Adipose-Derived Mesenchymal Stem Cell Features in Patients with a History of Head and Neck Radiation

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Objectives/Hypothesis: Radiation plays a prominent role in advanced stage head and neck tumors. Often, the radiated area includes adjacent nonmalignant mesenchymal tissue, which contains a mixture of cells that has been shown to accelerate wound healing. The purpose of this study is to determine the long-term effect of radiation on the expansion potential of adipose-derived stromal/stem cell (ADSC) tissue and on the ability of resident stem cells in this fraction to undergo phenotypic differentiation.

Study Design/Methods: After institutional review board approval, 12 patients with a history of head and neck radiation and pending surgery were enrolled. Adipose tissue was collected from irradiated tissue (XRT) and nonirradiated tissue (NRT) sites. Mesenchymal stem cells were isolated from these populations, with subsequent assessment of cellular kinetics and differentiation potential between harvest sites.

Results: Adipose-derived stromal/stem cells could not be isolated from XRT in six patients due to lack of in vitro cell proliferation. For the remaining six patients, overall cumulative population-doubling time was longer for XRT relative to NRT (29.3 vs. 11.5 days; P = 0.02). However, no significant differences were observed in cell generation time or viability. When XRT and NRT ADSC fractions were grown to standardized concentrations and incubated under conditions that induce phenotypic differentiation of resident stem cells, no significant changes in chondrogenic, adipogenic, or osteogenic differentiation were observed.

Conclusion: These preliminary observations suggest that irradiated ADSCs close to the surgical site undergo long-term changes in proliferative capacity. The potential for phenotypic differentiation is retained, however, in ADSCs that survive the irradiation process.

Key Words: Regenerative medicine, adipose-derived stem cell, radiation, stem cell, phenotypic, differentiation.

Level of Evidence: 2b.

INTRODUCTION

In recent years, mesenchymal stem cells (MSCs) have come to the forefront of experimental and clinical medicine with applications including wound healing,[1] treatment of osteoradionecrosis,[2] improved viability of cryopreserved ovarian grafts,[3] and de novo tissue engineering. This population of cells can be harvested from several body tissues and contains a variable percentage of stem cells that exhibit capacity for multilineage differentiation.[4] In adipose tissue, the stem cell fraction is esti-

mated to make up to 1% to 10% of the total nucleated cell population.[5] There is growing interest to translate the use of human adipose-derived stromal/stem cells (ADSCs) into an expanding range of therapeutic applications because of their pluripotency, ubiquitous distribution, less controversial status when compared to fetal stem cells, and resistance to unwanted transformation when compared to induced pluripotent stem cells. Nevertheless, the response of human ADSCs to xenobiotic insults such as radiation remains poorly characterized.

Radiation therapy is now a necessary consideration in over 50% of all new cancer diagnoses, including the majority of severe head and neck malignancies.[6] Because the irradiated area often includes mesenchymal tissue, resident stem cells may be affected. In vivo mouse data show that lethal or sublethal doses of radiation decrease the proliferation and differentiation of nonhematopoietic progenitors[7] and decrease the differentiation of preadipocytes into mature adipocytes. However, there is also evidence that bone marrow-derived human MSCs in culture exhibit resistance when exposed to ionizing radiation.[8] Despite this in vitro data, little is known about the long-term effects of radiation on human ADSCs in situ, particularly with respect to their proliferative and differentiation capacity. To further this understanding, we compared the expansion and differentiation of ADSCs prepared from patients with a history of head

Summary of Clinical and Biological Data for the Adipose Tissue Donors With Cells Who Progressed Through First Passage.

ID	Age	Sex	Chemotherapy	Primary Tumor	XRT Fat Site	NRT Fat Site	Radiation to Surgery (days)	Dose (cGy)	P1 CFLT NRT	P1 CFLT XRT*†
ADSC1	60	M	no	larynx	neck	chest	1,365	7,200	6	50
ADSC2	56	M	no	oral cavity	neck	leg	2,641	6,000	7	21
ADSC3	61	M	no	larynx	neck	chest	1,20	7,000	14	43
ADSC4	65	M	yes	oral cavity	neck	arm	2218	7,000	21	31
ADSC5	65	M	no	oral cavity	neck	chest	3,053	5,940	7	14
ADSC6	25	F	yes	lacrimal ACC	neck	leg	358	5,940	14	17
Mean ± SD	55.3 ± 15.2	–	–	–	–	–	–	6,513 ± 611	11.5 ± 5.89	29.3 ± 14.7

*$P = 0.02$ for XRT compared to NRT CFLT.
†$r = 0.933$; $P = 0.006$ dose vs. CFLT
ADSC = adipose-derived stromal/stem cell; F = female; M = male; NRT = nonirradiated tissue; P1 CFLT = passage 1 time to confluence; SD = standard deviation; XRT = irradiated tissue.

and neck irradiation from harvest sites in and out of the radiation field.

