

Ionizing Radiation Increases the Circulatory Endothelial Progenitor Cell Population in Glottic Cancer Patients

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Abstract

Background: Cancer of the throat or larynx is one of the predominant cancer types in India and Intensity Mediated Radiation Therapy (IMRT) is the most crucial treatment regimen against the disease. We aimed to examine the effect of Circulating Endothelial Progenitor (CEP) cells in Laryngeal cancer patients undergoing radiation therapy.

Patients: Five Laryngeal cancer patients were selected for the work. All patients were suffering from Squamous Cell Carcinoma (SCC) of the glottis and admitted to the institute with CT1 and CT2 stage of tumor with no lymph node metastasis (CN₀). After thorough check up the patients were subjected to conventional mononodal radiotherapy (IMRT), fractionated doses to the highest dose (1.8-2 Gy per fraction). Peripheral Blood Mononuclear Cells (PBMC) was isolated from all 5 patients and flow cytometric analysis (using anti CD 44 and CD309 monoclonal antibody) was performed before and after completion of the entire course.

Results: Results showed upregulation of the CEPs in all cases after exposure to radiotherapy. Additional immunofluorescence staining confirmed the rapid increase of these cells indicating a strong correlation between the exposure to ionizing radiation and stem cell expression in patients suffering from Glottic cancer.

Conclusion: Increase in the CEPs suggests that the tissue damage due to radiation exposure activate the stem cell niche and allures CEPs in the peripheral blood for angiogenesis. Further prospective assessments are warranted.

Keywords: Glottic cancer; Circulating endothelial progenitors; Intensity mediated radiation therapy

Introduction

The annual incidence of head and neck cancers worldwide is more than 550,000 cases with around 300,000 deaths each year [1]. Male to female ratio ranges from 2:1 to 4:1. About 90% of all head and neck cancers are squamous cell carcinomas (HNSCC). According to the American Cancer Society, about 13,560 new cases of laryngeal cancer (10,720 in men and 2,840 in women) has been documented in United States so far in 2015 (2014 Copyright American Cancer Society).

In the past decade, researchers have gained significant insights on the role of bone marrow (BM)-derived cells in adult neovascularization. A subset of BM-derived cells, called circulatory endothelial progenitor cells (CEPs), has been of particular interest, as these cells were suggested to home to sites of neovascularization and neoendothelialization and differentiate into endothelial cells (ECs) in situ, a process referred to as postnatal vasculogenesis. Therefore, EPCs were proposed as a potential regenerative tool for treating human vascular disease and a possible target to confine vessel growth in tumour pathology.

However, conflicting results have been reported in the field, and the identification, characterization, and exact role of EPCs in vascular biology is still a subject of much discussion. CEPs can be subdivided into two main categories, hematopoietic lineage CEPs and nonhematopoietic lineage CEPs. The hematopoietic CEPs originate from BM and represent a subpopulation of hematopoietic stem cells (HSCs) [2-6].

The hematopoietic CEPs can enter circulation on stimulation as cellular components of blood, negotiating a possibly heterogeneous cell population, represented by, for example, colony forming CEPs, noncolonyforming "differentiating" CEPs, myeloid CEPs, or angiogenic cells.

The nonhematopoietic CEPs are not HSC derived cells, which can be isolated from blood or tissue samples via the help of sequential culture and distinguished by their rather obvious embryonic cells (EC)-like phenotype [7] or differentiation capability into EC (-like) phenotype [8]. The origin of nonhematopoietic CEPs remains to be clarified, but they are generally thought to be derived from nonhematopoietic tissue prone lineage stem cells or organ blood vessels but not likely from HSC [9].

Another major problem with CEPs was its identification. Several literature has documented various markers for CEPs including CD133,

CDKDR, CD3, CD19, CD33 etc. However, there is still controversy on choosing the marker while working with CEPs. In our study we chose two different receptor markers for identification of CEPs-CD44 which is a well-known marker for stem cells as CEPs show stemness and Flk-1, which is type-II receptor for Vascular Endothelial Growth Factor (VEGFR-II), a protein expressed in endothelial cells through which vasculogenesis as well as angiogenesis occurs.

Previous works of Brunners et al. showed that Head and Neck Cancer patients show elevated level of EPCs whereas there was no alteration in Circulating Endothelial Cells (CECs) - endothelial cells that have been shed from the lining of the vascular wall into the blood stream [10].

