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Adipocyte Secretome Increases Radioresistance of Malignant Melanocytes by Improving Cell Survival and Decreasing Oxidative Status

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Radiotherapy is a treatment option for the majority of malignancies. However, because melanoma is known to be radioresistant, the use of ionizing radiation as an adjuvant therapy in cutaneous melanoma patients is ineffective. Obesity has now been recognized as a risk factor for melanoma. High adiposity is generally associated with a more pro-oxidative status. Oxidative stress is a major player in radiation therapy and also a common link between obesity and cancer. Several adipocyte-released proteins are known to have a role in controlling cellular growth and pro-survival signaling. For that reason, we investigated the influence of 3T3-L1 mature adipocyte secretome in B16-F10 malignant melanocyte radiosensitivity. We evaluated B16-F10 cell survival and redox homeostasis when exposed to four daily doses of ionizing radiation (2 Gy per day) up to a total of 8 Gy in a medical linear accelerator. B16-F10 melanocytes exhibited slight alterations in survival, catalase activity, nitrative stress and total oxidant concentration after the first 2 Gy irradiation. The motility of the melanocytes was also delayed by ionizing radiation. Subsequent irradiations of the malignant melanocytes led to more prominent reductions in overall survival. Remarkably, 3T3-L1 adipocyte-secreted molecules were able to increase the viability and migration of melanocytes, as well as lessen the pro-oxidant burden induced by both the single and cumulative X-ray doses. *In vitro* adipocyte-released factors protected B16-F10 malignant melanocytes from both oxidative stress and loss of viability triggered by radiation, enhancing the radioresistant phenotype of these cells with a concomitant activation of the AKT signaling pathway. These results both help to elucidate how obesity influences melanoma radioresistance and support the usage of conventional medical linear accelerators as a valid model for the *in vitro* radiobiological study of tumor cell lines.

INTRODUCTION

Melanoma is one of the world's most rapidly increasing malignancies (*1*) The number of reported cases increasing for the last few decades. Over 176,000 new cases of melanoma are reported annually in Europe and the U.S. (*2, 3*). Melanoma treatment has remained the same for the last few decades: surgical excision of the malignant mass is still the most effective treatment in primary melanomas (*4*).

Radiotherapy is frequently used to treat the majority of malignancies, and has a direct impact in the proliferative phenotype of both normal and cancer cells. Radiation-induced ionization of regulatory proteins and DNA might render the cells unviable and culminate in cellular death (*5*). Ionizing radiation can also indirectly cause cellular damage. The formation of highly reactive oxygen and nitrogen radicals increases the intracellular oxidative stress, depleting the antioxidant defenses, which subsequently react with many cellular components (DNA, proteins, lipids) leading to unrecoverable damage (*6*).

However, melanoma is known to be radioresistant, which discourages the use of ionizing radiation as an adjuvant therapy in melanoma patients (*7*). Recently, the use of radiation in higher delivered doses, hypofractionated and in combination with immunotherapy has led to some positive outcomes in melanoma metastasis treatment and palliation (*8–10*). Nonetheless, obesity, particularly high visceral adiposity, presents a problem in treatment planning and delivery of radiation to internal metastases. Generally, higher body adiposity is associated with both cancer initiation and progression (*11*). Obesity itself is a risk factor for several types of neoplasms, including melanomas (*12, 13*). High adiposity can be a contraindication for (and may limit the extent of) cancer surgery, since it contributes to the

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inadequate dosing of chemotherapeutic drugs and complicates the planning and delivery of radiation (*14–16*).

Adipocytes secrete a variety of factors that exert effects at both local and systemic levels (*17*). The grand majority of these factors are cytokines, chemokines and inflammatory mediators, but a role in growth regulation as a new aspect of adipokines has been revealed by novel adipocyte-released molecules (*18*). Fat-derived molecules stimulate melanoma progression and aggressiveness and act as mediators of proliferation in melanoma cells (*19, 20*).

Resistance to oxidative stress appears to be a key mechanism of tumor radioresistance (*21*). Obesity is linked to a more pro-oxidative status, with a concomitant systemic increase in reactive oxygen species (ROS), acting as an additional source of oxidants (*22*).