MATERIALS AND METHODS

Patient Selection

Upon approval from the institutional review board (IRB) (IRB#: 13-007986), Stem Cell Research Oversight Subcommittee, and Mayo Clinic Center for Regenerative Medicine, 12 patients were prospectively enrolled. All had a history of head and neck irradiation for treatment of malignancy and were being consented for multisite surgery such as a fibular-free flap or similar flap reconstruction. Data collected for each patient included time from radiation to surgery, type of surgery, primary cancer treated, amount of radiation received at harvest site, age at surgery, sex, secondary adipose collection site, and whether or not the patient received concomitant chemotherapy (Table I). At the time of surgery, 5 mL of adipose tissue was harvested from the previously radiated site, which in every case was the patient's neck; and an additional 5 mL of adipose tissue was collected from the secondary surgery site from which the patient's reconstructive tissue was being harvested (i.e., adipose tissue from the leg in the case of a fibular free flap).

Isolation, Culture, and Differentiation of the Adipose-Derived Stromal/Stem Cells

After surgery, the tissue was taken in sterile conditions and processed by briefly mincing with surgical scalpels, followed by digestion for 90 minutes at 37°C with 0.075% collagenase I (Worthington Biochemical, Lakewood, NJ) in Advanced MEM media (A-MEM, ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS). Digested tissue was centrifuged at 500× g for 5 minutes; the pellet was then washed with phosphate-buffered saline; and large particulate matter was removed with a 70-μm cell strainer (BD Biosciences, San Jose, CA). Following another round of centrifugation as above pellets were incubated overnight with A-MEM/10% (FBS, Thermofisher) in tissue culture containers to separate adhesive mesenchymal cells, including adipose stem cells, from nonadhesive tissue components of the stromal vascular fraction (SVF). Then, fresh media was added and cultures were checked daily for confluence with media changes being performed every third day. At 70% to 80% confluence, the cells were frozen and stored. This expanding ADSC fraction of adherent cells contains an overwhelming majority of adipose-derived stem cells (ADSCs), in addition to a small fraction of other cell types,[9] and thus con-

stituted the starting material for our experiments. Freshly thawed passage 1 cells were used to measure generation time, percent viable cells, and population-doubling time over the course of six more passages, as previously described.[4] Differentiation of passage 1 ADSCs was performed using StemPro media with appropriate supplements (ThermoFisher Scientific cat# A10071-01 for chondrogenesis, cat# A10070-01 for adipogenesis, and cat# A10072-01 for osteogenesis), according to the manufacturer's protocols. Adipogenic and osteogenic differentiation were assessed after 2 and 4 weeks, respectively. Partial chondrogenic differentiation in monolayer cell culture required 2 weeks of incubation with the chondrogenic media supplement.

Cell Proliferation Kinetics

Cells were grown to 70% to 80% confluence before passaging. Time to reach initial confluence during passage 1 was recorded. Cells were then passaged six additional times, recording population doubling, cellular viability, and generation time for each passage. Population doubling was calculated from the difference between the number of harvested and plated cells. Values were totaled to calculate cumulative population doubling (CPD). Generation time for each passage was taken as the average time between two cell doublings.[10] Area under the curve (AUC) was calculated for group comparison, and statistical significance was determined with z tests[11] All other group comparisons were made with Student's t test.

Cell Proliferation Kinetics and Statistical Analysis

Cells were grown to 70% to 80% confluence before passaging. Time to reach initial confluence during passage 1 was recorded. Cells were then passaged six additional times, recording population doubling, cellular viability, and generation time for each passage. Population doubling was calculated from the difference between the number of harvested and plated cells using the following formula.[10,12]

$$X = [log10(NH) - log10(N1)]/log10(2)$$

NH is the harvested cell number and N1 is the plated cell number. The population density (PD) for each passage was calculated and added to the PD of the previous passages to generate data for CPD.