In another study, Plaumbo et al. showed that radioiodine therapy causes temporary fall in CEP count [11]. When it comes to the treatment regimen, the most frequent tool for treating Glottic cancer is still radiotherapy at a dose range of 65-75 Gy, the highest range of radiation dose among all types of cancers. In this pilot study, we tried to examine the alteration in the CEP count present in peripheral blood of Glottic cancer patients undergoing radiotherapy.

Patient Samples

5 Patients undergoing IMRT (60 Gy, 2 Gy fractions) in Chittaranjan National Cancer Institute (CNCI), Kolkata, India, were screened from April, 2014 to March, 2015, and demographic details for all patients including age, sex, and personal history about smoking were recorded. All blood samples were collected on the day radiation treatment started (for pre-treatment) and after one week of completion of total radiotherapy cycle (for post-treatment).

All tumors were histopathologically categorized as squamous cell carcinoma of the larynx. Patients with recurrent glottic tumor and other types of laryngeal cancer (i.e., verrucous, sarcomatoid, and lymphoepithelial carcinoma) were excluded from the study. All works described here had been carried out in accordance with The Code of Ethics of the World Medical Association. (Declaration of Helsinki) and after getting formal consent from each patient.

Patient ID	Age (Years)	Sex	TNM Stage	Treatment Regimen
P1	63	Male	CT1N0Mx	IMRT (60 Gy, 30 cycles)
P2	56	Male	CT2N0Mx	IMRT (66 Gy, 33 cycles)
P3	50	Male	CT1N0Mx	IMRT (60 Gy, 30 cycles)
P4	66	Male	CT2N0Mx	IMRT (66 Gy, 33 cycles)
P5	67	Male	CT2N0Mx	IMRT (66 Gy, 33 cycles)

Flow cytometric analysis

PBMCs were isolated from the whole blood by Ficoll density gradient centrifugation. The peripheral blood was mixed with 1X Phosphate Buffer Saline (PBS) and the mixture was put dropwise on Ficoll in 4:3 ratios.

The samples were next centrifuged in 900 rpm for 30 minutes in a fixed angle rotor at room temperature and PBMCs were carefully collected from the buffy coat. 0.3×10^6 cells were taken for each experiment and after thorough washing using PBS; the cells were incubated with PE tagged anti CD 44 (ScBt) and FITC tagged anti Flk-1 antibodies (ScBt). After proper fixation with paraformaldehyde, the enumeration of CEP count was acquired using BD FACSCalibur Flow Cytometer.

Immunofluorescence staining

The PBMC suspension was poured on a slide (100 μ l) and fixed with Methanol for 15 minutes. Once fixation was done, cells were incubated with rabbit polyclonal antibody against CD 44 and Flk-1 (1:600 dilutions, ScBt) for 1 hour at room temperature.

FITC conjugated secondary rabbit polyclonal antibody (1:800 dilutions) were used and incubated at room temperature for 45 minutes. Diaminodiphenylindole (DAPI) was used for the nuclear staining and mounting. The cells were imaged under fluorescence microscope (Leica DM4000 B, Germany).

Result

Results showed marked upregulation in CD 44 and Flk-1 expression in all cases after completion of radiotherapy (Figure 1). When compared, the patients' pre-treatment status for CEPs increased almost up to 1.5 fold.

Surprisingly when the expression status of CD 44 and Flk-1 was examined in the monocyte population, post-radiation parameters showed slight decrease in those proteins' expression (Figure 2). Immunofluorescence staining clearly showed positive expression for CD 44 as well as Flk-1 in the PBMCs (Figure 3).

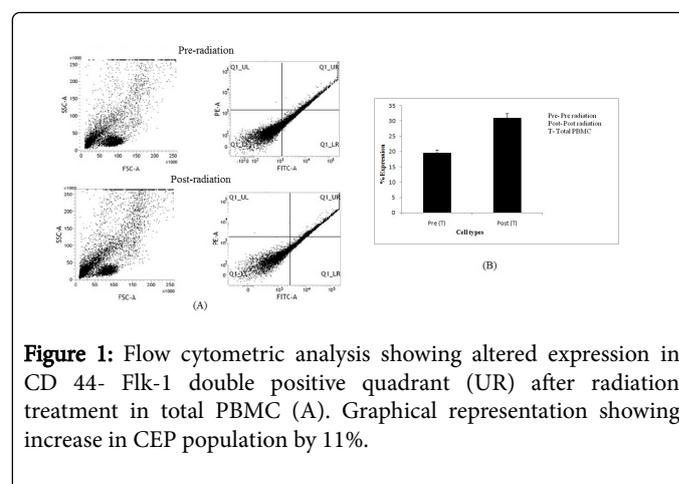


Figure 1: Flow cytometric analysis showing altered expression in CD 44- Flk-1 double positive quadrant (UR) after radiation treatment in total PBMC (A). Graphical representation showing increase in CEP population by 11%.

