Original Article

Noninvasive Radiofrequency Treatment Effect on Mitochondria in Pancreatic Cancer Cells

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BACKGROUND: The development of novel therapeutic approaches for cancer therapy is important, especially for tumors that have poor response or develop resistance to standard chemotherapy and radiation. We discovered that noninvasive radiofrequency (RF) fields can affect cancer cells but not normal cells, inhibit progression of tumors in mice, and enhance the anticancer effects of chemotherapy. However, it remains unclear what physiological and molecular mechanisms this treatment induces inside cells. Here, we studied the effect of RF treatment on mitochondria in human pancreatic cancer cells. **METHODS:** The morphology of mitochondria in cells was studied via electron microscopy. The alteration of mitochondrial membrane potential ($\Delta\psi$) was accessed using a Mitotracker probe. The respiratory activity of mitochondria was evaluated by analyzing changes in oxygen consumption rates determined with a Mito Stress Test Kit. The production of intracellular reactive oxygen species was performed using flow cytometry. The colocalization of mitochondria and autophagosome markers in cells was performed using fluorescence immunostaining and confocal microscopy analysis. **RESULTS:** RF fields treatment changed the morphology of mitochondria in cancer cells, altered polarization of the mitochondrial membrane, substantially impaired mitochondrial respiration, and increased reactive oxygen species production, indicating RF-induced stress on the mitochondria. We also observed frequent colocalization of the autophagosome marker LC3B with the mitochondrial marker Tom20 inside cancer cells after RF exposure, indicating the presence of mitochondria in the autophagosomes. This suggests that RF-induced stress can damage mitochondria and induce elimination of damaged organelles via autophagy. **CONCLUSION:** RF treatment impaired the function of mitochondria in cancer cells. Therefore, mitochondria can represent one of the targets of the RF treatment. *Cancer* **2014;120:3418-25.** *© 2014 American Cancer Society.*

KEYWORDS: radiofrequency, autophagy, mitochondria stress.

INTRODUCTION

The effects of electromagnetic fields on cancer cells led to discovery of major diagnostic tools such as X-ray imaging, computed tomography, and magnetic resonance imaging due to their ability to penetrate the human body. However, the energy produced by these ionizing radiation devices is generated by short electromagnetic waves (micrometer to nanometer length) with very high frequency at $10^{15}-10^{24}$ Hz (this range covers ultraviolet and gamma-rays) and is destructive not only for cancer, but for normal cells as well. Therefore, application of these electromagnetic fields is limited and can cause long-lasting toxic effects on vital organs, as happens in patients undergoing ionizing radiation treatment. Long electromagnetic waves within the range of meters, known as radio waves, have low frequency ($10^{10}-1.0$ Hz) and are produced by common home electronic devices such as radios and televisions. Electromagnetic fields produced by radio waves are recognized to be safe for humans because they have low absorbance rates by human tissues and cells when compared with those produced by short electromagnetic waves of high frequency.[1]

The ability of low-energy electromagnetic waves to affect cancer cells has been demonstrated in several in vitro studies.[2-7] Some studies verified their anticancer effect in vivo[2,3,8-10] or were studied in patients.[2,11,12] Molecular changes that can be induced in cancer cells after exposure to radiofrequency (RF) fields remain poorly understood. Most reports suggest that RF fields cause changes in the function of tubulin, the protein that plays an essential role in microtubule formation during cell division in cancer cells.[4-7] Several studies of the biological effects of electromagnetic fields with low frequency on cells indicate other changes, such as altering the function of ion channels on cell plasma membranes.[13,14] In our recent studies, we reported that noninvasive RF treatment at 13.56 MHz with the noninvasive field ranging from 1 KeV to 20 KeV/m^2 inhibits the growth of orthotopic hepatocellular carcinoma in mice[15] and enhances the anticancer effect of