Cell doubling, or generation time (X), was calculated for each passage using the formula:

$$X = (log2X\Delta t)/[\log{(NH)} - \log{(N1)}],$$

where Δt is the interpassage time.

Area under the curve for group comparison was calculated using Riemann sums,[13] and statistical significance was determined with z tests[11] All other group comparisons were made with Student t test using a $P < 0.05$ significance level.

Staining and Image Analysis

Biological stains were obtained from Sigma Aldrich (St. Louis, MO): Alcian Blue 8GX (cat# A3157) for acidic glycosaminoglycans in chondrocytes, Oil Red O (cat# O0625) for neutral fat and lipoproteins in adipocytes, and Alizarin Red (cat# A5533) for calcium deposits in osteocytes. Staining/destaining was performed using previously described protocols (Promo-Cell). To quantitate adipogenic and osteogenic differentiation by spectrophotometry, the stain was eluted in 1.0 mL isopropanol, or in ethanol-acetic acid as previously described.[14] To quantitate differentiated cells by image analysis, five representative fields were obtained for each sample, images were converted to grayscale, and intensity was analyzed using ImageJ software (NIH, Bethesda, MD).[15]

RESULTS

Lower Initial Proliferation Rates for Irradiated Tissue Adipose-Derived Stromal/Stem Cells Correlate With Radiation Exposure

Clinical and biological data are summarized in Table I. Following plating of SVF and subsequent removal of nonadherent cell populations, irradiated tissue (XRT) cells reached confluence after a mean 29.3 ± 14.7 days, and nonirradiated tissue (NRT) after a mean 11.5 ± 5.9 days ($P = 0.02$). Because initial plated cell counts could not be obtained, it remains unclear if the slower expansion of XRT ADSC reflects a lower starting cell count, some other long-term effect of radiation, or a combination of the two. For the XRT but not the NRT, there was a strong correlation between the dose of radiation and time to reach confluence ($r = 0.933$, $P = 0.006$), which is readily reflected in the disparity between those who received approximately 70 Gy (41.3 days) and those who received approximately 60 Gy (17.3 days). The XRT samples whose cell lines arrested during the first passage (Table II) generally came from older patients (mean 69.6) with higher doses of radiation

(mean 65 Gy). The time to confluence in the NRT group from this cohort was similar to the NRT group from patients whose XRT samples made it through the first passage (mean 17 days) (Table II).

Irradiated Tissue Adipose-Derived Stromal/Stem Cells Have Slower Proliferation Kinetics

Two features of cellular kinetics, CPD, and generation time (GT) were recorded along with the percent cellular viability (%V) at the end of each passage (Fig. 1). Overall group differences between XRT and NRT cells were calculated by comparing AUC values for CPD, %V, and GT as functions of the passage number. However, as shown in Figure 1D, a small but significant difference (22%, $P = 0.05$) in the CPD AUC persisted between XRT and NRT cells.

Irradiated Tissue Adipose-Derived Stromal/Stem Cells Retain Capacity for Induced Phenotypic Differentiation

