered five times a week. Patients received chest wall radiation, locoregional, or whole breast irradiation according to the pattern of surgical management. Patients operated by conservative surgery further received a boost.

Table 1. Participants' clinical characteristics

Characteristics	Breast cancer patients (n=45)
Age	
Mean ± S.D	49.76 ± 11.52
Range	33-75
Menopausal status	
Pre-	20 (44.4%)
Post-	25 (55.6%)
Nuclear grade	
I-II	26 (57.8%)
III	19 (42.2%)
Clinical Stage	
II	32 (71.1%)
III	13 (28.9%)
Tumor size (cm)	
≤5	20 (44.4%)
>5	25 (55.6%)
Axillary lymph node involvement	
Positive	33 (73.3%)
Negative	12 (26.7%)
ER status	
Positive	29 (64.4%)
Negative	16 (35.6%)
PR status	
Positive	27 (60%)
Negative	18 (40%)
Her-2/neu status	
Positive	24 (53.3%)
Negative	21 (46.7%)

ER: estrogen receptor, PR: progesterone receptor, Her-2/neu: human epidermal growth factor receptor 2

Blood Sampling

We obtained a total of 10 mL fasting heparinized blood sample from healthy controls and breast cancer patients. We collected the first blood samples from the patients before the start of radiotherapy and the second sample was

collected at the end of radiation course. Matched controls provided one blood sample.

We dispensed 5 mL of venous blood sample into EDTA vacutainers to separate plasma. The plasma was separated within three hours and stored at -80°C until analyzed. We measured TNF-α and INF-γ levels by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, USA). An antihuman TNF-α and INF-γ coating antibody was adsorbed onto microwells. Human TNF-α and INF-γ present in the sample or standard bound to antibodies adsorbed on the microwells. We further added a biotin-conjugated antihuman antibody which bound to human TNF-α and IFN-γ captured by the first antibody.

We dispensed 5 mL of peripheral blood samples into EDTA vacutainers and freshly devoted them for flow cytometric analysis of cytotoxic T cells and natural killer cells. This was done by flow cytometric enumeration of both cell types using relevant fluorescence and phycoerythrin-labelled monoclonal antibodies according to the method of Vaickus et al. (1991) and FACS caliber flow cytometer equipped with Cell Quest software.

Statistical analysis

The SPSS software version 17 (SPSS Inc., Chicago, IL, USA) conducted the statistical analyses. Mann-Whitney U test for non-parametric variables assessed the differences between groups. Statistical significance was set at $P \leq 0.05$.

Results

Patients' clinical characteristics

Clinical characteristics of the study participants are summarized in table 1.

Cytotoxic T cells (expressed as CD8+) in patients with breast cancer

We enumerated the cytotoxic T cells by flow cytometric study of their signature receptor CD8. Results were expressed as percent and summarized in table 2 and figure 1. The mean±SD of the percent of CD8+ cells in peripheral blood of patients with breast cancer before radiotherapy treatment was 32.51±5.30. This value decreased to 21.34±6.04 following radiotherapy while most healthy controls were 18.58±3.67. Statistical analysis of these results revealed a significant increase in the percent of CD8+ cells in breast cancer patients prior to radiotherapy compared to their corresponding controls ($P1<0.001$) and patients after treatment ($P2<0.001$). On the other hand, there was no significant difference between breast cancer patients after radiotherapy compared to controls in percent of CD8+ cells ($P1=0.064$) (Table 2 and Figure 1).

