Cell Injury, Repair, Aging and Apoptosis

Adipose Tissue Sensitivity to Radiation Exposure

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Treatment of cancer using radiation can be significantly compromised by the development of severe acute and late damage to normal tissue. Treatments that either reduce the risk and severity of damage or that facilitate the healing of radiation injuries are being developed, including autologous adipose tissue grafts to repair tissue defects or involutinal disorders that result from tumor resection. Adipose tissue is specialized in energy storage and contains different cell types, including preadipocytes, which could be used for autologous transplantation. It has long been considered a poorly proliferative connective tissue; however, the acute effects of ionizing radiation on adipose tissue have not been investigated. Therefore, the aim of this study was to characterize the alterations induced in adipose tissue by total body irradiation. A severe decrease in proliferating cells, as well as a significant increase in apoptotic cells, was observed *in vivo* in inguinal fat pads following irradiation. Additionally, irradiation altered the hematopoietic population. Decreases in the proliferation and differentiation capacities of non-hematopoietic progenitors were also observed following irradiation. Together, these data demonstrate that subcutaneous adipose tissue is very sensitive to irradiation, leading to a profound alteration of its developmental potential. This damage could also alter the reconstructive properties of adipose tissue and, therefore, calls into question its use in autologous fat transfer following radiotherapy. (Am J Pathol 2009, 174:44–53; DOI: 10.2353/ajpath.2009.080505)

Radiation therapy remains the cornerstone of modern cancer management, with an estimated half of all newly diagnosed cancer patients receiving radiotherapy at some point during the course of their disease. Compared with surgery, radiation therapy has the advantage of being potentially organ-preserving, although the functional

outcome might be negatively impacted by the side effects. Indeed, irradiation perturbs the homeostatic network linking parenchymal, mesenchymal, and vascular cells within tissues. Normal communication between cells through soluble, matrix- and cell-associated ligands and receptors is altered, as is an inexorable series of events leading to tissue regeneration and healing.^{1,2}

The use of radiation therapy to treat cancer inevitably involves the exposure of normal tissues that could develop complications. The damage in normal tissues differs depending on the target organ and cell type. Radiation injury is commonly classified into acute, consequential, or late effects, depending on the time before the appearance of symptoms. Acute (early) effects are those that are observed during the course of treatment or within a few weeks following the treatment. Acute radiation damage is most prominent in tissues with rapidly proliferating cells such as the epithelial surfaces of the skin or alimentary tract.^{3,4} Ionization events cause damage to vital cellular components, leading to cell death within the first few divisions following irradiation. Radiation also activates various cellular signaling pathways that lead to expression and activation of pro-inflammatory and pro-fibrotic cytokines, vascular injury, and activation of the coagulation cascade.⁴ Late reactions occur months to years following radiation exposure and are primarily the result of radiation-dependent depletion of tissue-specific stem cells or progenitors leading to fibrosis, organ dysfunction, and necrosis. In late-responding normal tissues, where cell death is not compensated for by rapid regeneration, this process unfortunately often culminates in the symptomatic complications of radiation exposure.^{5,6} Treatments that reduce the risk or the severity of damage to normal tissue, or that facilitate the healing of radiation injuries, are being developed. These treatments could greatly improve the quality of life of patients treated for cancer.

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Plastic and reconstructive surgical procedures are thus performed to repair tissue defects or involutonal disorders resulting from tumor resection. Different strategies have been used, including the use of autologous tissue transfer of tissues such as fat tissue.⁷ Adipose tissue is a highly specialized connective tissue whose primary function is to provide the body with an energy source. The primary cellular component for adipose tissue is a large collection of lipid-filled cells known as adipocytes. Other cellular components contained in adipose tissue are stroma-vascular cells, including endothelial and hematopoietic cells, and preadipocytes.^{8–11} Either preadipocytes or whole subcutaneous pads have been transplanted in patients to restore the volume of tissue lost at defect sites¹² or for the treatment of degenerative chronic lesions induced by oncologic radiation.^{13,14}

The sensitivity of healthy subcutaneous adipose tissue to radiation exposure has, however, never been studied. In other words, it is not known whether irradiated adipose tissue presents healing or reconstructive properties in autologous transplantation therapy, as healthy stromal cells do,¹⁵ or if irradiation of adipose tissue may be an issue for the patients who undergo total body radiotherapy.

Therefore, the aim of this study was to determine the characteristics of subcutaneous adipose tissue isolated from mice after total body irradiation (TBI). Proliferation and apoptosis were quantified *in vivo*. Phenotypic analysis of the stroma-vascular fraction was performed, and proliferation and differentiation potentials of progenitor cells were evaluated *in vitro*.

