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Low-Dose Radiation-Induced Senescent Stromal Fibroblasts Render Nearby Breast Cancer Cells Radioresistant

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Abstract

In addition to cell cycle arrest, DNA repair or/and apoptosis, ionizing radiation can also induce premature senescence, which could lead to very different biological consequences depending on the cell type. We show in this report that low-dose radiation-induced senescent stromal fibroblasts stimulate proliferation of cocultured breast carcinoma cells. Such effects of senescent fibroblasts appear to result from their ability to induce the expression in carcinoma cells of mitotic genes and subsequent mitotic division. The elevated proliferation of breast carcinoma cells correlates with resistance to radiation as well as to adriamycin. Of interest is the observation that exposure to lower doses (<20 cGy) augments the ability of senescent fibroblasts to promote the survival of cocultured breast carcinoma cells. The resistance appears to be mediated partially by the Akt pathway, because expression of a dominant negative Akt mutant in breast carcinoma cells results in a partial reversal of the radioresistance. The ability of fibroblasts to modulate the radiosensitivity of nearby carcinoma cells implicates the importance of targeting the stroma during therapy.

INTRODUCTION

Increasing evidence indicates that many cell types including fibroblasts respond to stresses, such as oxidative stress, inflammatory cytokines and various genotoxic agents, by engaging in a senescent-like phenotype, which differs from replicative senescence in that no telomere shortening is detected (1). With compelling evidence for the existence of senescence *in vivo* (1,2), this irreversible cell cycle arrest state has been considered as an important tumor suppressor mechanism (3). Paradoxically, abundant evidence indicates that senescent fibroblasts secrete degradative enzymes, cytokines and growth factors, which closely resemble activated those of fibroblasts (4–6). It is thought that the accumulation of senescent fibroblasts in stroma can serve as a means of stromal activation, which may constitute a permissive microenvironment to promote tumorigenesis (7,8).

Cells exist in a tightly regulated microenvironment where homeostatic processes dictate whether a given cell remains quiescent, proliferates, differentiates or undergoes apoptosis (9, 10). Like biological behaviors, cellular responsiveness to exogenous stimuli can also be affected by the tissue microenvironment. We recently showed that ionizing radiation was able to effectively induce premature senescence in human stromal fibroblasts, which was associated with significant alterations of the local microenvironment and of the biology of associated

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epithelial cells. We continued to explore the importance of the microenvironment in the present study by examining the effects of senescent stromal fibroblasts on the radiosensitivity of cocultured breast carcinoma cells.

MATERIALS AND METHODS

Cell Culture and Materials

Primary human mammary fibroblasts (HMFs, passages between 6 and 10, R. A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA), MCF-7 and MDA-MB-231 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. FITC-conjugated anti-pan-cytokeratin monoclonal antibody (mAb) and anti-fibroblast antigen Thy-1 mAb were purchased from Calbiochem (San Diego, CA), anti-phospho-histone H3 mAb from Upstate (Lake Placid, NY), Annexin V-FITC Apoptosis Detection Kit from Sigma (Saint Louis, MO), and Growth Factor Reduced (GFR) Matrigel and dispase from BD Biosciences (San Diego, CA). pLZRS.neo Akt (K179M) and pLZRS.neo were gifts from V. M. Weaver (University of Pennsylvania) and retrovirus-mediated gene transfer was performed as described (8).

Irradiation and Induction of Senescent-Pike Phenotypes

Confluent HMFs were irradiated with 5 cGy X rays from a Philips MG160 high-stability constant potential X-ray system (Philips, Hamburg, Germany) at dose rate of 1.84 cGy/min every 12 h until the indicated doses were achieved. The cultures were fed with fresh FBS DMEM twice a week. The majority (>95%) of the HMFs exhibited senescent-like phenotypes, as confirmed by the senescence-associated β-galactosidase (SA-β-gal) activity (11), when the total accumulated dose reached 10 Gy. More than two batches of the senescent-like fibroblasts were used for the coculture experiments with normally growing fibroblasts as a control.

Two-Dimensional and Three-Dimensional (3D) Coculture Assay

Because senescent cells may exhibit a secretary phenotype and affect nearby epithelial cells through a mode of paracrine-mediated action (4–6), we cocultured fibroblasts and MDA-MB-231 cells on plastics in low-serum (1% FBS) medium (LSM) to facilitate the detection of the potential paracrine activity. Normally growing fibroblasts or senescent-like fibroblasts (3×10^5) were seeded in a 60-mm dish and gradually arrested after 3 days when maintained in LSM (not shown). MDA-MB-231 cells (10^5) were allowed to adapt to LSM for 3 days and were then plated on top of the fibroblast lawn. The coculture was maintained in LSM for the indicated time before subsequent assays. The 3D heterotypic coculture was established by a modified cell overlay method as described previously (8,12).