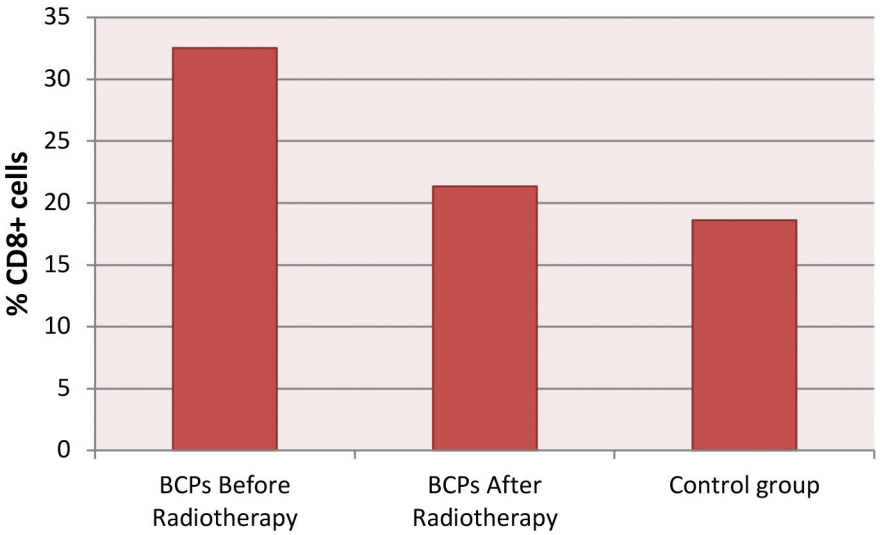

Figure 1. Bar chart illustrating the mean percent of cytotoxic T cells (expressed as CD8+) in patients with breast cancer before and after radiotherapy treatment as compared to control subjects.

Table 2. Statistical analysis of the mean percent of cytotoxic T cells (expressed as CD8+) in patients with breast cancer before and after radiotherapy treatment compared to control subjects

	Breast cancer patients		Control group
	Before Radiotherapy (n=45)	After Radiotherapy (n=45)	(n=45)
Percent of CD8 + cells			
Range	22.20 – 43.10	13.0 – 31.30	12.0 – 27.0
Mean ± SD	32.51 ± 5.30	21.34 ± 6.04	18.58 ± 3.67
Median	32.65	20.10	18.50
*P*1	0.001*	0.064	
*P*2	0.001*		

*P*1: *P* value comparing the control group with each studied group; *P*2: *P* value comparing the breast cancer patients before and after treatment; *: Statistically significant at $P \leq 0.05$.

Natural killer cells (expressed as CD56+) in patients with breast cancer

We enumerated the natural killer cells by flow cytometric analysis of CD56+ specific for natural killer cells (NK) cells. Results were expressed as percent and summarized in table 3 and figure 2. The mean±SD of the percent of CD56+ cells in peripheral blood of cancer breast patients before radiotherapy was 12.32±4.0. This value was reduced down to 8.04±2.39 after radiotherapy compared to 10.98±2.88 in healthy controls. Statistical analysis of these data revealed a significant reduction in the percent of CD56+cells in breast cancer patients after radiotherapy compared to the before treatment ($P2<0.001$) and healthy controls ($P1=0.001$). On the other hand, we found no significant difference between patients before radiotherapy and healthy controls ($P1=0.099$) (Table 3 and Figure 2).

Plasma levels of TNF- α in patients with breast cancer

ELISA quantified the TNF-α cytokine in the plasma of breast cancer patients and healthy controls. Results are summarized in table 4 and figure 3. The mean±SD of TNF-α was 19.83±7.47 in breast cancer patients before radiotherapy treatment. This value increased up to 27.50±4.73 following treatment while it was 6.50±3.32 in healthy controls. Statistical analysis of these results showed a significant increase in TNF-α in plasma of breast cancer patients after receiving radiotherapy compared to the corresponding values prior to radiotherapy ($P2=0.001$) and healthy controls ($P1<0.001$). Moreover, a significant increase occurred in TNF-α in breast cancer patients prior to radiotherapy compared to