Materials and Methods

Materials and Antibodies

For adipose tissue digestion, bovine serum albumin and collagenase were purchased from Sigma Aldrich (St. Quentin Fallavier, France). Culture medium and newborn calf serum were provided by Invitrogen (Cergy-Pontoise, France). The methylcellulose used was Methocult M3534 (StemCell Technologies, Vancouver).

For fluorescence-activated cell sorter (FACS) analysis, we used directly conjugated primary murine monoclonal antibodies (all BD Biosciences, Heidelberg, Germany, unless indicated) against mouse CD34-fluorescein isothiocyanate (FITC) or -phycoerythrin (clone RAM34), mouse CD45-peridinin chlorophyll-a protein (PerCP) (clone 30-F11), mouse CD90-allophycocyanin (clone 53-2.1), mouse Ly-6A/E (Sca-1)-phycoerythrin (clone D7), and mouse CD31-allophycocyanin (clone MEC13.3). We used directly conjugated rat immunoglobulin (BD Biosciences) for isotype controls.

Animals

Lethal (10Gy) or sublethal (7Gy) irradiation, given in one dose (BIOBEAM 8000, Cs137, 4.4 Gy/min), was performed on 5- to 6-week-old C57Bl/6 mice (Harlan,

France). Animals were housed in a controlled environment (12 hours light/dark cycle at 21°C), and maintained on acidified water and autoclaved food for 7 days. At the end of the experiments, the mice were euthanized by cervical dislocation under CO₂ anesthesia. Blood samples were immediately drawn and tissues were quickly removed and processed for analyses as described.

Analysis of Peripheral Blood

Before and 7 days after irradiation, 200 μ l of peripheral blood was collected from the retro-orbital plexus and immediately transferred into vials containing heparin. Blood cell counts were performed automatically using a hematology analyzer. The differential of the nucleated cells was determined automatically by the analyzer (Micros OT 60 ABX, No. 21CFR864.5220 Montpellier, France).

Paraffin-Embedded Sections of Adipose Tissue

Subcutaneous adipose tissue was removed, fixed on 95% ethanol, and embedded in paraffin. Sections were deparaffinized in xylene, and rehydrated in descending grades (100% to 50%) of ethanol with a final wash in tris-buffered saline (TBS). Slides were then either stained with May-Grünwald Giemsa or processed for Ki-67 staining or terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analysis. Slides stained with May-Grünwald Giemsa were then used for determination of mature adipocyte size, by using image J software (NIH, USA).

Immunostaining for Ki-67

For Ki-67 staining, sections were incubated in 10 mmol/L citrate buffer (pH 6.0), for antigen retrieval. Endogenous peroxidase activity was removed by incubating with H₂O₂, 3% in TBS for 15 minutes. Ki-67 protein was detected using a 1:25 dilution of rat monoclonal antibody (Clone TEC3, Dakocytomation, Denmark). Histofine simple stain mouse "MAX PO" anti-rat (Microm, Francheville, France) was used as a secondary antibody. Finally, sections were stained using 3-Amino-9-ethylcarbazole (AEC), and counterstained using hematoxylin. Control experiments were performed using purified rat IgG. Resulting immunostaining was observed using an optical microscope (DMRB Leica) and quantified using Visilog 6.3 image analyzer software (Noesis, France). For each group of three mice, 15 fields were analyzed.

TUNEL Assay

Apoptotic cells were detected with a commercial *in situ* cell-death detection kit, POD (Roche DIAGNOSTICS, Mannheim, Germany) according to the manufacturer's protocol. Slides were washed in TBS and incubated in 20 mmol/L citrate buffer (pH 6.0) under 750W microwave irradiation for 1 minute. Non-specific sites were blocked

with Tris-HCl buffer (100 mmol/L Tris-HCl, 3% bovine serum albumin, 10% newborn calf serum) for 30 minutes. The tissue section was covered with 50 μ L of TUNEL reaction mixture containing dUTP-fluorescein (2'-deoxyuridine 5'-triphosphate) supplemented with TdT (terminal deoxynucleotidyl transferase) for 60 minutes at 37°C. After three washes with TBS, endogenous peroxidase activity was removed by incubating with H₂O₂, 3% in TBS for 10 minutes. Non-specific sites were blocked again and 50 μ L of Convert-peroxidase (Roche Diagnostics, Mannheim, Germany) diluted at 1:2 in Tris-HCl buffer was applied to each slide for 30 minutes at 37°C. Finally sections were stained using 3,3'-Diaminobenzidine (DAB) and counterstained using hematoxylin. Control experiments were performed using dUTP-fluorescein without TdT. Staining was observed under an optical microscope (DMRB Leica) and quantified using Visilog 6.3 image analyzer software (Noesis, France). For each group of three mice, 15 fields were analyzed.