Well-established protocols exist for the induction of adipogenic, osteogenic, and chondrogenic differentiation of ADSCs.[16,17] To determine if radiation alters the differentiation ability of adipose tissue-derived stem cells, ADSC fractions were incubated under adipogenic, osteogenic, and chondrogenic conditions and stained appropriately, as indicated in Materials and Methods (Fig. 2). Two strategies were used to obtain quantitative measurements for comparing XRT and NRT cells. For adipogenic-differentiated cells, the dye was eluted with isopropanol[18]; supernatants were collected by brief centrifugation; and optical density was measured at 405 nm. Qualitative and quantitative comparison of XRT and NRT cells showed a similar extent of adipogenic, osteogenic, and chondrogenic differentiation (Fig. 2A, 2B, 2C). For osteogenic differentiation, we combined direct ImageJ (NIH) analysis of the stained cells with spectrophotometric measurement of the eluted dye[14] and found good correlation between these measurements (Fig. 2B). For this reason, ImageJ (NIH) analysis alone was used to determine chondrogenic differentiation. As before, no statistically significant differences were observed.
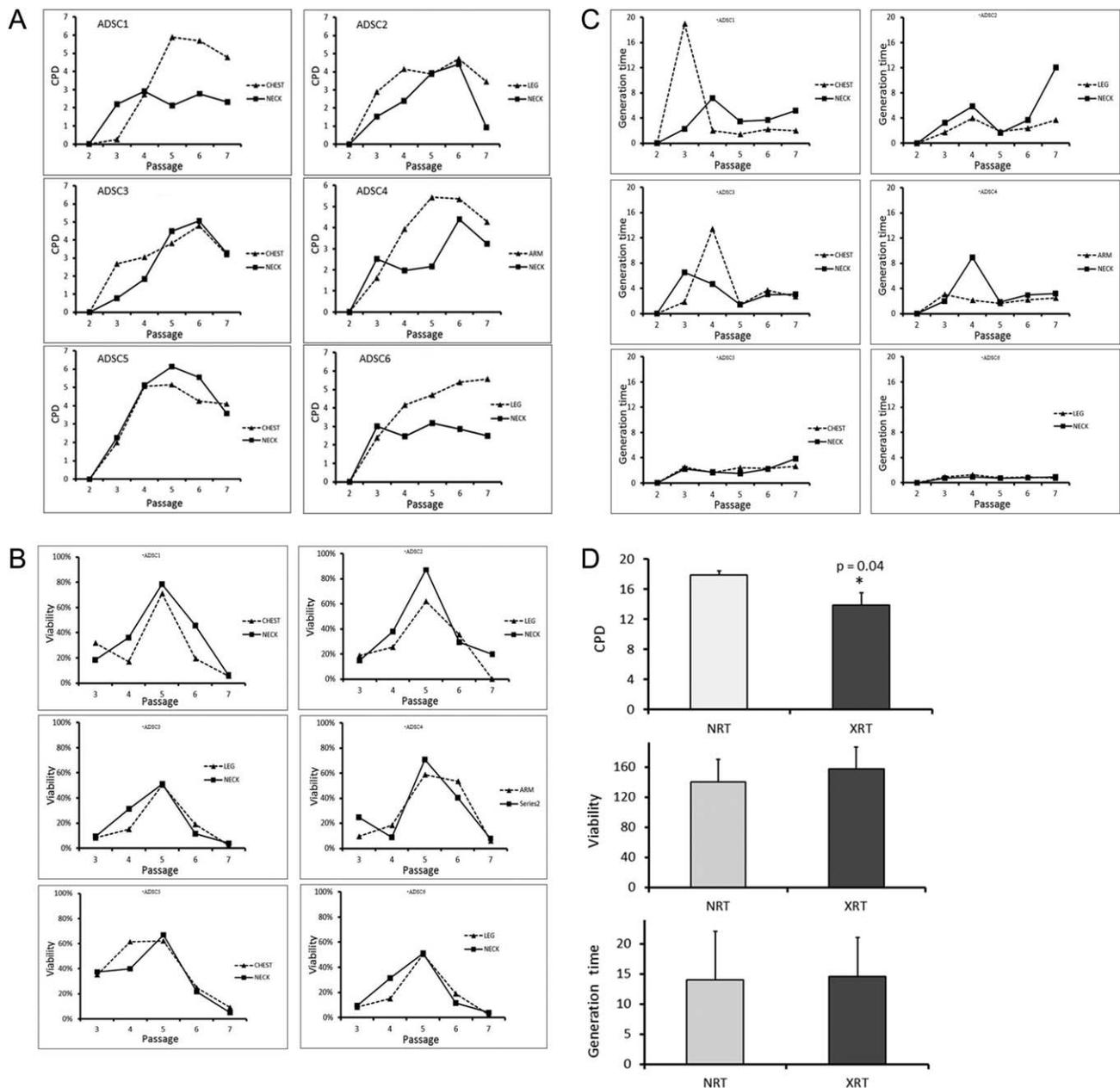
Fig. 1. Cellular kinetics of XRT and NRT cells from all donors during six successive passages. 2×10^5 fresh frozen cells containing the ADSC fraction were plated in 10-cm culture dishes and grown to confluence before re-passaging. A) Cumulative population doubling; (B) percent viability; (C) generation time; (D) AUC analysis. AUC was calculated for each donor cell population belonging to the NRT and XRT groups as, described in Methods. XRT ADSCs from the head and neck area are labeled "Neck" in all cases.
ADSC = adipose-derived stromal/stem cell; AUC = area under curve analysis; CPD = cumulative population doubling; NRT = nonirradiated tissue; XRT = irradiated tissue.