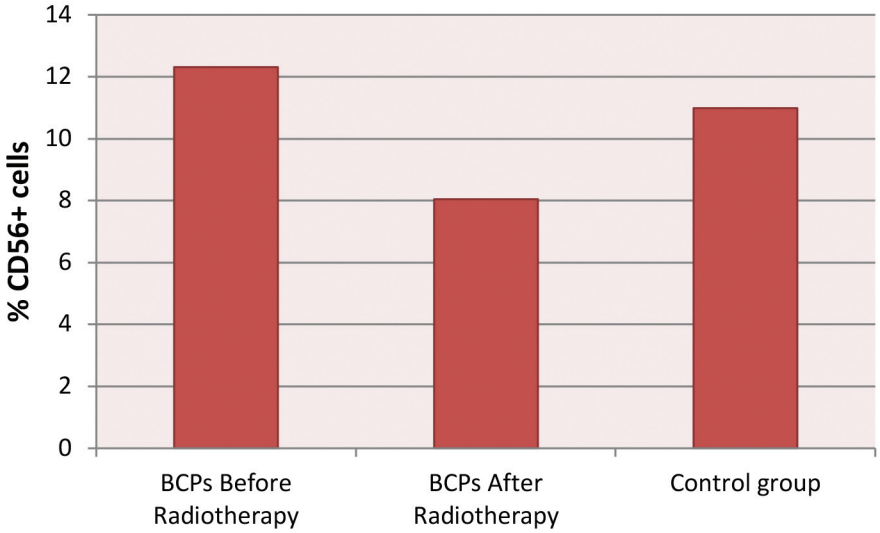

Figure 2. Bar chart illustrating the mean percentage of natural killer cells (expressed as CD56+) in patients with breast cancer before and after radiotherapy treatment compared to control subjects.

Table 3. Statistical analysis of the mean percent of natural killer cells (expressed as CD56+) in patients with breast cancer before and after radiotherapy treatment compared to control subjects

	Breast cancer patients		Control group (n=45)
	Before Radiotherapy (n=45)	After Radiotherapy (n=45)	
Percent of CD 56+ cells			
Range	7.50 – 23.50	4.90 – 12.40	5.50 – 18.0
Mean ± SD	12.32 ± 4.0	8.04 ± 2.39	10.98 ± 2.88
Median	12.10	7.30	10.10
P1	0.099	0.001*	
P2	0.001*		

*P*1: *P* value comparing the control group with each studied group; *P*2: *P* value comparing breast cancer patients before and after treatment; *: Statistically significant at $P \leq 0.05$.

healthy controls (*P*1<0.001) (Table 4 and Figure 3).

Plasma levels of INF- γ in patients with breast cancer

By use of ELISA, we quantified INF-γ cytokine in the plasma of breast cancer patients. Results are summarized in table 5 and expressed as (ng/mL). The mean ± SD of INF- γ was 149.33±13.86 in breast cancer patients before radiotherapy treatment. This value increased up to 175.17±21.04 after treatment, while it was 63.42±9.05 in healthy controls. Statistical analysis of these results showed a significant increase in INF-γ in breast cancer patients before radiotherapy treatment, compared to healthy controls (*P*1<0.001); moreover, INF-γ levels significantly increased in patients after receiving radiotherapy (*P*2<0.001). In addition, after radiotherapy treatment, patients still had significantly higher levels of INF-γ in comparison to the healthy controls (*P*1<0.001) (Table 5 and Figure 4).

Discussion

The current study showed significant changes in the cytokine levels in the plasma of breast cancer patients after receiving radiotherapy. TNF-α cytokine significantly increased in the plasma of breast cancer patients following radiotherapy to compared to pretreatment and healthy controls. The present results suggested the activation of cytokines cascade after exposure to radiotherapy. This greatly influenced cellular radiosensitivity and the onset of tissue complications.

Previous data highlighted radiation-induced changes in cytokines, chemokines, and T-cells

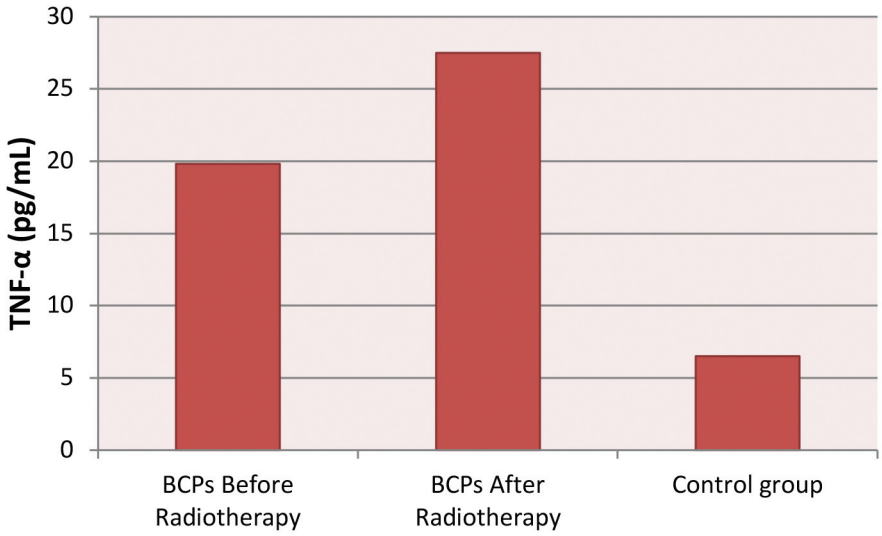

Figure 3. Bar chart illustrating the mean serum levels of TNF-α (pg/mL) in patients with breast cancer before and after radiotherapy treatment compared to control subjects.