RNA Extraction and Quantitative PCR Analysis

For PCR analysis, subcutaneous adipose tissue was excised and stored at -80°C. Total RNA from tissue was prepared using RNeasy Lipid Tissue Mini Kit (Qiagen, France) according to the manufacturer's recommendations, and 1 μ g was reversed transcribed with the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in the presence of random primers. Quantitative PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye on an ABI PRISM 7000 Sequence Detector System (Applied Biosystems) according to the manufacturer's instructions. The nucleotide sequences of the PCR primers used were as follows: 36B4: Forward (5'-AGTCG-GAGGAATCAGATGAGGAT-3'), Reverse (5'-GGCTGACT-TGGTTGCTTTGG-3'); superoxide dismutase (MnSOD): Forward (5'-CTTACAGATTGCTGCCTGCTCTAA-3'), Reverse (5'-AATCCCCAGCAGCGGAAT-3'); nicotinamide-adenine dinucleotide phosphate oxidase (NADPH)ox: Forward (5'-ACCAAATGTTGGGCTAGGAT-3'), Reverse (5'-AAAA GGATGAGGCTGCAGTTGA-3').

Each PCR was performed in duplicate in a 25 μ L volume using SYBR Green I Master Mix Plus (Applied Biosystems), 0.3 μ mol/L of each primer, for 15 minutes at 95°C for initial denaturing step, followed by 40 cycles of 95°C for 30 seconds, and 60°C for 30 seconds in the ABI Prism 7000 sequence Detector System. To exclude contamination by nonspecific PCR products such as primer dimers, melting curve analysis was applied to all final PCR products after the cycling protocols. Values for each gene were normalized to the expression levels of the 36B4 mRNA. Relative quantification of studied genes was calculated by using δ Ct formula, as recommended by the manufacturer (Applied Biosystems). Results were expressed relative to the control condition, which was arbitrarily assigned a value of 1.

Aconitase Activity Measurement

Aconitase enzymatic activity was measured using a Bioxytech aconitase-340 assay kit (OxisResearch, Foster City, CA, USA) on tissue homogenates. The assay is based on measurement of concomitant formation of NADPH from NADP⁺ when isocitrate (produced by aconitase) is decarboxylated by isocitrate dehydrogenase. Briefly, around 50 mg inguinal adipose tissue removed from control, sublethally, or lethally irradiated mice was minced and homogenized in 500 μ L assay buffer provided by the kit with tissue lyser beads (Qiagen, France). After elimination of lipids by 10 minutes centrifugation at 380g at 4°C, the enzymatic reaction was started by mixing 200 μ L of homogenate (about 2 mg proteins/ml) with 200 μ L citrate, 200 μ L isocitrate dehydrogenase, and 200 μ L NADP⁺. Absorbance was recorded during 40 minutes at 340 nm at 37°C. Then, the slope was estimated in the linear part of the curve, and aconitase activity was calculated according to the supplier's instructions with normalization to the protein concentration of the sample measured by Biorad DC protein assay (BioRad, Marne la Coquette, France).

Isolation of Mature Adipocytes and Stroma-Vascular Fraction

Cells were isolated according to Björntorp et al with minor modifications.¹⁶ Inguinal subcutaneous adipose tissue was dissected from visible blood vessels and ganglions and was digested at 37°C in phosphate buffer PBS containing 0.2% bovine serum albumin and 2 mg/ml collagenase for 30 minutes (collagenase A, Roche Diagnostics, Meylan, France). After elimination of undigested fragments by filtration through 25 μ m filters, mature adipocytes were separated from the pellets of the stroma-vascular fraction (SVF) by centrifugation (600 $\times$ g, 10 minutes). SVF cells were incubated for 5 minutes in hemolysis buffer (140 mmol/L NH₄Cl and 20 mmol/L Tris, pH 7.6) to eliminate red blood cells and washed by centrifugation in PBS. The number of isolated SVF cells was then counted and their viability assessed by trypan blue exclusion. SVF cells were either used for flow cytometry analyses or plated *in vitro*. Mature adipocytes were directly counted on thoma grid.