For the current study, we hypothesized that adipocytes might lead to two antagonistic outcomes towards melanocyte radiosensitivity. Although the fat-derived growth factors might protect melanocytes from radiation-induced loss of survival by stimulating their overall proliferation, the adipocyte-generated oxidants can further increase the oxidative burden, aggravating the radiation-induced damages. In this study, we irradiated cell cultures at standard doses to investigate the action of the adipocyte secretome in melanoma radioresistance.

MATERIALS AND METHODS

Cell Cultures

B16-F10 melanocytes and 3T3-L1 pre-adipocytes were maintained in high-glucose Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich® LLC, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B (Sigma-Aldrich). Cells were culture at 37°C in an incubator with a 5% CO_2 humidified atmosphere.

B16-F10 melanocytes were cultured in 96-well plates (1×10^4 cells/ml) for viability determination and in 24-well plates (10×10^4 cells/ml) for the other assays. All treatments were performed in serum-free conditions and after a 16 h FBS-starving period.

Adipocytes Differentiation and Conditioned Media Collection

Two days after 3T3-L1 pre-adipocyte cultures reached confluence, a mixture of 2 μ*M* insulin (Sigma-Aldrich), 1 μ*M* dexamethasone (Sigma-Aldrich) and 0.25 m*M* isobutylmethylxanthine (Fluka™/Honeywell Research Chemicals, Morris Plains, NJ) was added to the cultures. The media was replaced and the cells maintained with 2 μ*M* insulin every two to three days. On day 7 after induction, cultures were washed with phosphate buffered saline (PBS) and incubated in serum-free DMEM. On the next day, the conditioned media, enriched with adipocyte secretome, was harvested from the adipocyte cultures, centrifuged for 5 min at 300*g* and the supernatant was collected for the subsequent treatments.

Irradiation of Cells

Prior to irradiation, computed tomography scans were performed to obtain three-dimensional (3D) images and calculate the density of both the 24- and 96-well plates. To simulate a biological structure, the plates were placed between two 5-cm-height water phantoms (Supplementary Fig. S1; http://dx.doi.org/10.1667/RR14551.1.S1).

The 3D conformal radiation dosimetric plan comprised two fields (one anteroposterior and one posteroanterior). Irradiations were performed using the software XIO-Release version 4.70.02 (Supplementary Fig. S2; http://dx.doi.org/10.1667/RR14551.1.S1). A total radiation dose of 8 Gy in four daily fractions of 2 Gy, prescribed to the isocenter, was delivered in a PRIMUS™ linear accelerator (Siemens) with a 6 MV photon beam.

Metabolic Activity Assay

The (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) was used to assess the metabolic activity of malignant melanocytes (B16-F10) irradiated under the influence of the adipocyte secretome (3T3-L1). B16-F10 cells were seeded in 96-well plates and cultured for 24 h. Afterwards, cells were treated with the mature 3T3-L1 adipocyte conditioned media (CM) and irradiated. Next, 20 μl of MTT was added to each well and incubated for 3 h. After this period, the violet formazan precipitate was dissolved in 100 μl of dimethyl sulfoxide (Merck & Co., Kenilworth, NJ) and the absorbance at 550 and 650 nm recorded. Metabolic activity was calculated by the formula $ABS_{(final)} = ABS_{(550)} - ABS_{(650)}$, and normalized by dividing over the absorbance of the control.

Total Antioxidant Status Determination

The *in vitro* determination of the total antioxidant status (TAS) was done with the TAS kit (Randox Laboratories, Crumlin, UK) following the manufacturer's instructions. Briefly, 5 μl of the sample were mixed with 200 μl of the chromogenic solution and the substrate was added to a final volume of 250 μl. The appearance of the radical cation $ABTS^{\bullet+}$ was monitored for 15 min at 600 nm. Antioxidants suppress the blue-green color production to a degree that is proportional to their concentrations. Calibration was performed using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards and the results were expressed as $TAS_{(mmol/ml)} = |\text{Trolox standards}|/(\Delta ABS_{(samples)} - \Delta ABS_{(Trolox)})$, where $\Delta ABS = ABS_{final} - ABS_{initial}$, according to the manufacturer instructions.