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gemcitabine chemotherapy in a murine model of orthotopic pancreatic cancer.[16] Investigation of the RF-induced cell death mechanism in cells indicated its ability to affect cancer cells via autophagy.[16] Importantly, this effect was not observed in normal cells. Autophagy is one of the mechanisms used by cells for processing damaged organelles. Mitochondria are highly sensitive to external and internal stresses, which perhaps explains the short life time and rapid turnover of these organelles. Even in nonproliferating tissues, mitochondria turn over constantly, with a half-life of approximately 10-20 days.[17] Autophagy of outworn or damaged mitochondria is known as mitophagy. Therefore, in this study, we focused on studying the RF field effect on mitochondria and its function in cancer cells. Because we previously obtained positive results for this novel noninvasive method for the treatment of pancreatic cancer, one of the deadliest types of cancer with very limited therapeutic options, we used human pancreatic cancer cells in the current study. We discovered that our RF treatment caused a substantial change in morphology and function of mitochondria in cancer cells.

MATERIALS AND METHODS

Cell Culture

Human pancreatic cancer cells (AsPC-1 and Panc-1) were acquired from the American Type Culture Collection (Manassas, VA). Cells were maintained in standard growth conditions (37°C, 5% CO_2, Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin [Gibco, Grand Island, NY]) and authenticated by the Characterized Cell Line Core Facility (STR DNA fingerprinting, MD Anderson Cancer Center).

RF Treatment

For in vitro studies, cells were seeded at 0.1×10^6 cells/well in 2 mL of media into 12-well plates and after overnight incubation were exposed for 5 minutes to the RF field at 900 W at a frequency of 13.56 MHz (Therm Med LLC, Erie, PA) as described elsewhere.[18] Before each experiment, the device was calibrated for a heating profile using gold nanoparticles. After RF treatment, cells were placed back into the tissue culture incubator until analysis. Cells that remained unexposed to the RF field were used as a control.

High-Resolution Transmission Electron Microscopy Imaging of Autophagy In Vitro

Cells were exposed to the RF field for 5 minutes and fixed on the following day with 1 M cacodylate buffer (pH 7.4) containing 3% glutaraldehyde and 2% paraformaldehyde for 24 hours. Samples were then washed with 0.1% cacodylate buffered tannic acid and treated with 1% osmium tetroxide. Finally, samples were stained with 1% uranyl acetate, dehydrated with ethanol, and embedded in LX-112 medium. After polymerization, samples were cut on a microtome and double-stained with uranyl acetate/lead citrate. Imaging was performed using a JEM1010 transmission electron microscope (Jeol USA, Inc., Boston, MA) equipped with the AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

MitoTracker Staining

Prewarmed at 37°C, MitoTracker Red FM reagent (Molecular Probes, Inc., Eugene, OR) was added to cancer cells immediately after RF exposure at a final concentration of 0.5 mM. Untreated cells were used as a control. All cells were placed into the tissue culture hood for 30-minute incubation. After that, the cells were washed with warm phosphate-buffered saline (PBS) and fixed with 2% formaldehyde. Fixed cells were analyzed under an Olympus IX81 fluorescent microscope (Olympus America Inc., Center Valley, PA). Quantitative analysis of fluorescence intensity was performed on 3 randomly selected microscopic fields using Image-Pro software (Media Cybernetics, Rockville, MA).

OCR Measurement

The Seahorse XF96 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA) was used to obtain real-time measurements of oxygen consumption rate (OCR) in cells. The Mito Stress Test Kit (Seahorse Biosciences) was used to measure basal respiration, adenosine triphosphate (ATP) turnover, proton leak, and spare respiratory capacity. The preparation of cells and analysis for the assay was performed according to the manufacturer's instructions. Briefly, RF-treated cancer cells and untreated cells that were used as a control were harvested, reconstituted in the equilibrated assay medium to a final concentration of 0.1×10^6 cells/mL, and seeded in quintuplets in 96-well cartridges. The cartridge with cells was placed for 30 minutes into the Seahorse XF96 Analyzer incubator unit before running a program for equilibration. Basal rates of oxygen consumption were measured 4 times during the first 35 minutes. If fluctuations in OCR levels during this time did not exceed 10%, the assay continued and other chemicals (Oligomycin, carbonyl cyanide-ptrifluoromethoxyphenylhydrazone (FCCP), and a mixture of antimycin A with rotenone) were subsequently added to cells. After 2 minutes of mixing, postexposure OCR measurements were performed 4 times.