Cell Phenotyping

Isolated cells were analyzed by flow cytometry (FACS) using the following protocol. Freshly-isolated SVF cells were stained in staining buffer consisting of phosphate-buffered saline containing 0.5% new calf serum and FcR Block reagent (StemCell Technologies, Vancouver, Canada). Cells were incubated with anti-mouse monoclonal antibodies (mAb) or rat immunoglobulins (isotypes) conjugated with FITC, phycoerythrin, PerCP, or allophycocyanin for 30 minutes at 4°C. Triple or quadruple staining was performed by incubating cells with FITC, phycoerythrin, PerCP, and allophycocyanin-conjugated primary antibodies in one step, to precisely characterize the

Table 1. Physiological Parameters and Characteristics of Inguinal Adipose Tissue 7 days after Sublethal or Lethal Total Body Irradiation

	Control	Sublethal Irradiation	Lethal Irradiation
Mice weight (g)	22.14 ± 0.39	20.44 ± 0.18**	18.57 ± 0.18***
Leucocyte blood count (10 ³ /μl)	3.6 ± 0.29	3.42 ± 0.51	0.125 ± 0.01***
Fat pad weight (g)	0.231 ± 0.014	0.165 ± 0.003**	0.15 ± 0.016 **
Mature adipocyte number (×10 ⁶)/mice	0.577 ± 0.078	0.32 ± 0.03**	0.360 ± 0.007*
Mature adipocyte mean diameter (μm)	65.63 ± 3.22	39.38 ± 1.93***	22.26 ± 1.57***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ in irradiated versus control mice.

different cell populations. Cells were washed in staining buffer and then analyzed on a FACS (FACS Calibur, Becton Dickinson, Mountain View, CA). Data acquisition and analysis were then performed (Cell Quest Pro software, Becton Dickinson).

Colony Forming Unit-Fibroblasts Assay

To evaluate the frequency of mesenchymal-like progenitors in the inguinal SVF, cells were cultured in T-25 flasks at a final concentration of 16 or 32 cells/cm² in Dulbecco's Modified Eagles Medium (DMEM) F12 10% newborn calf serum and incubated at 37°C, 5% CO₂. The medium was renewed every 2 days. After 14 days, the cells were washed with PBS and fixed with methanol for 15 minutes. For scoring the colony forming unit-fibroblasts (CFU-f), flasks were stained with Giemsa 6% for 30 minutes. Plates were scored under an optical microscope, and colonies were considered aggregates of more than 50 cells.

In Vitro Adipogenesis Assay and Triglyceride Content Measurement

For adipogenic differentiation, cells were plated at a density of 8000 cells/cm² in DMEM:F12 supplemented with 10% newborn calf serum, biotin (16 μmol/L), panthotenic acid (18 μmol/L), ascorbic acid (100 μmol/L), and amphotericin (25 μg/ml), streptomycin (10 mg/ml), and penicillin (10000 U/ml). At confluence, adipogenic differentiation was induced by adding dexamethasone (33 nmol/L), insulin (2 nmol/L), 3, 3', 5-tri-iodo-L-thyronin (T3; 2 nmol/L) and transferrin (10 μg/ml) for 10 days. Medium was changed every 2 days. Adipocytes were characterized by oil red O staining. Differentiation was quantified by cellular triglyceride (TG) content measurement by using a commercially available test combination (Triglycerides enzymatiques PAP 150, Biomerieux) after cell lysis in 0.1N NaOH. The TG content was calculated per μg of proteins. The protein content was determined by using the DC Protein Assay Kit (BioRad, Marne la Coquette, France).

Methylcellulose Cultures

SVF cells from each fat pad were isolated as described above and plated at 7×10^3 cells/ml in 1.5 ml of methylcellulose. Colony number was assessed at day 21 after

plating. Adipocyte colonies were identified as lipid droplet-containing cells.

Statistical Analyses

All statistical analyses were done by unpaired *t*-test using Prism software (GraphPad software, San Diego, CA). Results are expressed as mean ± SEM.

Results

Total body irradiation induces changes in blood and tissue cell-composition, depending on the dose of ionizing agents and on the sensitivity of the tissue considered. The sensitivity of adipose tissue to irradiation was investigated 7 days after sublethal (7Gy) or lethal (10Gy) total body irradiation.

Blood numeration was used as a control of the irradiation efficiency. As expected, leukocyte number was severely decreased by day 7 in lethally irradiated mice ($5.62 \pm 0.86\%$ of control values), although sublethal irradiation induced a slight and non-significant decrease in leukocyte blood cell count ($75.61 \pm 14\%$ of control values; Table 1).