Table 4. Statistical analysis of the mean plasma levels of TNF-α (pg/mL) in patients with breast cancer before and after radiotherapy treatment compared to control subjects

	Breast cancer patients		Control group
	Before Radiotherapy (n=45)	After Radiotherapy (n=45)	(n=45)
TNF-α (pg/mL)			
Range	14.0 – 40.0	20.0 – 36.0	2.0 – 14.0
Mean ± SD	19.83 ± 7.47	27.50 ± 4.73	6.50 ± 3.32
Median	17.0	28.0	6.0
*P*1	0.001*	0.001*	
*P*2	0.001*		

*P*1: *P* value comparing the control group with each studied group; *P*2: *P* value comparing breast cancer patients before and after treatment; *: Statistically significant at $P \leq 0.05$.

subsets. Some of these changes were attributed to the expansion of tumor reactive T-cells, improved clinical response, and increased survival.[20] Therefore, the analysis of cancer cytokine signature is a topic of interest with regard to understanding the roles of cytokines in cancer care.[21]

In agreement with the present study, Akmansu et al.[22] measured serum TNF-α and showed that radiation affected their levels in a series of 19 head and neck cancer patients undergoing radiotherapy at a mean dose of 65 Gy. All patients showed increased TNF-α after receiving radiotherapy. Furthermore, Sathishkumar et al.[23] reported that high-dose spatially-fractionated radiation resulted in a significant induction in TNF-α measured in the serum of some patients 24-72 hours following radiation. In contrast to our results, Yamamoto et al.[24] investigated basal and radiotherapy induced changes in the secretion of certain cytokines in oral squamous carcinoma cells. They showed that the basal TNF-α was higher in oral squamous carcinoma cells compared to the normal non-inflamed keratinocytes from the gingiva. After irradiation, cytokine secretion in the tumor cells moderately decreased. Many different factors are able to influence the cytokine profiles produced following radiation exposure. For instance, radiation dose and tissue type affected the local response and the antitumor effect.[24] In addition, it should be noted that in vivo and in vitro cytokine expression profiles change greatly.[25]

In this study, we quantified INF-γ cytokine in the plasma of breast cancer patients. Its level significantly increased in breast cancer patients

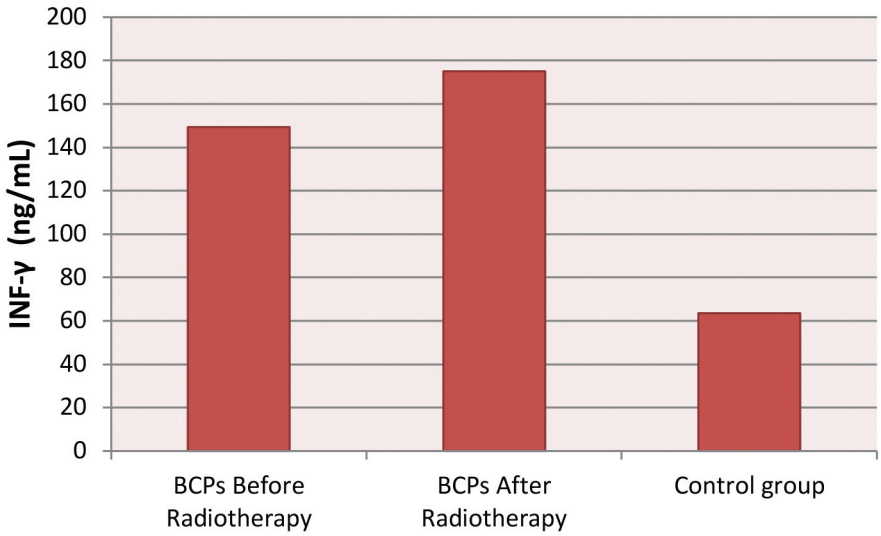

Figure 4. Bar chart illustrating the mean serum levels of IFN-γ (ng/mL) in patients with breast cancer before and after radiotherapy treatment compared to control subjects.