Catalase Activity

Catalase activity was determined based on the rate of decomposition of H_2O_2, which is proportional to the reduction of the absorbance at 240 nm. A volume of 100 μl of each sample was added to 400 μl of 5 m*M* hydrogen peroxide substrate solution in 0.05 *M* phosphate buffer (pH 7.0). The decay kinetics for absorbance was determined in a Jenway 6505 Uv/Vis spectrophotometer for 1 min. Results are expressed as $|\Delta ABS/min| \times 10^6$.

TBARS Determination

To determine thiobarbituric acid-reactive substances (TBARS), 100 μl of thiobarbituric acid 10% (w/v) (Merck & Co) were added to 500 μl of cellular extract and placed in a boiling water bath for 30 min. Afterwards, the amount of malondialdehyde (MDA) present was determined by measuring the absorbance at 535 nm. The standard curve was performed with standard solutions of malonaldehyde bis(dimethyl acetal) (Sigma-Aldrich).

Nitrotyrosine Quantification

B16 cell lysates were prepared in PBS and sonicated for 10 min (SilentCrusher S; Heidolph North America, Elk Grove Village, IL). Proteins and other cellular components were precipitated with 15% (v/v) ice-cold trifluoroacetic acid upon centrifugation at 10,000 rpm for 10 min. Chromatographic detection of 3-nitrotyrosine (3-NT) in the supernatants was performed as described by Teixeira *et al.* (*23*) (see Supplementary Table S1 for detailed chromatographic conditions; http://dx.doi.org/10.1667/RR14551.1.S1). 3-NT standards were pre-

pared from lyophilized 3-Nitro-L-tyrosine (Santa Cruz Biotechnology® Inc., Dallas, TX). The reverse-phase HPLC VWR-Hitachi Elite LaChrom (VWR) chromatographic system that was used consisted of a quaternary pump, model HTA L-2130, an autosampler L-27200, a ODS-Hypersil C18 analytical column (200 cm × 4 mm i.d.; 5 μm particle size) (Merck & Co.) in a L-2300 column oven and a diode array detector, model L-2455. The resulting chromatograms (Supplementary Fig. S3; http://dx.doi.org/10.1667/RR14551.1.S1) were analyzed using Agilent EZChrom Elite software version 3.3.2 (Agilent Technologies Inc., Palo Alto, CA).

In Vitro Scratch Assay

Using a pipette tip, an injury was inflicted in B16-F10 confluent cultures. Cells were then incubated after 3T3-L1 conditioning and/or irradiation. At 4, 12 and 24 h after treatment, the migrated distance was photographed under an inverted microscope (Nikon® Instruments Inc., Melville, NY) at a 200× magnification and the scratch closure was determined by measuring the injury width with ImageJ software (National Institutes of Health, Bethesda, MD). Shown are the normalized values (±SEM) of nine measurements.

Western Blotting Analysis

Proteins were extracted using RIPA buffer from B16 cell lysates. Equal volumes of protein extracts were loaded onto a 10% SDS-PAGE with a 5% stacking gel. After electrophoretic separation, proteins were blotted into a nitrocellulose membrane (Amersham Life Science Inc., Cleveland, OH). Immunodetection for total AKT, phosphorylated AKT (Ser473), and β-actin (all from Cell Signaling Technology® Inc., Danvers, MA) was accomplished with enhanced chemiluminescence (Clarity™ ECL kit; Bio-Rad® Laboratories Inc., Hercules, CA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software Inc., LaJolla, CA). Differences between treatments were evaluated by Student's t test or two-way ANOVA with Sidak multiple comparisons test accordingly to the number of conditions and treatments. Significance level was set to $P < 0.05$.

RESULTS

3T3-L1 CM Protects B16-F10 Cells from Radiation Damage

To access the susceptibility of B16-F10 cells to the direct effects of ionizing radiation, B16-F10 melanocytes were irradiated with a single 2 Gy dose of X rays and the cellular viability was then evaluated by the MTT assay. Upon irradiation, B16-F10 cells showed a slight (10%) decrease in their metabolic activity. However, 12 h later the metabolic activity of the irradiated melanocytes was improved to control values (Fig. 1A). When cultured with 3T3-L1 CM the malignant melanocytes significantly increased their metabolic activity by more than 30% whether irradiated or not (Fig. 1A).