Seven days after irradiation, mice weight was significantly decreased, by 8 and 16%, in sublethally and lethally irradiated mice, respectively, as compared with controls (Table 1). The subcutaneous (inguinal) fat pad weight was significantly decreased in irradiated mice compared with controls, regardless the dose of irradiation (Table 1). Additionally, inguinal fat pads removed from irradiated mice presented morphological changes as revealed by May-Grünwald Giemsa coloration (Figure 1). Indeed, 7 days after irradiation, the size of mature adipocytes was reduced (Table 1, Figure 1D), and the presence of non-adipose cells was more apparent (Figure 1). These modifications were emphasized after lethal irradiation compared to sublethal irradiation. Indeed, the number of small adipocytes (diameter <50 μm) was increased by up to five in inguinal fat pads removed from irradiated mice as compared with controls (Figure 1D). In contrast, no large mature adipocytes (diameter >100 μm) were observed in irradiated samples, although they represented $11.02 \pm 3.62\%$ of total adipocytes in control mice (Figure 1D). In addition to decreased adipocyte size, the total number of mature adipocytes per fat pad was also decreased in irradiated mice compared with controls, and this decrease was not irradiation dose-

Figure 1. Morphological characterization of subcutaneous adipose tissue after irradiation. May-Grunwald Giemsa staining was performed on white adipose tissue isolated from control mice (A) or mice 7 days after sublethal (B) or lethal total body irradiation (C). Mature adipocytes diameter (D) was measured on adipose tissue slides. Three populations have been distinguished according to their diameter: less than 50 μm; from 50 to 100 μm; and greater than 100 μm. The quantification was performed in control, sublethally (7Gy), and lethally (10Gy) irradiated mice. **P* < 0.05.

dependent (Table 1). The presence of numerous small mature adipocytes in inguinal fat pads after irradiation compared to controls induced an apparent increase of

adipocyte number, as appears in Figure 1. However, the total adipocyte number has to take into account the fat pad weight, which is severely decreased in irradiated animals. The total number of adipocytes in fat pads after irradiation is thus significantly lower than in control animals.

We next investigated the cellular mechanisms involved in the effects of radiation in adipose tissue. Inguinal fat pads isolated from either control, sublethally, or lethally irradiated mice were processed for immunohistochemistry 7 days after irradiation and were stained with an antibody against the nuclear antigen Ki-67 to detect proliferating cells (Figure 2, A–C) or processed for TUNEL analysis to quantify apoptotic cells (Figure 2, D–F). Quantification of immunostaining demonstrated the presence of $3.9 \pm 0.7\%$ of proliferating cells in control adipose tissue (Figure 2A). Irradiation induced a total loss of these proliferating cells at sublethal and lethal doses (Figure 2, B,C). Control adipose tissue also harbored numerous apoptotic cells ($5.5 \pm 1.3 \times 10^3$ nuclei/cm²; Figure 2D). This percentage was significantly increased after sublethal ($11.18 \pm 1.1 \times 10^3$ nuclei/cm²; factor 2.01 ± 0.20 ; Figure 2E) or lethal ($25.79 \pm 5.4 \times 10^3$ nuclei/cm²; factor 4.65 ± 0.98 ; Figure 2F) irradiation. Most of the apoptotic cells were SVF cells, localized between mature adipocytes (Figure 2, D–F, black arrows). However, some mature adipocytes were also positive for TUNEL (Figure 2, D–F, red arrow). We thus showed that control adipose tissue harbored both proliferating and apoptotic cells, suggesting a high cell dynamic. In addition, ionizing radiation significantly altered this parameter in subcutaneous adipose tissue.

To investigate whether oxidative stress and especially radical oxygen species (ROS) production may lead to irradiation damage in adipose tissue, we measured by quantitative reverse transcription PCR the expression level of genes encoding ROS metabolism enzymes and quantified aconitase activity, considered to be a marker of oxidative stress.¹⁷ NADPH oxidase (NADPHox) is a major source of ROS in various cells including adipose derived cells,¹⁸ and its expression was significantly increased after lethal irradiation, although a sublethal dose gives rise to a slight but insignificant rise in its expression level (Figure 3A). In parallel, we measured the expression of an antioxidant enzyme, MnSOD, and showed that its expression level was reduced in adipose tissue of irradiated mice (Figure 3A). These results suggest that irradiation leads to an increase in ROS production via the activated NADPHox pathway and impaired antioxidant defense system. To confirm the presence of oxidative stress in adipose tissue following ionizing radiation, we quantified aconitase activity. Aconitase is an essential mitochondrial enzyme known to be particularly susceptible to oxidative damage.¹⁷ Similarly to MnSOD expression, aconitase activity was reduced in adipose tissue after irradiation, and the magnitude of the decrease was dependent on the dose of radiation (Figure 3B). This result thus confirms the presence of oxidative stress in adipose tissue after irradiation, particularly after lethal irradiation.