Table 5. Statistical analysis of the mean plasma levels of IFN- γ (ng/mL) in patients with breast cancer before and after radiotherapy treatment compared to control subjects

	Breast cancer patients		Control group (n=45)
	Before Radiotherapy (n=45)	After Radiotherapy (n=45)	
INF-γ (ng/mL)			
Range	136.0 – 190.0	150.0 – 220.0	50.0 – 80.0
Mean ± SD	149.33 ± 13.86	175.17 ± 21.04	63.42 ± 9.05
Median	144.0	171.0	65.0
*P*1	0.001*	0.001*	
*P*2	0.001*		

*P*1: *P*-value comparing the control group with each studied group; *P*2: *P*-value comparing the breast cancer patients before and after treatment; *: Statistically significant at $P \leq 0.05$.

after exposure compared to healthy controls and pretreatment. Type 2 IFN-γ are produced primarily by Th1 cells. These cells represent the subsets of CD4+ that play an important role in antitumor immune response. IFN-γ is a potent activator of macrophage, NK and neutrophil phagocytic activity. Also, it promotes the synthesis of Class I and II major histocompatibility complex (MHC) molecules that enhance antigen presentation.[26]

Similar to our findings, Reits et al.[9] showed that tumor-specific CTLs more effectively eliminated tumor cells with upregulated MHCI molecules compared to control cells. One possible explanation for increased MHCI expression on irradiated tumor cells is that ionizing radiation can induce IFN-γ secretion in the tumor microenvironment, mainly by infiltrating T lymphocytes and NK cells.[27]

Also, Ma JL[28] measured serum IFN-γ in a series of 63 esophageal carcinoma patients treated with RT alone at 60-66 Gy. IFN-γ levels increased in a dose-dependent manner in 88% patients who responded to therapy. However, in the remaining seven (12%) patients, in whom tumors recurred locally despite radiation, IFN-γ levels remained relatively constant throughout therapy.[28]

We measured the percentage of CD8+ cells and CD56+ specific for NK cells in the peripheral blood of patients with breast cancer. Our findings revealed a significant reduction in their percentage compared to their corresponding values prior to treatment as well as healthy controls. The percentage of CD8+ cells and CD56+ in the peripheral blood of patients with breast cancer was lower after RT exposure. This suggests that these changes are related to higher radiosensitivity of circulating lymphocytes.

In accordance with this study, Standish et al.[29] reported that women with stage II-III breast cancer showed lymphopenia and low NK cell activity after six weeks of RT. The decreases observed in NK cell activity after RT could be due to the reduction in NK cell numbers in the peripheral blood and/or the functional activity of NK cell. This remains an important question to be addressed in future studies. Quinfeng et al.[30] investigated CD4+ and CD8+ infiltration in tumor biopsies from cervical cancer patients, before and after radiotherapy. They found similar levels infiltration of both cells before radiotherapy. However, after irradiation, their levels significantly decreased, showing a radiation-induced rebalance in the lymphocytes within the tumor.

These novel data emphasize the importance of research on immunomodulators to maintain white cell functions in breast cancer patients. Immune therapies that increase lymphocyte counts, NK cell activity and phagocytic activity may be warranted in the post-RT period.[30]

In contrast to our results, Lim JY et al.[31] suggested that localized tumor irradiation enhanced both the generation of specific T cells and their localization to the tumor sites. They observed that improvement occurred with both fractionated and single high-dose treatment. They further characterized the frequency of CD4+ and CD8+ T cells within the tumor-draining lymph node. The number of CD8+ T cells increased in

lymph nodes draining irradiated tumors, suggesting higher antigen presentation.

These conflicting results imply that the immunological profile of different tumors is tissue-specific, where local microenvironmental parameters (such as grade of hypoxia, tissue pH, tumor stroma architecture, and the local cytokine milieu) are key factors in determining local and systemic immune responses after radiotherapy.[32]

The immune modulatory potential of radiation and its out-of field systemic effects can help to individualize treatment and guide the selection of beneficial radiation-based regimens in combination with chemotherapies, immune checkpoint-blocking agents, or immune-stimulating agents.[33]

Conclusion

Radiotherapy induced modifications of the soluble and cellular mediators of the antitumor immune response.

The percentage of CD8+ and CD56+ specific for NK cells in the peripheral blood of patients with breast cancer was significantly lower after radiotherapy compared to the before treatment.

Circulating cytokines were measured following exposure to radiation in breast cancer patients. Plasma levels of both TNF-α and INF-γ cytokines significantly increased after receiving radiotherapy.