Ionizing radiation imbalances the cellular redox homeostasis (*6*). Accordingly, we next evaluated oxidative stress markers and antioxidant status of the irradiated B16-F10 melanocytes.

Lipid oxidation by ROS gives rise to a number of byproducts. MDA is one of the principal end products of fatty acid peroxidation in cells (*24*). We evaluated the MDA levels by the TBARS assay but found no significant differences in lipid peroxidation with either irradiation or 3T3-L1 secreted factors at the two time points studied (Fig. 1B).

Catalase is an oxidative stress protective enzyme that neutralizes hydrogen peroxide formed during oxidative stress. The activity of this enzyme in B16-F10 melanocytes was not altered after exposure to ionizing radiation. However, a significantly lower activity level was observed when cells were treated with 3T3-L1-secreted molecules (Fig. 1C). Incubation with adipocyte 3T3-L1 CM resulted in a significant increase in catalase activity in the melanocytes 4 h postirradiation.

Although the total antioxidant capacity of 3T3-L1 CM is significantly greater than fresh culture media (Fig. 1D), when fed to B16-F10 cultures and irradiated, the number of antioxidants present in the 3T3-L1 CM is no different from the control 12 h after treatment (Fig. 1E). Interestingly, the intracellular production of redox scavenger mechanisms is stimulated by the adipocyte secretome and counterbalances the lower radiation-induced antioxidant defense status (Fig. 1F).

To address the ability of melanocytes to migrate, a mechanical damage was inflicted to confluent melanocyte cultures and the scratch closure was microscopically inspected (Fig. 2B). Melanocytes rapidly occupied the injured area when incubated with 3T3-L1 CM. Exposure to radiation (2 Gy) slowed down B16-F10 motility towards the injury void space, particularly 4 h postirradiation, but this effect was reversed when 3T3-L1 CM was also present (Fig. 2A).

These results indicate that adipocyte-released molecules can protect melanoma cancer cells from X-ray radiation damages, both reducing antioxidant status and enhancing cell migration.

Adipocyte Secretome Contributes to Melanoma Radioresistance

As radiation dose increases, the amount of direct and indirect oxidative stress-mediated cellular damages increases proportionally. Our next approach was to determine the effects of cumulative radiation of B16-F10 melanocytes. A total dose of 8 Gy was delivered in four daily 2 Gy irradiations to melanoma cells in the presence of the 3T3-L1 adipocyte-derived factors.

The metabolic viability of the irradiated melanocytes displayed a constant decrease of approximately 20%, for radiation doses greater than 4 Gy. However, 3T3-L1 CM treatment of B16-F10 cells significantly increased the survival of the melanocytes suppressing the radiation-induced cytotoxic effects (Fig. 3A). Catalase activity followed a similar trend: radiation promotes a diminished basal catalase activity but when in the presence of the 3T3-L1 CM, catalase activity is highly enhanced (Fig. 3B).