Figure 2. Effect of irradiation on proliferation and apoptosis in inguinal adipose tissue. Ki-67 staining (A–C) or TUNEL assay (D–F) were performed on paraffin embedded adipose tissue isolated from control mice (A, D) or mice 7 days after sublethal (B, E) or lethal (C, F) irradiation. Ki-67 staining was revealed by AEC, and TUNEL positive cells were revealed by DAB (dark nuclei, **black arrows**). In both cases, slides were counterstained with hematoxylin to visualize negative nuclei (**blue arrows**). **Red arrows** point on mature adipocytes positive for TUNEL.

To analyze the potential changes at a cellular level in adipose tissue following irradiation, we focused on lethal irradiation and analyzed the proportion and phenotype of the different cell subsets present in SVF. We next investigated the proliferation and differentiation potentials of

progenitors. Flow cytometry was performed by using different cell surface markers previously shown to be expressed in SVF and allowed the identification of cell subsets. CD45 was used to discriminate between hematopoietic and non-hematopoietic cells present in SVF. As shown in representative dot plots, the percentage of CD45-positive hematopoietic cells in the inguinal SVF (region R1) was significantly reduced (two fold) 7 days after lethal irradiation (Figure 4).

As already described,⁹ a large majority of CD45-negative (non-hematopoietic) cells present in control adipose tissue expressed CD90, CD34, and Scal (Figure 4A). Multiple staining showed that all of the CD90-positive cells were also positive for CD34 and Scal (Figure 4A). This CD45–/CD90+/CD34+/Scal+ population was similar in control and irradiated mice and represented 80% to 90% of the CD45-negative population (Figure 4B). Additionally, endothelial cells characterized by the expression of both CD34 and CD31 were found in the CD45-negative population and represented 20% to 30% of the non-hematopoietic cells in control and lethally irradiated mice (Figure 4). This data suggested that irradiation did not modify the phenotype or the percentage of the different cell subsets present in the non-hematopoietic compartment of the SVF.

SVF cells isolated from control or irradiated mice were then plated at the same density, either under clonal conditions or in liquid medium under adipogenic conditions. Culture under clonal conditions gives rise to a CFU-f formation that gives a quantitative measure of the stromal progenitor population and its ability to proliferate. After irradiation, the number of CFU-f was severely decreased (Figure 5, A–C), suggesting that the number of progeni-

Figure 3. Effect of irradiation on radical oxidative stress in inguinal adipose tissue. NADPH oxidase and MnSOD gene expression were quantified by quantitative reverse transcription PCR (A), and aconitase activity (B) was measured in subcutaneous fat pad removed from control (white bars), sublethally (gray bars) or lethally (black bars) irradiated mice. * $P < 0.05$; ** $P < 0.01$; $n = 3$.

Figure 4. Effect of irradiation on SVF cell subsets. Flow cytometry was performed to compare the percentage of cells expressing the pan-hematopoietic cell surface marker CD45 in combination with specific markers for endothelial cells (CD31/CD34) or preadipocytes (CD34/CD90/ScaI) in total SVF from control (**A**) or lethally irradiated (**B**) mice. Dot plots were representative of 8 to 10 experiments.

tors and/or their ability to proliferate in clonal conditions were impaired by radiation. Proliferation of SVF cells in liquid medium and adipocyte differentiation were then assessed. A morphologically homogeneous population of fibroblast-like cells with 80% to 90% confluence was observed after 3 to 4 days. No morphological difference was observed between cultures of cells isolated from irradiated and non-irradiated mice (data not shown). In contrast, the cell number in cultures of SVF isolated from irradiated mice was significantly lower than control as early as 4 days after the plating (confluence), and up to 2 weeks in culture (Figure 5D). These data suggest that irradiation impaired SVF cell proliferation in adipose tissue.