DISCUSSION

Adipose tissue is a rich source of mesenchymal cells with significant therapeutic potential in wound healing and regenerative medicine. Human ADSCs have not been previously characterized with respect to in situ changes in proliferation kinetics and pluripotency as a result of radiation exposure due to postsurgical oncology management. With these cells having received high doses of radiation, the goal of the present work was to compare their proliferative and differentiation potential

relative to ADSCs from nonirradiated harvest sites. Out of 12 patients recruited for this study, only six were capable of growing ADSCs from their XRT harvest sites in laboratory culture conditions. The inability to culture cells from the radiated tissue of half of the enrolled patients is a testament to the arduous process of reaching the first passage in radiated samples. In the six patients whose cells could be cultured, we found that XRT ADSCs proliferate slower compared to NRT cells on the first passage, but they appear to proliferate similarly
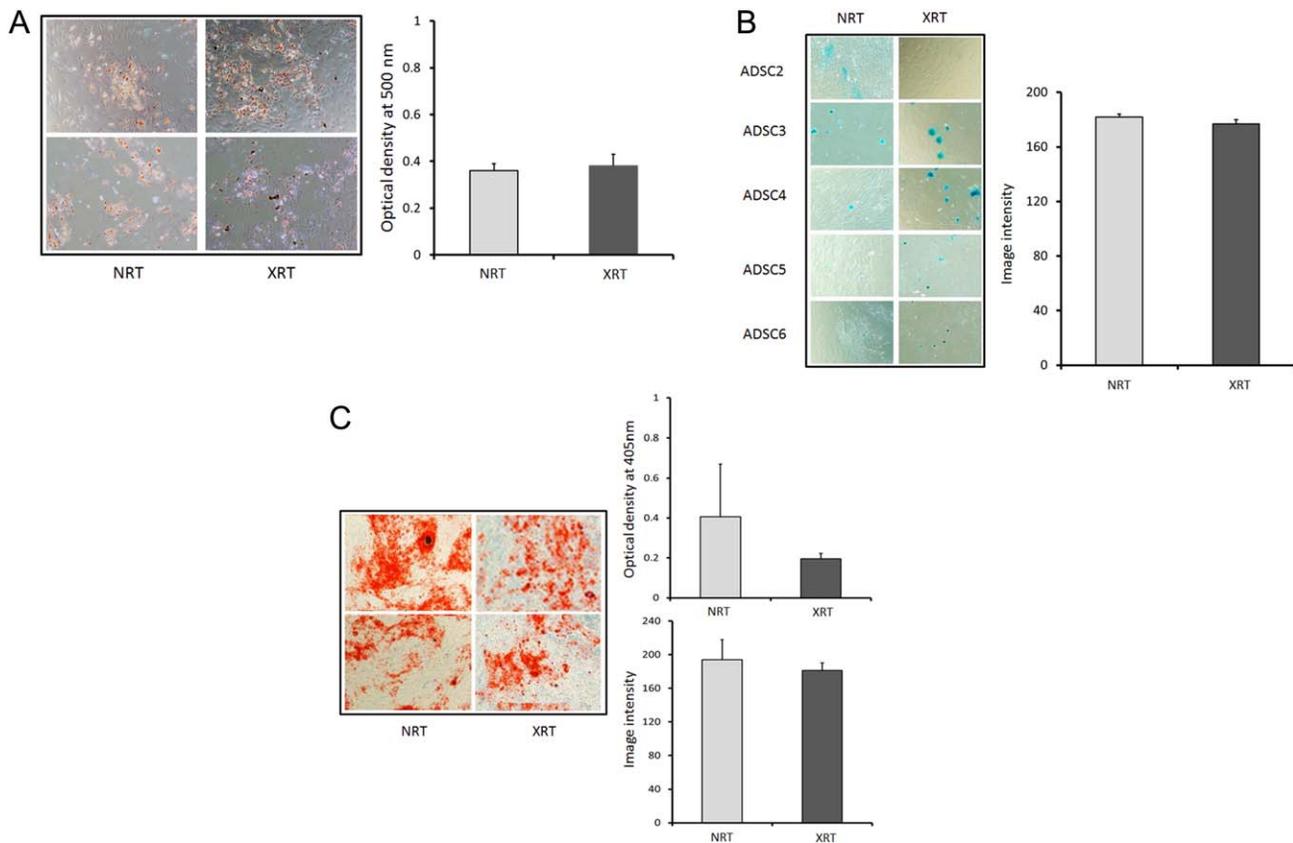
Fig. 2. Comparison of differentiation features of XRT and NRT ADSCs. (Left) Representative photographs of stained cells after induction of differentiation (20× magnification). (Right) Quantitation of eluted dyes by spectrometry and/or ImageJ analysis. CT = NRT cells. (A) Adipogenic differentiation; (B) osteogenic differentiation; (C) chondrogenic differentiation.
ADSC = adipose-derived stromal/stem cell; NRT = nonirradiated tissue; XRT = irradiated tissue.