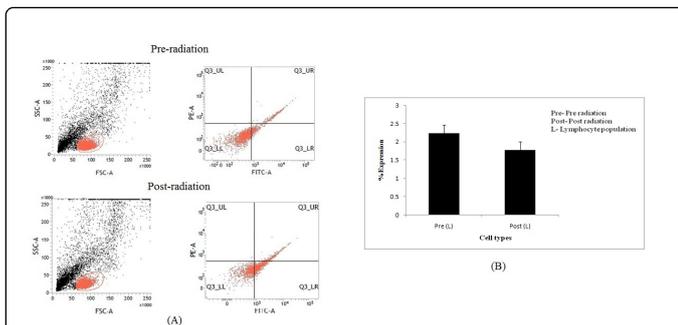


Figure 2: Flow cytometric analysis showing altered expression in the CEP marker (CD 44-Flk-1) quadrant (UR) after radiation treatment in the lymphocyte population (A). Graphical representation showing decrease in CEP population by 1%.

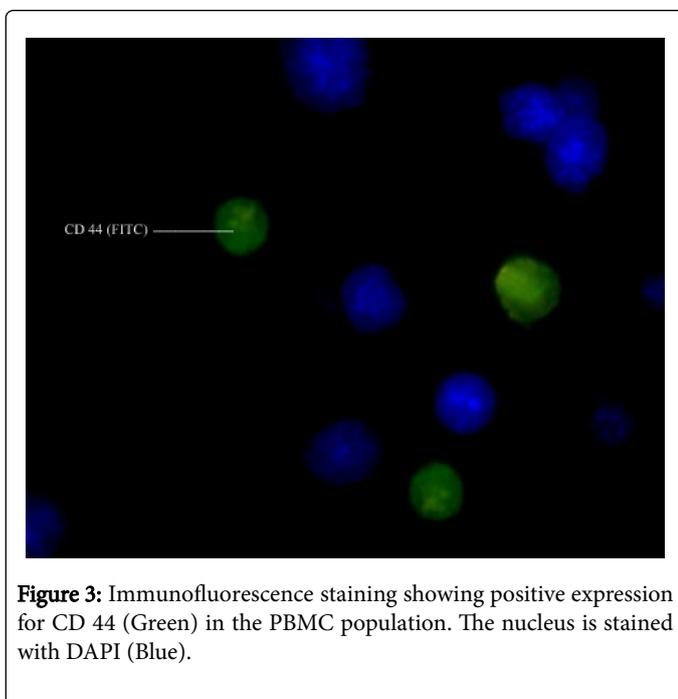


Figure 3: Immunofluorescence staining showing positive expression for CD 44 (Green) in the PBMC population. The nucleus is stained with DAPI (Blue).

Discussion

Recent study suggests that endothelial cells are the principal target of radiation injury in the intestinal mucosa and elsewhere [12,13]. Apoptosis of endothelial cells in the microvasculature occurs first, whereas death of neighboring epithelial cells is a secondary event leading to clinical symptoms associated with mucositis, colitis, and other organ toxicities. Although radiotherapy is known to reduce the pool of hematopoietic stem cells [14], so far, there have been only a few clinical reports on the detection of circulating CEPs and most of these studies have evaluated the significance of CEPs in non-cancer conditions [15-17]. The significance of circulating CEPs in cancers has been reported in case of multiple myeloma [18,19].

Recent studies have also demonstrated that the number of CEPs in the peripheral blood of cancer patients is altered by disease status and treatment, such as chemotherapy and anti-angiogenic therapies [20]. Accordingly, a potential value of CEPs as biomarker in the disease

course and responses to newly introduced chemotherapeutic drugs is increasingly being discussed. Recently, a series of investigations have suggested that endothelial progenitor cells (CEPs) derived from the bone marrow are present in peripheral blood and these cells get recruited to denuded areas for incorporation into the nascent endothelium [21,22]. New therapies targeting to induce these progenitor cells, which are fundamental to radiation induced injury repair, can significantly improve clinical treatment of cancer. Therefore, it is of paramount importance to identify subpopulations of cells exhibiting stem marker expression and behavior. Our study showed that Glottic cancer patients undergoing IMRT has an increased amount of CEPs as response to radiation which may be used as a marker for further characterization of the disease.

Conflict of Interest

The authors have declared no conflict of interest exists.

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