Eight days after confluence and induction of adipocyte differentiation, triglyceride (TG) content was quantified in both cultures as an index of adipocyte differentiation. As shown in representative phase contrast pictures, adipocyte differentiation was severely impaired in cultures obtained from irradiated animals (Figure 6A,B). This observation was confirmed by TG content measurement. Indeed, the accumulation of TG in cultures of SVF cells isolated from irradiated animals was significantly lower than in cultures from control mice (Figure 6C). In addition the number of adipogenic clones was decreased in irradiated samples compared with controls when SVF cells

Figure 5. Effect of irradiation on cell proliferation. Cells isolated from inguinal adipose tissue from control (white symbols) or lethally irradiated (black symbols) mice were cultured either in clonal conditions to obtain CFU-F (**A–C**) or in liquid medium (**D**) as described in Material and Methods. For CFU-F quantification, Giemsa staining was performed in the flask of control cells (**A**) or cells isolated from irradiated mice (**B**), and the number of CFU-F was quantified (**C**). For culture in liquid medium (**D**), at indicated time, cells were trypsinized and counted. The y axis shows cell number expressed as a percentage of control values. The results represented the mean $\pm$ SEM of 4 to 6 cultures. * $P < 0.05$; *** $P < 0.001$.

were plated in 3-dimensional medium such as methylcellulose (Figure 6D), as previously described.⁹

Discussion

In this study, we demonstrate that radiation exposure induces an acute response in subcutaneous adipose tissue. Indeed, irradiation induces morphological changes, as well as alteration in both proliferation and differentiation potentials of precursor cells present in SVF, at least in part via oxidative stress. These damages were observed following sublethal or lethal irradiation, suggesting that cells harbored in adipose tissue are very sensitive to ionizing radiation.

Irradiation induces major modifications in adipose tissue within 7 days by inducing apoptosis and metabolic changes in both mature adipocytes and cells present in

Figure 6. Adipocyte differentiation of SVF cells isolated from inguinal adipose tissue of control and lethally irradiated mice. Phase contrast pictures of differentiated cultures obtained from control (**A**) and lethally irradiated (**B**) mice. Quantification of triglyceride content (**C**) in each culture was performed 8 days after induction of adipocyte differentiation, and was expressed as fold increased in differentiated versus confluent cultures. **D:** Adipogenic clone number in methylcellulose cultures were counted and expressed per well. White bar = control mice; black bars = irradiated mice. The results are expressed as the mean $\pm$ sem of 4–6 cultures. * $P < 0.05$.

the SVF. Indeed, the resident hematopoietic population was decreased after irradiation while adherent stromal cells presented alteration in proliferation and differentiation potentials. This must be noted since only tissues with high proliferative capacity are supposed to support the acute effects of radiation exposure.^{3,4} Indeed, in bone marrow, stromal cells display a very low turnover rate *in vivo*; therefore they are far less sensitive than hematopoietic cells to irradiation. In contrast, we showed here that in control adipose tissue, numerous proliferating or apoptotic cells are present, suggesting a continuous turnover that could explain the severe damage induced by irradiation in this tissue. These observations are consistent with a very recent study that reveals, contrary to a commonly held view, a relatively intense cell turnover in adipose tissue.¹⁹

Previously, it was proposed that most of the damage observed after irradiation was caused by indirect effects, for example through ROS, that can be amplified and persist.¹ Our results and the fact that adipose lineage cells are very sensitive to oxidative stress^{20,21} are in agreement with this proposal and suggest that oxidative

stress may contribute to the damage induced by irradiation in adipose tissue. In addition, the magnitude of oxidative stress (evaluated by the increase in NADPHox expression and the decrease in MnSOD expression and aconitase activity) appeared to correlate to the dose of irradiation as well as the percentage of cell death in the tissue, suggesting again a causal link between these two events.

Irradiation induces a decrease in both number and mean size of mature adipocytes, suggesting a metabolic effect. This observation is in agreement with old studies performed on Wistar rats that describe a rapid (within hours) increase in free fatty acids in adipose tissue as well as in serum of irradiated rats, suggesting an increased mobilization of energy sources from adipose tissue.²² More recently, long-term effects of total body irradiation or local abdominal irradiation on adipose tissue have been described in ob/ob mice.^{23,24} In both cases, as in our study, radiation exposure arrests body weight gain and reduces adiposity. However, by 3 months after radiation exposure, there was no significant difference in fat pad weight or in the size or appearance of mature adipocytes between the irradiated and non-irradiated groups.²³ This disparity with our results could be explained by two factors. First, the time course is different, since we were interested in acute effects (7 days) versus long term effects (3 months) in these studies. Second, it must be noted that these observations were obtained in obese mice for which leptin expression is blunted and adipose metabolism is impaired.