ROS Production Measurement

Triplicates of cells (1×10^6 cells/well seeded in 12-well-plate) were exposed to the RF field for 5 minutes or remained untreated and were trypsinized immediately after the end of treatment. To inhibit cytotoxic effect of trypsin cells were first resuspended in 1 mL of serum-containing culture media. After centrifugation media over the cells was substituted with 1 mL PBS containing 1 μg/mL of hydroethidine and 6 μg/mL of 2′,7′-dichlorodihydrofluorescein diacetate were added to Panc-1 and AsPC-1 cells, respectively. Untreated unstained cells were used as a negative control. Cells were incubated for 30 min at 37°C. After washing with PBS, cells were analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA).

Fluorescent Immunocytochemistry Staining for Mitochondria and Autophagy Markers

Cells were fixed 5 minutes after RF exposure with 2% paraformaldehyde overnight and then stained with Tom20 (F10) antibody (Santa Cruz Biotechnology, Inc., Dallas, TX) as a marker for mitochondria or anti-LC3B antibody (Cell Signaling Technology, Danvers, MA) as a marker for autophagosomes. Fluorescently labeled secondary antibodies were added to track the presence of the primary antibodies bound with the targets, fluorescein isothiocyanate–labeled secondary antibody was used for binding with LC3B, and Texas Red secondary antibody was used for binding with Tom20. Images of cells were taken using Olympus IX81 inverted fluorescent microscope (Olympus America Inc.).

Statistics

Results from experiments are presented as means with standard deviations. GraphPad Instat 3 software (GraphPad Software Inc., La Jolla, CA) was used for evaluation of distribution assumption of analysis and validation of the test type for statistical analysis. All results showed a normal pattern of value distribution and were analyzed using a 2-sided Student *t* test; $P<.05$ was considered statistically significant. Flow cytometry analysis involved data from at least 10,000 events that was repeated 3 times, and representative histograms were selected for publication.

RESULTS

Exposure of Cancer Cells to the RF Field Induces Morphological Changes of Mitochondria

AsPC-1 and Panc-1 human pancreatic cancer cells were exposed to the RF field for 5 minutes and fixed 24 hours after treatment as shown in Figure 1. In our previous studies, we already observed the ability of RF treatment to change cellular morphology by causing shrinking and detachment of cancer cells, which was followed by significant reduction of their viability.[16] Here examination of cellular organelles under electron microscopy revealed the most significant morphological changes in mitochondria (Fig. 2). Many cells had enlarged bloated mitochondria with altered cristae shapes.

Figure 1. Cell treatment in the RF field. Cells were seeded in culture dishes and placed between the transmission top head and reciprocal bottom platform of the RF unit. Treatment was performed for 5 minutes at 13.56 MHz and at a power of 900 W.

RF Treatment Alters Mitochondrial Membrane Potential ($\Delta\psi$) in Pancreatic Cancer Cells

To further validate the effect of RF field on mitochondria in cancer cells, we investigated whether it altered membrane polarization of mitochondria. To this end, we stained cells before and after RF exposure with Mitotracker Deep Red FM, a red fluorescent dye that stains mitochondria in live cells and for which accumulation is dependent upon membrane potential. Treatment of AsPC-1 cancer cells in the RF field for 5 minutes caused 6-fold elevation of red fluorescent signal from 6.2 ± 3.2 to 48.2 ± 12.3 ($P=.007$) when compared with untreated cells (Fig. 3). In Panc-1 cells RF treatment was followed by 3-fold increase in fluorescence signal, from 7.7 ± 0.7 to 23.6 ± 10.8 ($P=.04$), indicating an RF-induced alteration of $\Delta\psi$.