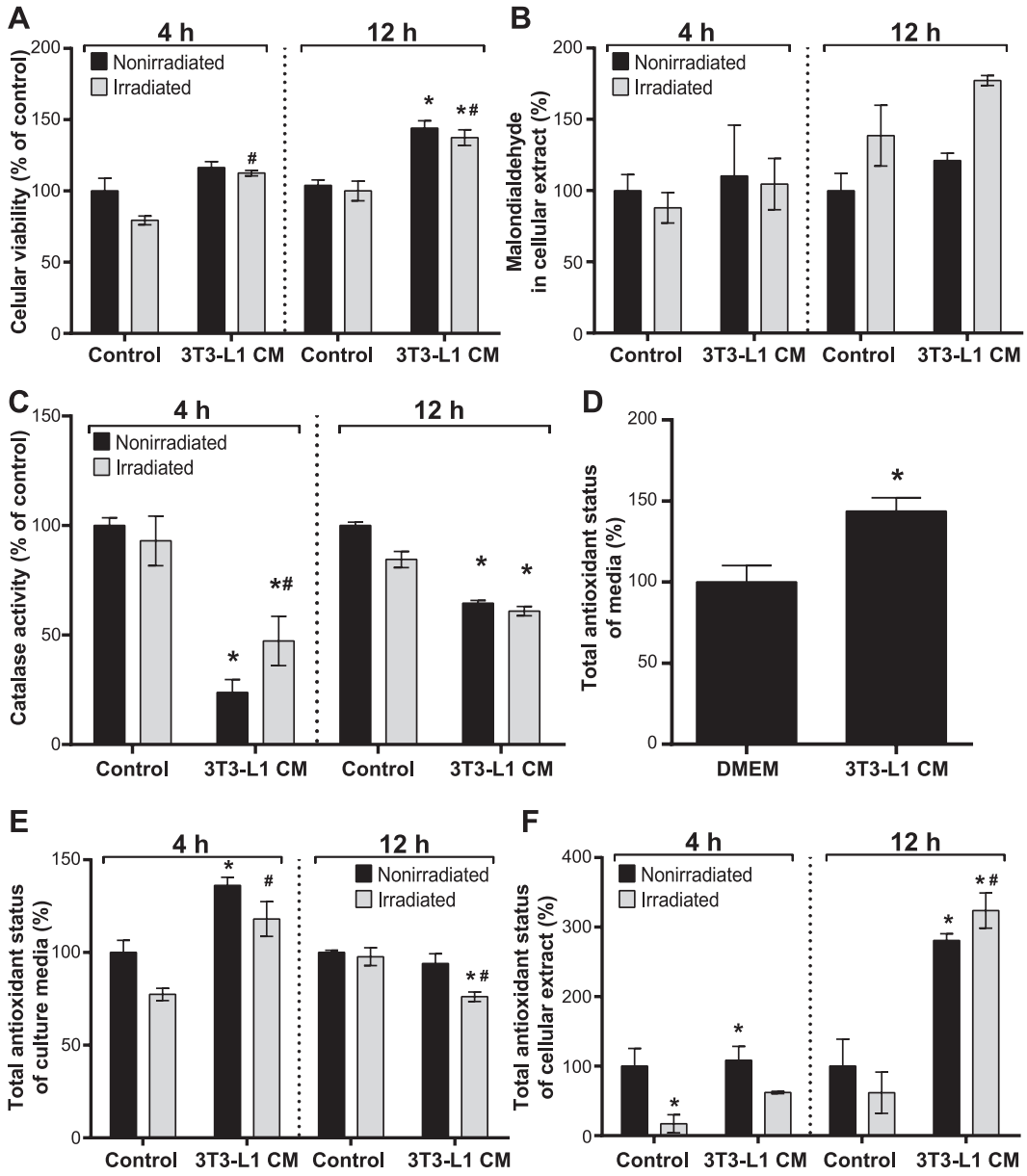

FIG. 1. B16-F10 survival and antioxidant status upon single 2 Gy irradiation and treated with 3T3-L1 CM. Controls were treated with serum-free DMEM. Panel A: Metabolic activity of melanoma cells by MTT reduction assay. Results represent the percentage of viable cells normalized by the absorbance of control. Panel B: Levels of MDA by TBARS determination in B16-F10 cell lysates. Panel C: Catalase activity was determined based on the reduction of absorbance at 240 nm. Total antioxidant concentration in fresh 3T3-L1 CM and DMEM (panel D) and in the treated B16-F10 culture supernatant (panel E) and cellular extract (panel F) was determined enzymatically by the TAS assay. Shown are mean values (±SEM) of three measurements for each time point/condition. Results are normalized as a percentage of the control treatment (*$P < 0.05$ vs. control treatment; #$P < 0.05$ vs. irradiation).

Reactive nitrogen species (RNS) lead to oxidative protein modifications. Free 3-NT is a biomarker of the turnover of radiation-induced nitrated proteins (*25*). The levels of 3-NT rapidly increased with the first cumulative radiation doses, but by the end of the experiment the 3-NT levels approximated those of the control group. 3T3-L1-released factors protected irradiated B16-F10 cells from oxidative nitration primarily in the first radiation fractions (Fig. 3C).