Recommendations

Future studies should focus on underlying immune mechanisms, including larger numbers of patients with variable cancers, and if possible, be further validated by relevant clinical studies.

Future research should also define optimal radiotherapy protocols on one hand and optimal, tumor-specific immune therapeutic approaches on the other hand, so as to achieve the highest level of synergy.

Conflict of Interest

None declared.

References

1. Eriksson D, Stigbrand T. Radiation-induced cell death mechanisms. *Tumour Biol*. 2010;31(4):363-72. doi: 10.1007/s13277-010-0042-8.
2. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. *Nat Rev Immunol.* 2009;9(5):353-63. doi: 10.1038/nri2545.
3. Reynders K, Illidge T, Siva S, Chang JY, De Ruysscher D. The abscopal effect of local radiotherapy: using immunotherapy to make a rare event clinically relevant. *Cancer Treat Rev.* 2015;41(6):503-10. doi: 10.1016/j.ctrv.2015.03.011.
4. Schaue D, Kachikwu EL, McBride WH. Cytokines in radiobiological responses: a review. *Radiat Res.* 2012;178(6):505-23. doi: 10.1667/RR3031.1.
5. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Intrferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol.* 2004;75(2):163-89. doi: 10.1189/jlb.0603252.
6. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammatory, the seventh hallmark of cancer:links to genetic instability. *Carcingenesis*. 2009;30(7):1073-81. doi: 10.1093/carcin/bgp127.
7. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immune-editing. *Nat Rev Immunol.* 2006;6(11):836-48. doi:10.1038/nri1961.
8. Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature.* 2009;457(7225):102-6. doi: 10.1038/nature07623.
9. Reits EA, Hodge JW, Herberts CA, Groothuis TA, Chakraborty M, Wansley EK, et al. Radiation modulates the peptide repertoire, enhances MHC class I expression, andinduces successful antitumor immunotherapy. *J Exp Med.* 2006; 203(5):1259-71. doi:10.1084/jem.20052494.
10. Song KH, Kim MH, Kang SM, Jung SY, Ahn J, Woo HJ, et al. Analysis of immune cell populations and cytokine profiles in murine splenocytes exposed to whole-body low-dose irradiation. *Int J Radia Biol.* 2015;91(10):795-803. doi: 10.3109/09553002.2015.1068461.
11. Pandey R, Shankar BS, Sharma D, Sainis KB. Low dose radiation induced immunomodulation: effect on macrophages and CD8+T cells. *Int J Radiat Biol.* 2005;81(11):801-12. doi:10.1080/09553000500531886.
12. Deorukhkar A, Krishnan S. Targeting inflammatory pathways for tumor radiosensitization. *Biochem Pharmacol.* 2010; 80(12):1904-14. doi: 10.1016/j.bcp.2010.06.039.
13. Levy A, Chargari C, Cheminant M, Simon N, Bourgier C, Deutsch E. Radiation therapy and immunotherapy: Implications for a combined cancer treatment. *Crit Rev Oncol Hematol.* 2013;85(3):278-87. doi: 10.1016/j.critrevonc.2012.09.001.
14. Demaria S, Formenti SC. Radiation as an