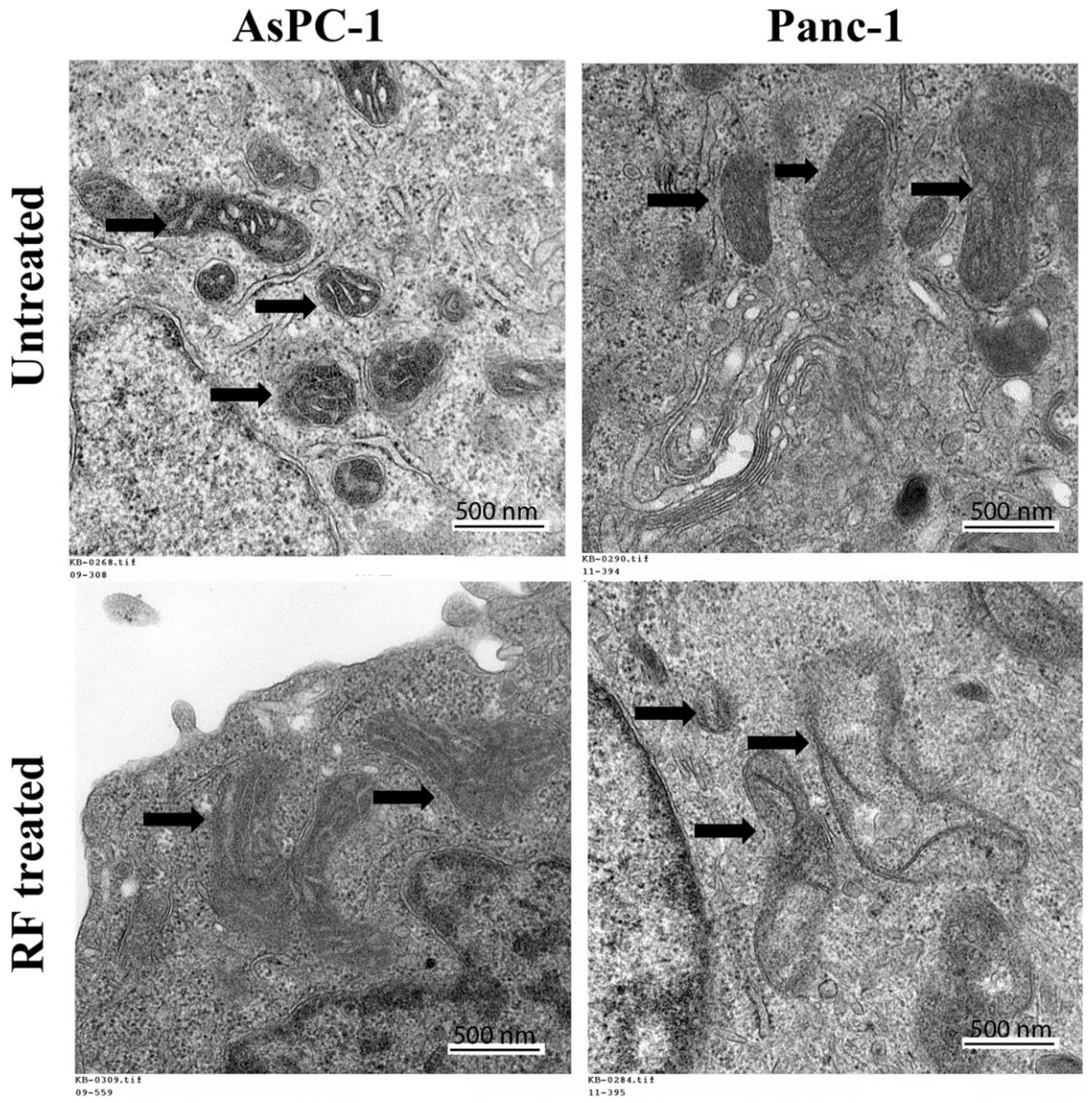

Figure 2. RF treatment altered the morphology of mitochondria in pancreatic cancer cells. Cells were fixed for the transmission electron microscopy imaging 24 hours after RF treatment. Untreated cells were used as a control. The mitochondria are indicated by arrows.