Activation of the PI3K-AKT signaling pathway has been correlated with radiation resistance (*26, 27*). To investigate whether these radioresistant inducer effects of 3T3-L1 CM were due to activation of the PI3K-AKT signaling pathway, immunoblotting for AKT and Ser473 phospho-AKT was performed in B16-cell lysates. The expression of the active form of AKT was only detected in treatment groups containing the 3T3-L1 CM (Fig. 3D), confirming the stimulation of this pathway by adipocyte-released factors.

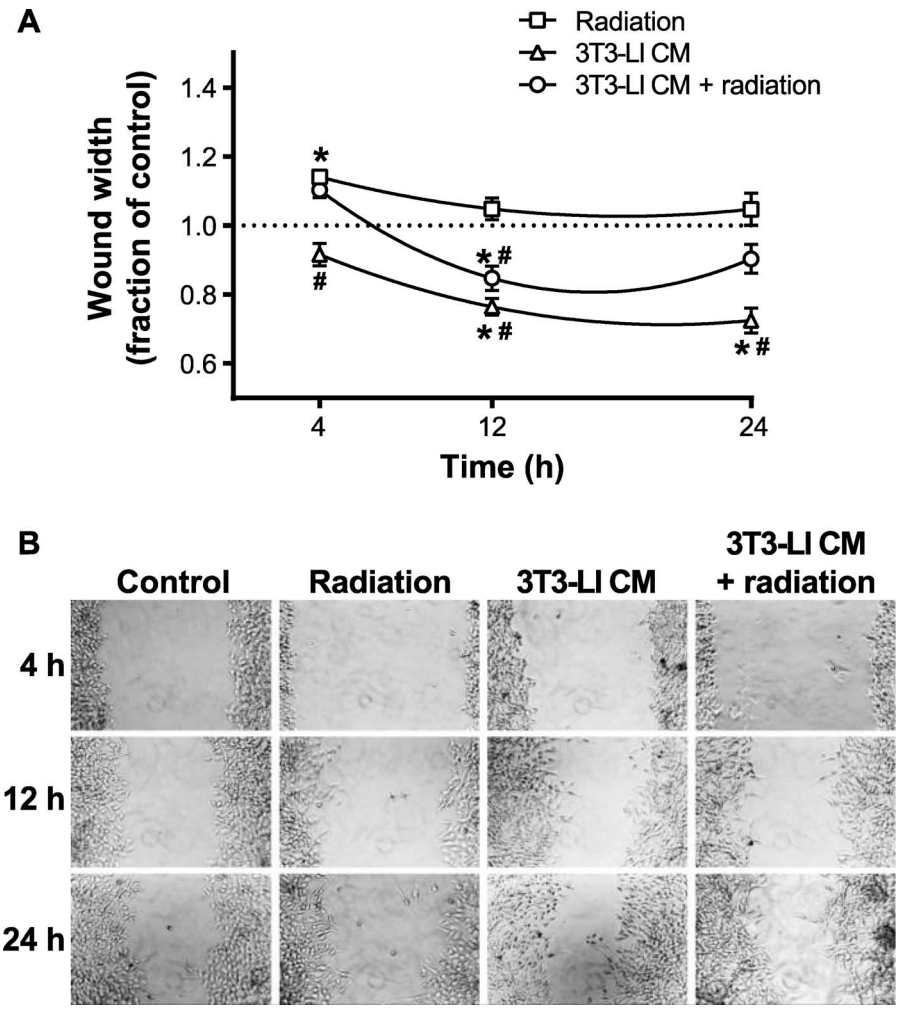

FIG. 2. Migration of B16-F10 melanocytes exposed to 3T3-L1 CM and/or radiation (2 Gy). Panel A: Injury closure fraction was determined by diving over the width of void space of the control. Bars show mean values (±SEM) of nine measurements for each time point/condition. (*$P < 0.05$ vs. control treatment; #$P < 0.05$ vs. irradiation). Panel B: Representative microscopy images of the injuries for each treatment and time point (200×).

These findings further underscore the protective effects of the adipocyte secretome in shielding melanocytes from oxidative stress and loss of viability triggered by repetitive fractions of ionizing radiation.