Cell Isolation and Gene Expression Profiling

Breast cancer cells (MDA-MB-231 or MCF-7) were cocultivated with normally growing fibroblasts or radiation-induced senescent-like fibroblasts for 24, 72 or 120 h, after which they were isolated from the coculture and total RNA was extracted. To isolate breast carcinoma cells from fibroblasts, the coculture was treated with dispase for 1 h at 37°C and the resultant heterotypic cell aggregates were trypsinized and resuspended as single cells. Fibroblasts in the cell suspension were then labeled by incubation with MACS Anti-Fibroblast MicroBeads (Miltenyi Biotech, Auburn, CA) at 4°C for 30 min. The magnetically labeled HMFs were depleted from the cell suspension by the MidiMACS Separation Unit. The cell suspensions were kept on ice throughout the separation process to avoid potential transcriptional alterations of the cells. The purity of breast carcinoma cells in the resultant eluent was verified by immunofluorescently staining the cells with anti-pan-cytokeratin mAb. Under the optimal conditions, an approximate 95% recovery with a purity close to 100% was obtained. Gene

expression levels were determined by cDNA microarrays with the capacity to display transcript levels of 7,680 known human genes (glass spotted nucleotide arrays provided by Dr. Yao-Yu Eric Chuang, National Cancer Institute) as described previously (13). Homotypically cultured breast carcinoma cells were used as the source of reference RNA, and average linkage clustering was carried out using the Cluster software (14). qRT-PCR was performed as described previously (23). Quantitative RT-PCR was performed with primers corresponding to each gene using the QuantiTeetSYBR Green PCR Kit (Qiagen) and the Opticon Monitor Thermycycler (MJ Research, Inc.). mRNA levels were calculated relative to that of the *GADPH* gene.

Clonogenic and Apoptosis Assays

To determine the long-term clonogenic potential of cells after irradiation, breast cancer cells isolated from the coculture were seeded at 1,000 cells per 60-mm dish and grown in DMEM plus 10% FBS medium for 14 days. The number of colonies containing 50 or more cells was then determined by a computer-assisted algorithm (K. Y. Tsai, National Taiwan University, Taiwan).

To measure the extent to which cancer cells undergo apoptosis in the cocultures, the cultures were fixed and costained with anti-pan-cytokeratin and Annexin V-FITC. Apoptosis was calculated as the percentage of Annexin V-positive breast carcinoma cells relative to the total number of breast carcinoma cells (cytokeratin-positive) present in the coculture.

RESULTS

Our previous study showed that radiation-induced senescent-like fibroblasts promote invasive growth of cocultured MDA-MB-231 cells (8). We asked in the present study whether such an effect of senescent-like fibroblasts could affect the radio- and chemosensitivity of breast cancer cells. The factors we previously identified as mediating the effects of radiation-induced senescent-like fibroblasts are secretory matrix metalloproteinases (MMPs), which degrade extracellular matrix (ECM) and thereby indirectly influence surrounding epithelial cells. To determine whether factors other than MMPs could mediate the effects of fibroblasts on epithelial cells, we first cocultured fibroblasts and breast carcinoma cells in the absence of ECM. MDA-MB-231 cells were labeled with GFP and fibroblasts were immunostained with an anti-fibroblast antibody so that the two cell types could be distinguished visually under a fluorescence microscope. With available evidence indicating a secretary phenotype of senescent fibroblast that could affect nearby epithelial cells, we maintained confluent cultures of human mammary normally growing fibroblasts or senescent-like fibroblasts in low-serum (1% FBS) medium (LSM) to facilitate detecting the activity of secreted factors. Under these conditions, normally growing fibroblasts were gradually arrested after 3 days (not shown). MDA-MB-231 cells were also adapted in LSM for 3 days and then plated on top of the fibroblast lawn. The cocultures were maintained for an additional 7 days. Visual inspection under a phase-contrast microscope revealed a significant increase in cell numbers in the coculture with senescent-like fibroblasts compared to that with normally growing fibroblasts (Fig. 1A, left panels). This increase in cell numbers appeared to result from an increase in MDA-MB-231 cells as shown by marked increase of green (GFP-labeled) cells (Fig. 1A, right panels). To determine the carcinoma cell numbers, MDA-MB-231 cells were isolated from cocultures by magnetic cell sorting (MACS) and cells were counted using a Coulter counter. The results indicated a significant increase in the MDA-MB-231 cell population in a time-dependent manner, with an increase in carcinoma cell numbers of approximately twofold at day 3 and fourfold at day 7 in the cocultures with senescent-like fibroblasts relative to those with normally growing fibroblasts (Fig. 1B). The increased cell numbers appeared to result from an elevated cell proliferation, because MDA-MD-231 cells exhibited a greater