RF Treatment Decreases OCR in Pancreatic Cancer Cells

We also analyzed the pattern of OCR in cancer cells in response to the RF-induced stress using the MitoStress Kit, which directly measures of the activity of electron transport chains in mitochondria. As seen in Figure 4, RF treatment caused significant decline in the OCR levels from 103 ± 12 pmoles/min in untreated AsPC-1 cells to 43 ± 22 after treatment (P=.0001). Similarly, in Panc-1 cells, average OCR levels in untreated cells remained at 203 ± 15 pmoles/min and decreased to 143 ± 35 pmoles/min (P=.0018) after RF exposure.

The ATP coupler oligomycin is used to prevent phosphorylation respiration in cells. Treatment of cells with oligomycin permits evaluation of oxygen consumption levels devoted for ATP synthesis in cells. As expected, levels of OCR in cells that were not exposed to the RF treatment decreased after exposure to oligomycin as shown in Figure 4. RF treatment had some additive effect on oligomycin-induced decline of OCR in AsPC-1 cells

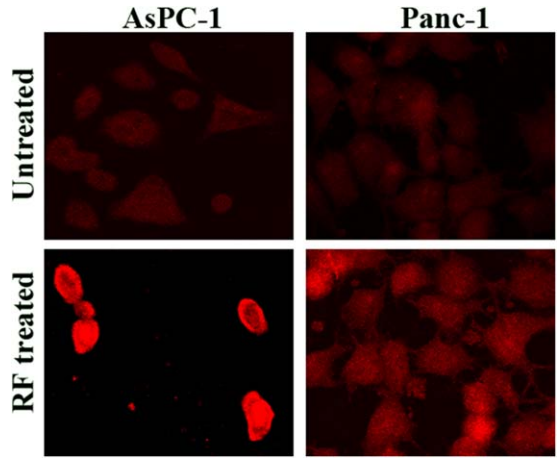

Figure 3. MitoTracker Red FM fluorescent staining was increased in pancreatic cancer cells after RF exposure. Cells were exposed to the RF field for 5 minutes. MitoTracker reagent was added immediately after the end of treatment, and cells were incubated for 30 minutes. After washing, cells were fixed and imaged for fluorescence intensity in red spectrum.

and insignificant effect in Panc-1 cells, though in both cases exposure of cells to the RF caused reduction of ATP production.

The recovery of mitochondrial function after oligomycin treatment can be achieved by addition of the uncoupling FCCP reagent; this agent is used to determine maximum respiration in cells. OCR levels in untreated and RF-treated cancer increased. However, addition of FCCP to cells that were exposed to the RF field did not achieve maximal respiration levels, which were recorded for those cells that were not exposed to the RF. The average maximal OCR values in RF-treated cells after addition of FCCP reagent were 1.5- to 2-fold lower when compared with those in RF-untreated cells.

Finally, treatment of cells with rotenone and antimycin A caused functional arrest in mitochondrial complexes I and II, shutting down mitochondrial respiration completely. The results in Figure 4 demonstrate that RF treatment did not alter the cells' response to these reagents.

RF Treatment Stimulates ROS Production in Pancreatic Cancer Cells

Frequent response of mitochondria to stress is revealed by elevation of ROS production. Therefore, we used oxidant-sensing fluorescent probes to determine the levels of ROS production in pancreatic cancer cells after RF-induced stress. We observed the shift of mean fluorescence intensity from 1.2×10^3 in untreated AsPC-1 cells to 1.6×10^3 immediately after 5-minute exposure to RF