DISCUSSION

Cell survival response and (anti)oxidant status are good indicators of radiation susceptibility. Our results disclosed an increased metabolic activity of irradiated melanocytes when treated with adipocyte conditioning media (3T3-L1 CM). Low-dose irradiation (2 Gy) was only able to induce significant alterations in B16-F10 viability in the early hours after treatment. However, repeated irradiation of melanocytes, up to a total of 8 Gy, led to a constant decrease in metabolic activity. On the other hand, when 3T3-L1-adipocyte secreted molecules were present, radiation-induced loss of viability was overturned and melanocytes had higher metabolic activities.

Melanocytes are considered to be resistant to radiation damage (*28*). Ionizing radiation increases the oxidative burden in tumor cells (*29*). The mild decrease in catalase activity and antioxidant capacity combined with the temporary increase in protein nitrative stress observed corroborate the low radiosensitivity of B16-F10 melanocytes. Nevertheless, adipocyte-released factors enhanced the capacity of melanocytes to fight radiation-induced oxidative stress, further contributing to melanoma radioresistance.

In obese patients there is a shift in the delivery of external-beam radiation, resulting in the target location not receiving the full dose (*30*). In addition to the physical constraints to dose planning and delivery, our data further reinforce that the adipokine-rich environment might further contribute to the radioresistance of the melanocytes, culminating in a global underdosing effect. In addition to careful planning and dose delivery, which must take into account the higher body mass index of the patients, consideration must also be given to the more pro-