immunological adjuvant: current evidence on dose and fractionation. *Front Oncol.* 2012;2:153. doi: 10.3389/fonc.2012.00153.
15. Golden EB, Formenti SC. Is tumor (R)ejection by the immune system the "5th R" of radiobiology? *Oncoimmunology*. 2014;3(1):e28133.
16. Dewan MZ, Galloway AE, Kawashima N, Dewyngaert JK, Babb JS, Formenti SC, et al. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. *Clin Cancer Res*. 2009;15(17):5379-88. doi: 10.1158/1078-0432.CCR-09-0265.
17. Schaue D, Ratikan JA, Iwamoto KS, McBride WH. Maximizing tumor immunity with fractionated radiation. *Int J Radiat Oncol Biol Phys.* 2012;83(4): 1306-10. doi: 10.1016/j.ijrobp.2011.09.049.
18. Filatenkov A, Baker J, Mueller AM, Kenkel J, Ahn GO, Dutt S, et al. Ablative tumor radiation can change the tumor immune cell microenvironment to induce durable complete remissions. *Clin Cancer Res*. 2015;21(16):3727-39. doi: 10.1158/1078-0432.CCR-14-2824.
19. Dovedi SJ, Adlard AL, Lipowska-Bhalla G, McKenna C, Jones S, Cheadle EJ, et al. Acquired resistance to fractionated radiotherapy can be overcome by concurrent PD-L1 blockade. *Cancer Res.* 2014;74(19):5458-68. doi: 10.1158/0008-5472.
20. Minafra L, Bravatà V. Cell and molecular response to IORT treatment. *Transl Cancer Res.* 2014;3(1) :32-47. doi: 10.3978/j.issn.2218-676X.2014.02.03.
21. Scola L, Vaglica M, Crivello A, Palmeri L, Forte GI, Macaluso MC, et al. Cytokine gene polymorphisms and breast cancer susceptibility. *Ann N Y Acad Sci.* 2006;1089:104-9. doi:10.1196/annals.1386.017.
22. Akmansu M, Unsal D, Bora H, Elbeg S. Influence of locoregional radiation treatment on tumour necrosis factor-alpha and interleukin-6 in the serum of patients with head and neck cancer. *Cytokine.* 2005;31(1):41-5. doi:10.1016/j.cyto.2005.02.009.
23. Sathishkumar S, Dey S, Meigooni AS, Regine WF, Kudrimoti MS, Ahmed MM, et al. The impact of TNF-alpha induction on therapeutic efficacy following high dose spatially fractionated (GRID) radiation. *Technol Cancer Res Treat.* 2002;1(2):141-7. doi:10.1177/153303460200100207.
24. Yamamoto T, Kimura T, Ueta E, Tatemoto Y, Osaki T. Characteristic cytokine generation patterns in cancer cells and infiltrating lymphocytes in oral squamous cell carcinomas and the influence of chemoradiation combined with immunotherapy on these patterns. *Oncology.* 2003;64(4):407-15.
25. Chiang CS, Chen FH, Hong JH, Jiang PS, Huang HL, Wang CC, et al. Functional phenotype of macrophages depends on assay procedures. *Int Immunol.* 2008;20(2):215-22. doi:10.1093/intimm/dxm137.
26. Venkatesh GH, Manjunath VB, Mumbrekar KD, Negi H, Fernandes DJ, Sharan K, et al. Polymorphisms in radio-responsive genes and its association with acute toxicity among head and neck cancer patients. *PLoS One*. 2014;9:e89079. doi: 10.1371/journal.pone.0089079.
27. Lugade AA, Sorensen EW, Gerber SA, Moran JP, Frelinger JG, Lord EM. Radiation-induced IFN-c production within the tumor microenvironment influences antitumor immunity. *J Immunol*. 2008; 180(5):3132-9. doi:10.4049/jimmunol.180.5.3132.
28. Ma JL, Jin L, LiYD, He CC, Guo XJ, Liu R, et al. The intensity of radiotherapy-elicited immune response is associated with esophageal cancer clearance. *J Immunol Res.* 2014;794349. doi:10.1155/2014/794249.
29. Standish LJ, Torkelson C, Hamill FA, Yim D, Hill-Force A, Fitzpatrick A, et al. Immune defects in breast cancer patients after radiotherapy. *J Soc Integr Oncol.* 2008;6(3):110-21.
30. Quinfeng S, Depu W, Xiaofeng Y, Shah W, Hongwei C, Yili W. In situ observation of the effects of local irradiation on cytotoxic and regulatory T lymphocytes in cervical cancer tissue. *Radiat Res*. 2013;179(5):584-9. doi: 10.1667/RR3155.1.
31. Lim JY, Gerber SA, Murphy SP. Type I interferons induced by radiation therapy mediate recruitment and effector function of CD8+ T cells. *Cancer Immunol Immunother.* 2014;63(3):259-71 doi: 10.1007/s00262-013-1506-7.
32. Komatsu N, Hori S. Full restoration of peripheral Foxp3 regulatory T cell pool byradioresistant host cells in scurfy bone marrow chimeras. *Proc Natl Acad Sci.* 2007;104(21):8959-64. doi:10.1073/pnas.0702004104.
33. Lumniczky K, Sáfrány G. The impact of radiation therapy on the antitumor immunity: local effects and systemic consequences. *Cancer Lett.* 2015;356(1)114-25. doi: 10.1016/j.canlet.2013.08.024.