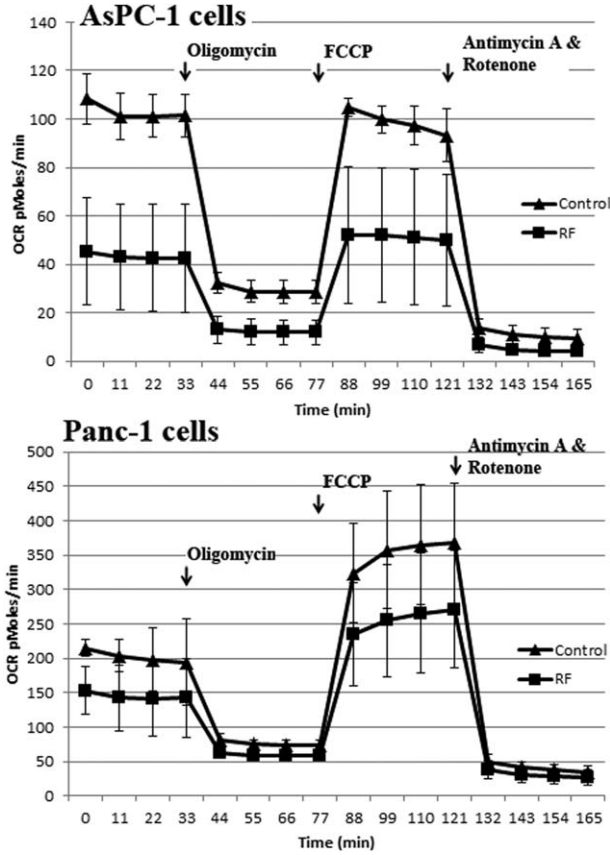

Figure 4. RF treatment decreased oxygen consumption rates (OCR) in pancreatic cancer cells. AsPC-1 cells (top panel) or Panc-1 cells (bottom panel) were harvested immediately at the end of 5 minutes of RF field exposure and left for equilibration in a Seahorse X96 incubator. OCR measurements were performed with the Mito Stress Test Kit and were analyzed by the Seahorse Instrument Software as described in the Materials and Methods.

(Fig. 5). The same pattern was noticed in Panc-1 cells that showed fluorescence elevation from 1.3×10^3 in untreated state to 1.0×10^4 after RF treatment. The elevation of fluorescence intensity in both cases indicated an elevation of ROS production in cancer cells after RF exposure.

RF Treatment Increased Colocalization of Mitochondria with Autophagosomes

Previously, we were able to show that RF treatment can induce autophagy in pancreatic cancer cells.[16] Damaged organelles, including mitochondria, can be processed in cells via autophagy.[19] Data from our experiments indicate that RF treatment induced substantial stress on mitochondria, which may be damaging. The first step of processing damaged mitochondria is uptake inside autophagosome vesicles. This event in cells can be verified by fluorescent

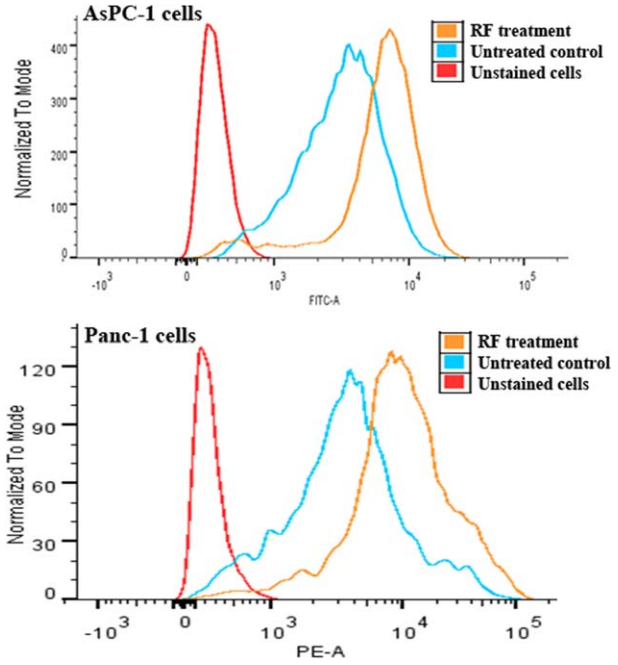

Figure 5. RF treatment enhanced ROS production in pancreatic cancer cells. AsPC-1 cells (top panel) or Panc-1 cells (bottom panel) were harvested immediately at the end of 5 minutes of RF field exposure and treated with HE or DCFH-DA, respectively, as described in the Materials and Methods. Stained cells were analyzed via flow cytometry.

colocalization of LC3B as a marker for autophagosomes membrane with the mitochondria marker Tom20, the protein expressed on the outer membrane of mitochondria. Increased levels of LC3B green fluorescent puncta were noticed only in cells exposed to the RF field, indicating an increased presence of autophagosomes in cells after RF exposure (Fig. 6) and correlating with our previous studies in which we performed a thorough investigation of RF-induced autophagy.[16] Red fluorescence staining with Tom20 antibody designating mitochondria was similar in both treated and untreated cells. Colocalization of the LC3B (red) and TOM20 (green) signals revealed negligible colocalization of Tom20 with LC3B in AsPC-1 and Panc-1 cells, but large areas of colocalization (orange/yellow color) in these cells were observed after RF exposure, suggesting that autophagy-mediated mitochondrial degradation occurs in pancreatic cancer cells upon RF treatment.