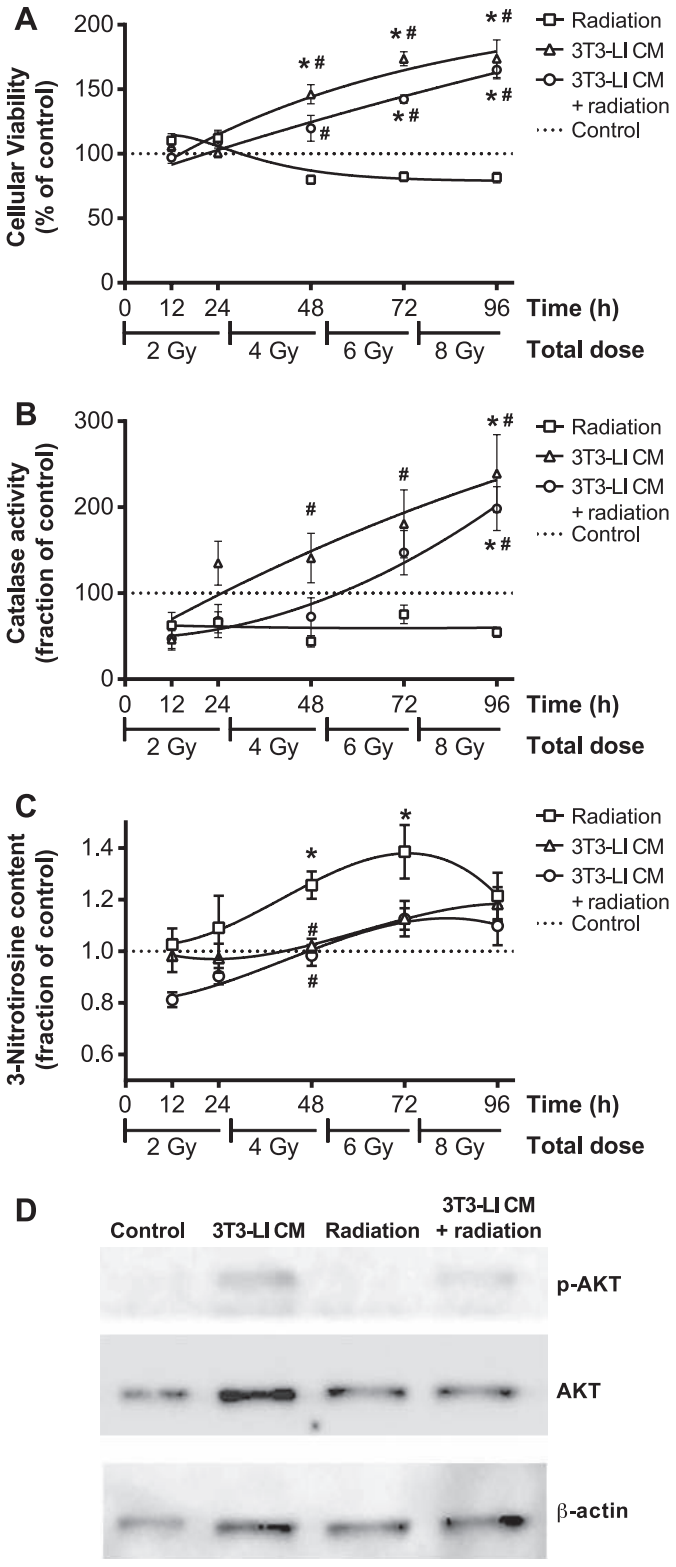

FIG. 3. Adipocyte-released factors protect B16-F10 melanocytes from fractionated radiation damage. Melanocytes were treated with 3T3-L1 CM, irradiated daily (2 Gy) for four consecutive days or received both treatments (3T3-L1 CM + radiation). At 12, 24, 48, 72 and 96 h after the first irradiation, cellular metabolic activity (panel A), catalase activity (panel B) and 3-nitrotirosine content (panel C) were determined. Results are represented as percentage of control. Bars show mean ± SEM (*$P < 0.05$ vs. control treatment; #$P < 0.05$ vs. irradiation; n = 6). Panel D: Immunoblot detection of AKT and Ser473 phosphorylated-AKT. Beta-actin was used as a loading control. A representative Western blot is shown.

inflammatory status and the adipokine and chemokine-rich environment that can protect malignant cells from radiation damage.

Oxidative stress has been proposed as a common link between obesity and cancer. The increased levels of circulating oxidants and ROS produced from peritumoral adipose tissue accelerate oxidative stress within tumor cells and might contribute to the increased risk for cancer progression in obese patients (*22*). ROS production also increases along the differentiation of 3T3-L1 cells into mature adipocytes (*31*), however, our results showed a higher antioxidant capacity of 3T3-L1 CM. Given the short half-life of radicals, this ROS-mediated effect might be less significant in our cell culture model. In fact, numerous 3T3-L1 adipocyte-secreted proteins have implications in growth regulation and act as cell mitogens (*18, 20, 32*). We believe these pro-proliferative and anti-apoptotic effects overcome the adipocyte-derived ROS.

Several signaling pathways involved in the radioprotective mechanisms of melanoma have been identified. MEK, ERK and PI3K-AKT cell survival cascades are known to play important roles in overcoming radiation-induced damage (*33*) and have implications for radioresistance mechanisms in cancer (*26, 27*). Blockage of these pathways has been shown to radiosensitize melanocytes to ionizing radiation (*34*). We have already demonstrated that 3T3-L1 CM is rich in numerous growth factors (*20*), such as insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF), which have been linked to cancer radioresistance. Downregulation of insulin-like growth factor 1 (IGF-1) receptor in mouse melanoma B16-F1 cells improved radiosensitivity (*35*) and, in some malignancies, radioresistant phenotypes were aggravated by HGF (*36*). IGF-1 and HGF signaling activates PI3K-AKT pro-survival pathways (*37*). These findings led us to assume that the growth-factors present in the 3T3-L1 CM and the upregulation of the AKT signaling cascade are contributing to the radioresistant phenotype of B16-F10 cells observed in our results. Nevertheless, given the vast number of growth factors and bioactive molecules released by adipocytes, it is our belief that a ''one mechanism fits all'' approach is unlikely to exist and other signaling pathways certainly are co-activated and contribute to the enhanced radioresistance of melanoma.

Radiation also induces vascular damage that potentiates tumor hypoxia (*38*). In a recently published study, our group showed that adipocyte secretome enhances vasculogenic mimicry in B16-F10 cells (*20*). Therefore, reducing the vascular network dependence of the tumor for blood supply, which allied with the radiation-desensitizing effect, plays a cumulative role towards melanoma aggressiveness.

In summary, our results indicate that an environment rich in adipocyte-released factors contributes to the protection of melanocytes from radiation-induced oxidative stress and viability loss, circumventing the efficacy of radiation, with a contribution of adipocyte-released factors PI3K-AKT activation.

Although further *in vivo* studies are of paramount importance to elucidate the molecular players involved, the current study demonstrates the importance of taking into consideration the role of adiposity when planning radiotherapy regimens.

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