Irradiation induces a rapid loss of the hematopoietic cells resident in adipose tissue. A similar decrease has been described in peripheral blood and in bone marrow following irradiation,^{25,26} suggesting that the hematopoietic cells resident in adipose tissue behave similarly to their counterparts in other tissues. In parallel, no change in either the phenotype or the percentage of non-hematopoietic cells was observed 7 days following irradiation. It is well known that irradiation damages hematopoietic cells and their microenvironment in different ways (direct and indirect effects). Indeed, in hematopoietic cells, irradiation activates cell-cycle checkpoints and apoptotic programs, leading to cell death. Concomitantly, cells in the microenvironment such as stromal cells respond to ionizing radiation by altering their production of soluble growth factors, cytokines, reactive oxygen species, and extracellular matrix proteins, but without immediate cell death. In addition, alteration in these activities can modify cell behavior. For example, in response to ionizing radiation, fibroblasts and macrophages can permanently remain in an activated state that continuously generates growth factors and ROS, and these factors can thus affect the function of normal hematopoietic cells.¹

Although the phenotype of non-hematopoietic cells was not altered in irradiated mice, the proliferation and differentiation potential of progenitors was severely impaired. Indeed, precursors obtained from irradiated mice present a lower proliferation capacity, in clonal conditions or in liquid medium, as well as a lower adipogenic differentiation potential. These limited proliferation and differentiation potentials of progenitor cells must be related to

injury in marrow stroma following a high dose of TBI in humans. Indeed, the frequency of CFU-f is severely impaired early after radiation exposure.^{27–29} Similar observations were performed *in vitro* after irradiation of mesenchymal stem cell cultures.³⁰ This putative decrease in progenitor number after irradiation may have consequences for the use of adipose-derived cells for tissue regeneration. Indeed, the existence of stem/stromal cells in adipose tissue has been reported by several groups in rodents as in humans, and their use in tissue repair, seems promising.^{15,31} According to our results, it is likely that a previous irradiation of adipose tissue may compromise the reconstructive properties of adipose-derived cells. Further experiments are needed to finely investigate this point.

The decrease in the adipogenic differentiation of the SVF cells isolated from irradiated mice shown in this study may reflect a deficit in the number of preadipocytes and/or a functional defect of cells with adipogenic lineage due to radiation exposure. Our *in vitro* results demonstrate that both effects occurred, since we showed a decrease in preadipocyte number (assessed by adipogenic clones) and a decrease in TG content. This result is in agreement with conclusions drawn in obese mice, for which the long term effect of TBI on body weight or adiposity is not due to a limited ability of the individual adipocytes to accumulate lipids, but rather to a limited ability of preadipocytes to proliferate and/or to differentiate.²⁴ This impaired differentiation is also consistent with other studies on different models and other differentiation programs, although most of them were performed *in vitro*. Indeed, using human or rodent cells, differentiation toward osteoblasts, adipocytes, myotubes, chondrocytes, or functional endothelial cells was significantly altered by radiation exposure.^{30,32–36}

In addition, a close relationship between cell proliferation and differentiation has been established early during the adipocyte differentiation program. Indeed, on reaching confluence, proliferating preadipocytes in culture become growth-arrested at the G1/S phase of the cell cycle by contact inhibition, and this growth arrest is a required first step in the commitment of all cultured preadipocytes toward terminal differentiation.^{37,38} The lower proliferation potential and thus the subsequent decreased level of confluence observed in preadipocytes isolated from irradiated mice could also result in the decreased adipogenesis. However, although confluence represents an important transitional event between proliferation and adipocyte differentiation, it is not a prerequisite for adipose conversion.³⁸

These alterations in the stromal compartment of adipose tissue may have severe late consequences. Indeed, in bone marrow, it is proposed that the stromal microenvironment is unable to self-repair the injury suffered, leading to quantitative as well as qualitative long-lasting effects. Depletion of the stromal compartment leads to a loss of the precursor reservoir and thus compromises its ability to maintain tissue homeostasis.^{27–29} In addition, neighbors of irradiated cells respond with so-called stress proteins as if they were exposed, contributing to the long-lasting effects.³⁹ *In vivo*, ionizing radiation

produces rapid and persistent remodeling of the extracellular matrix in mouse fat pad stroma that can contribute to neoplastic progression.⁴⁰

In conclusion, irradiation damaged subcutaneous adipose tissue by altering both mature adipocyte and SVF cell functions. The opposite effect of irradiation on both proliferation and apoptosis demonstrates that the developmental potential of subcutaneous adipose tissue is profoundly altered following irradiation, which may be an issue for patients that undergo total body irradiation as radiotherapy. Indeed, in terms of therapeutics, these acute effects may modify the reconstructive capacity of adipose tissue and therefore its use in autologous fat tissue transfer after irradiation. They may also contribute to metabolic dysfunction, which could be deleterious to patients presenting with malignant diseases.

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