DISCUSSION

Mitochondria play an important role in different physiological and pathological processes in eukaryotic cells. Studies unraveling the detailed mechanisms of their function in cancer cells provided the unique targets for cancer cell suicide and lead to the development of the innovative class of anticancer drugs, the so-called mitochondrion-targeted agents.[20] Multiple chemotherapeutic and non-chemotherapeutic modalities that cause cancer cell death were shown to disrupt the function of mitochondria by affecting their phosphorylating respiration as an initial step in the subsequent cascade of apoptosis-inducing events. In the current study, we were able to demonstrate the ability of a novel noninvasive method of cancer treatment based on the use on RF fields to alter the function of mitochondria. Along with morphological changes that were observed in mitochondria of pancreatic cancer cells in response to RF treatment, we observed alteration of mitochondrial $\Delta\psi$ and a significant 1.5- to 2-fold decrease of oxygen consumption and ATP production. Finally, mitochondria is the major source of ROS production during cellular stress. During normal metabolism, ROS are produced in small amounts as by-products and are quickly deactivated or reduced by special intracellular enzymes and small antioxidant molecules, thus avoiding harm to the cell. In stressful environments, synthesis of ROS can be increased dramatically and cause mitochondria damage followed by induction of programmed cell death. We demonstrated the induction of ROS production in pancreatic cancer cells in response to RF-induced stress. In our previous studies, we observed that similar or lower doses of RF treatment were sufficient to induce cell death or inhibit proliferation in different types of malignant cells, including pancreatic and liver cancer cells in vitro and in vivo.[15,16] Our results in the current study suggest that cytotoxic effect of RF treatment may be, at least in part, mediated by affecting mitochondria function.

Recent studies by other investigators have demonstrated that deprivation of ATP production can stimulate an autophagy mechanism in cells for alternative energy sources.[21,22] In our previous studies, we were able to show that RF treatment of cancer cells was followed by induction of autophagy.[16] Data obtained in the current study indicate that substantial reduction of oxygen consumption in RF-treated cancer cells could be one of the initiating signals for autophagy activation, as seen by the increase in green LC3B puncta signals in Fig. 6. Moreover, exposure of mitochondria to the RF field could be devastating and could lead to mitochondrial damage followed by mitophagy in cancer cells. Increased colocalization of the mitochondria marker Tom20 with the autophagosome marker LC3B that we observed in cancer cells after RF treatment indicate the ability of RF treatment to cause mitochondria damage leading to elimination of these damaged structures by autophagosomes. We