proliferation rate when cocultured with senescent-like fibroblasts than with normally growing fibroblasts (Fig. 1C). The number of either senescent-like fibroblasts or normally growing fibroblasts in the cocultures was not detectably different (not shown).

To probe this MMP-independent growth-promoting activity of radiation-induced senescent fibroblasts, we profiled the gene expression pattern of MDA-MB-231 cells. When we compared the gene expression profiles of MDA-MB-231 cells that were cocultivated with either normally growing fibroblasts or senescent-like fibroblasts, we identified 71 genes that were differentially (more than twofold) expressed. This set of genes was grouped into six clusters according to their distinct temporal expression patterns (not shown). Interestingly, seven out of the 14 genes that showed an early induction (subcluster 1) of genes encoding proteins important for the execution of mitosis, including HEC, a gene involved in spindle checkpoint signaling (15), MPHOSPH1, a gene encoding a kinesin-related protein required for cytokinesis (16), ECT2, a gene encoding a transforming protein that regulates cytokinesis (17), TOP2A, a topoisomerase (18), SMARCE1, a gene involved in chromatin remodeling (19), and CENNFs, a gene responsible for chromosome segregation during mitosis (20). We focused on this group of genes for further validation. Using quantitative RT-PCR to determine the mRNA levels, we verified the increased expression of these genes (Fig. 2A). The increased expression of these genes would predict an accelerated cell cycle progression and cell division in MDA-MB-231 cells. To test this, we identified mitotic populations of MDA-MB-231 cells by immunostaining with the mitotic marker phospho-histone H3. The results revealed a marked increase in the percentage of mitotic MDA-MB-231 cells in cultures that were cocultured with senescent-like fibroblasts relative to those with normally growing fibroblasts (Fig. 2B), confirming the data obtained from gene profiling. The increased number of mitoses is consistent with the elevated proliferation rate of MDA-MB-231 cells grown in the presence of senescent-like fibroblasts.

We next asked whether the growth promoting activity of senescent-like fibroblasts could modulate the sensitivity of cocultured breast cancer cells to either radio- or chemotherapy. MDA-MB-231 cells were cultured alone (homotypic culture) or with either normally growing fibroblasts or senescent-like fibroblasts in LSGM for 3 days and then treated with varying doses of radiation. The cocultures were allowed to recover for 24 h and then the breast cancer cells were isolated from the cocultures for the standard colony formation assay. The results indicated that senescent-like fibroblasts exerted a radioprotective effect on the cocultured MD-MB-231 cells, as shown by an apparent difference in the slopes of the radiation survival curves (Fig. 3A). Notably, low doses of radiation (<20 cGy) appeared to stimulate the survival of carcinoma cells in the presence of senescent-like fibroblasts (Fig. 3A). A similar result was seen in the cocultures of cells of another breast cancer cell line, MCF-7 (Fig. 3B), indicating that the protective effect of senescent fibroblasts is not limited to one cell type. To examine whether this protective effect extended to chemotherapeutic agents, we cocultured MDA-MB-231 cells with either normally growing or senescent-like fibroblasts for 3 days and then treated them with adriamycin for 72 h. The cultures were fixed and stained with the Annexin V-FITC Apoptosis Detection Kit (Sigma, St. Louis, MO). MDA-MD-231 cells were differentiated from fibroblasts by staining with an epithelial cell marker (cytokeratin). Quantitative analysis of apoptosis indicated that, in the presence of senescent-like fibroblasts, MDA-MB-231 cells were less sensitive to adriamycin than MB-23 cells cocultured with normally growing fibroblasts (Fig. 3C). The protective effect was also evident, albeit to a lesser extent, when conditioned medium was used (Fig. 3D), supporting a critical role of secreted factors from senescent fibroblasts. The clonogenic cell survival assay showed that senescent-like fibroblasts rendered cocultured MDA-MB-231 cells more resistant to adriamycin (Fig. 3E). Taken together, these results indicate that senescent fibroblasts render nearby malignant MECs resistant to cancer therapeutic agents.