on subsequent passages. Likewise, phenotypic differentiation along osteogenic, adipogenic, and chondrogenic lineages appears to be similar. It can therefore be inferred that if the cells are present in the radiated adipose tissue and have the capacity to proliferate, albeit slow, they ultimately behave similarly to cells from nonradiated adipose tissue. Thus, subsequent passages and ultimate phenotypic differentiation are similar when directly compared to adipose tissue that has not been previously radiated. Despite this, the unreliability of cellular growth, with only half of the radiated samples even producing cells, brings into question the viability of ADSCs in this tissue and thus calls to question their utility for regenerative efforts. The negative effect of radiation on the reliable first passage of cells is propounded by several observations in this study, including the higher average dose of radiation sustained in the group with arrested XRT samples and the longer P1 time to confluence seen in the XRT samples from patients with higher doses of radiation. The effect of chemotherapy is difficult to assess given the small sample size.

To investigate cellular kinetics, we used benchmarks of cell health such as cumulative population doubling, cellular viability, and generation time. A possible explanation for the slower expansion rate of XRT cells during passage 1 is the presence of fewer cells per mL volume of starting SVF material relative to SVF collected from NRT sites. This could result from the onset of fibrosis displacing the cellular component of the SVF. This possibility is supported by observations that similar doses of radiation to those received by the patients in the study were shown to induce tissue fibrosis.[19] In addition to the irradiated tissues possibly providing a lower starting cell count, ionizing radiation has also shown to have long-term effects on the growth of mesenchymal cells in previous work. Bone marrow-derived mesenchymal stromal cells exposed ex vivo to 30 Gy to 60 Gy of radiation initially had longer cumulative population doubling times than nonirradiated cells; however, a small subset of cells continued to proliferate with a constant level of cell doublings. Likewise, this dosage of radiation was found to be fatal to many of the cells upon exposure.[20] Similarly, a dose as small as 15 Gy was shown to increase cumulative population doubling in vitro.[21] These results are consistent with our observations regarding differences in CPD between XRT and NRT cells at passages 0 to 1 (XRT taking longer) and at passages 2 to 6 (similar rates). Ionizing radiation is reported to induce a host of changes in cell cycle regulatory factors and widespread epigenomic remodeling with promoter hypermethylation as a main mechanism of gene

silencing.[19] The present study, although limited to six cases, is one of the few available in the literature where the effect of radiation exposure on pluripotency and cellular kinetics of human ADSCs is investigated long after the initial irradiation event. In this work, radiation did not affect ADSC pluripotency because staining for adipogenic, osteogenic, and chondrogenic biomarkers was similar between XRT and NRT cells. This further reinforces already available evidence that pluripotent stem cells are more resistant to radiation exposure than stromal cells.

CONCLUSION

These preliminary observations suggest that irradiated adipose mesenchymal cells close to the surgical site undergo long-term changes in proliferative capacity. However, the potential for phenotypic differentiation of ADSC pluripotent stem cells is retained. As the therapeutic range of ADSCs continues to expand into the fields of tissue engineering and immunomodulation, careful site selection out of the radiation field should be deeply considered when employing these therapies in head and neck cancer patients.

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