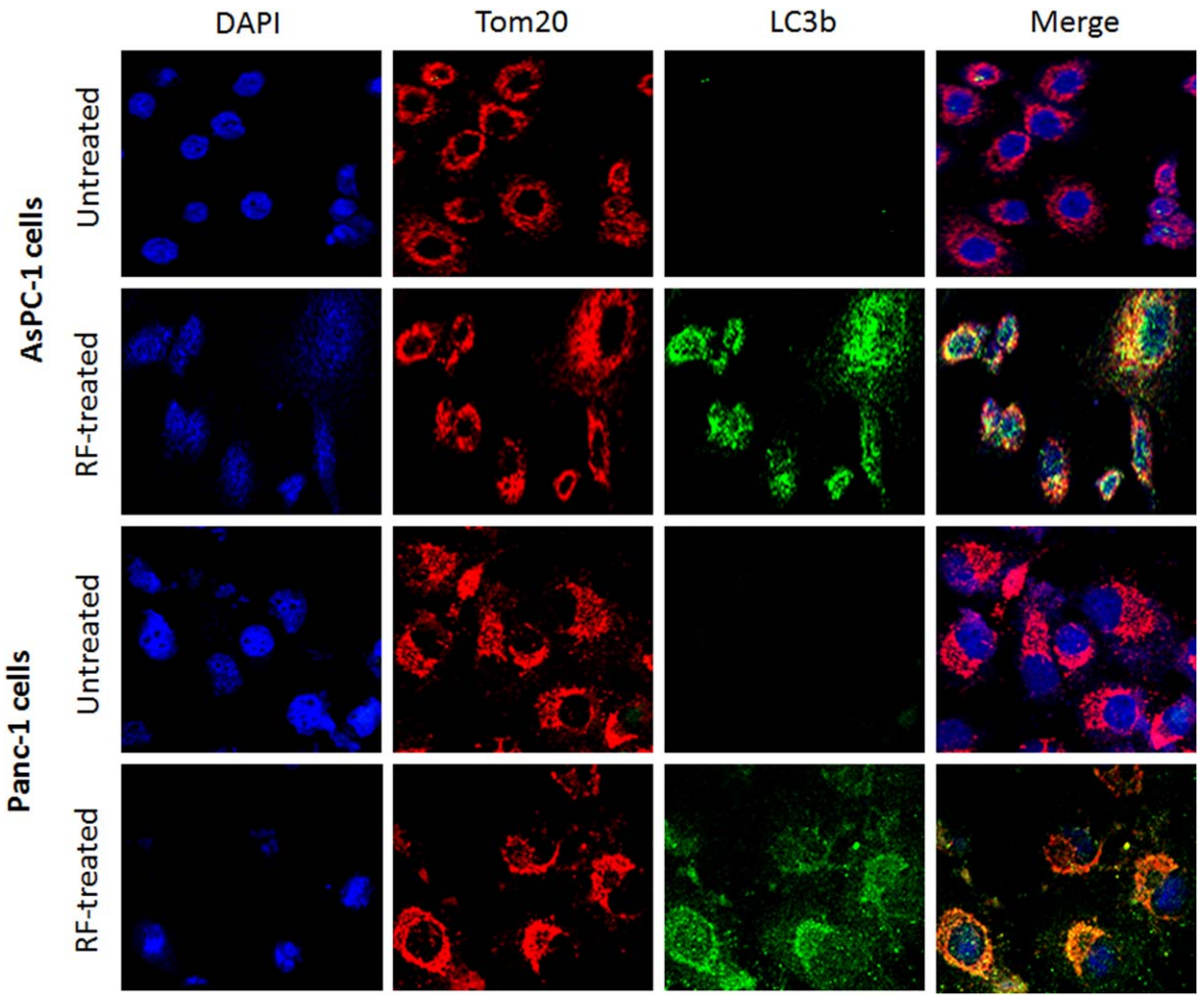

Figure 6. RF treatment enhanced colocalization of damaged mitochondria with autophagosomes. Cells were treated in the RF field for 5 minutes and fixed after 48 hours. Fluorescent immunocytochemical staining of cells was performed with Tom20 antibody (red) as a mitochondria marker and with LC3B antibody (green) as an autophagosome marker. 4',6-diamidino-2-phenylindole (DAPI) was used for nuclei staining (blue). Images were obtained using fluorescent confocal microscopy.The efficacy of innovative noninvasive methods of cancer treatment based on the use of electromagnetic radiofrequency fields treatment has been reported in previous studies; however, its mechanism of action remains poorly understood. We demonstrate the ability of radiofrequency fields treatment to affect mitochondria in cancer cells.

are now identifying mitophagy markers that elucidate this mechanism and will provide a detailed understanding of the processing of mitochondria-containing autophagosomes in cancer cells after RF exposure.

In conclusion, the results obtained in the current study demonstrate that RF treatment can affect mitochondria in cancer cells by weakening their oxygen consumption and ATP production and causing mitochondrial damage followed by induction of substantial colocalization of mitochondria in autophagic vesicles. This reveals mitochondria as a potential target in cancer cells for RF treatment and warrants further investigation.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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