To further substantiate the findings described above, we employed a three-dimensional coculture system that allows recapitulating cell-cell and cell-extracellular matrix (ECM) interactions to assess the influence of senescent stromal fibroblasts on the radiosensitivity of cocultured breast carcinoma cells in a tissue-like setting. MDA-MB-231 cells were cocultivated with normally growing or senescent-like fibroblasts in a 3D matrix for 4 days so that the interaction between epithelial cells and stromal fibroblasts became fully developed. The cocultures were then treated with single doses of radiation and maintained for an additional 10 days. Consistent with our previous results (8), senescent-like fibroblasts induced invasive growth of cocultured MDA-MB-231 cells in 3D ECM. Radiation did not significantly inhibit such invasive growth unless the dose was greater than 0.5 Gy (Fig. 4A). To analyze the effects quantitatively, single cells were recovered from the ECM gels after digestion with dispase and trypsin. MDA-MB-231 cells were isolated from HMFs using MACS and the cell numbers were quantified. The results indicate that the MDA-MB-231 cells cocultured with senescent-like fibroblasts displayed reduced sensitivity to radiation compared with the cells cocultured with normally growing fibroblasts. Of interest is that low-dose radiation (<20 cGy) appeared to stimulate the survival of cocultured MDA-MB-231 cells with senescent-like fibroblasts (Fig. 4B). Again, a similar protective effect from senescent fibroblasts was also observed in MCF-7 cell cocultures (Fig. 4D).

Our previous study showed that senescent-like fibroblasts induced invasive growth of MDA-MB-231 cells through the activation of the PI3K/Akt pathway (8). Given the available evidence indicating that PI3K/Akt pathway enhances the viability of irradiated cells (21,22), we asked whether the PI3K/Akt-dependent survival could contribute to the senescent-like fibroblast-induced radioresistance of MDA-MB-231 cells. For this, we tested cells of a MDA-MB-231 cell line overexpressing a dominant-negative Akt (K179M), which blocked the phosphorylation of Akt (Fig. 4C) and also partially attenuated the radioprotective effects of senescent-like fibroblasts (Fig. 4B). We did not observe any detectable effect of dominant negative Akt in cocultures of normally growing fibroblasts with MDA-MB-231 (not shown). Together, our results indicate that the radio-responsiveness of cancer cells can be significantly modulated by alterations in the stromal microenvironment and that senescent stromal fibroblasts can markedly decrease the radiosensitivity of associated breast carcinoma cells.

DISCUSSION

We reported previously that radiation-induced prematurely senescent fibroblasts caused significant perturbation of the local microenvironment at least in part because of marked morphological changes and up-regulation of multiple matrix metalloproteinases (MMPs) in senescent fibroblasts. Such changes profoundly affected the biology of the surrounding epithelial cells as highlighted by the disruption of mammary epithelial duct formation in MCF-10A cells and the invasive growth of MDA-MB-231 breast carcinoma cells (8). As a continuation of that study, we examined how the senescent stromal fibroblasts could modulate the sensitivity of cocultured breast cancer cells to anti-cancer therapies. Our results indicate that senescent fibroblasts can stimulate proliferation of surrounding breast carcinoma cells. Stromal cells can be induced by various stress signals to undergo premature senescence; these senescent cells accumulate in aged people, resulting in significant alterations of the microenvironment. Such changes may have a strong impact on cancer cells during metastasis when cancer cells breach the basement membrane and interact with the stromal cells. It has been well documented that senescent fibroblasts secrete many factors that affect surrounding cells and the microenvironment (4–6). Consistent with the secretary phenotype is the observation that the growth-promoting activity of senescent fibroblasts was manifested under the low-serum culture conditions. To gain insight into the effects of senescent fibroblasts, we examined the gene expression profiles of breast cancer cells cocultured with senescent fibroblasts. Using a hierarchical clustering algorithm to analyze the temporal profiles of gene

expression under the different coculture conditions, we identified six distinct clusters. A significant enrichment of mitotic genes that were up-regulated in MDA-MB-231 cells cocultured with senescent fibroblasts correlated with a significant increase of the mitotic cell population, which may be responsible at least in part for the elevated proliferation rate of cocultured cancer cells with senescent fibroblasts. We showed previously that the PI3K/Akt pathway in MDA-MB-231 cells is activated by cocultured senescent fibroblasts (8), which would promote cell proliferation. However, we showed that the PI3K/Akt pathway is activated by integrins in response to proteolytic degradation of extracellular matrix. The increased proliferation we observed in the present study is induced by senescent fibroblasts in the absence of ECM, implicating the effects of the secreted factors. The correlation between the cell proliferation rate and the sensitivity of breast carcinoma cells to radiation and adriamycin suggests that the senescent stroma increases the resistance to cancer therapies at least in part by promoting proliferation of cancer cells. The PI3K/Akt pathway is important for cell survival, and it has been associated with increased radioresistance in numerous cancer cell types (21, 22). Suppression of Akt by ectopic expression of the dominant negative mutant restored the radiosensitivity, albeit partially, suggesting that the Akt pathway can be stimulated by secreted factors through an integrin-independent pathway. Of interest is the observation that exposure to low-dose radiation (<20 cGy) augments the effect of senescent stroma in promoting cell survival. In their review, Deschavanne and Fertil (24) examined the effects of feeder cell coculture on radiosensitivity. Most of those studies were done with feeder cells that were homologous to the cells examined. In the presence of feeder cells, most cells examined appeared to exhibit increased radiosensitivity. Of interest is that the effect was more visible in the low-dose range. While we also observed more pronounced effects in the low-dose range, the breast cancer cell lines tested were more resistant when cocultured with fibroblasts, especially senescent fibroblasts. Senescent fibroblasts promote breast cancer cells to proliferate, which would result in less time available for cells to repair potentially lethal damages because of increased cell cycle progression. Senescent fibroblasts also activate the Akt pathway, which promotes cell survival through multiple mechanisms. The observed radioresistance of breast cancer cells implicates a dominant pro-survival effect of cocultured senescent fibroblasts. Further studies are required to delineate the mechanism underlying this effect. Collectively, our data show an important role for the microenvironment in modulating the responsiveness of cancer cells, implicating the tumor microenvironment as a potential therapeutic target.

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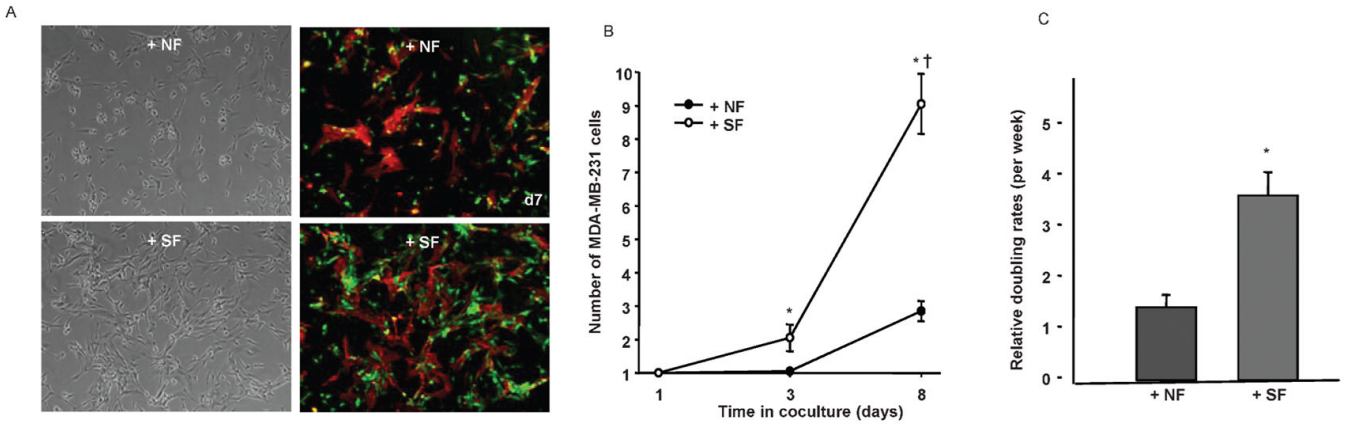

FIG. 1.

Senescent-like fibroblasts promote proliferation of cocultured MDA-MB-231 cells. Panel A: Representative images of cocultures of MDA231 cells with either normally growing fibroblasts or senescent-like fibroblasts as visualized under a phase-contrast microscope (left panels) or a fluorescence microscope (right panels). MDA231 cells were labeled by infection with a retroviral vector-expressing green fluorescence protein [pBABE(GFP)*puro*] and HMFs were labeled by immunostaining with anti-fibroblast antibody. A total of 3×10^5 normally growing or senescent-like fibroblasts were seeded on 60-mm culture dishes, and the cultures were maintained in serum-free medium for 3 days, after which 10^5 MDA-MB-231 cells were seeded on top of the fibroblast lawn. The cocultures were maintained in serum-free medium for 1 week before analysis. Panel B: A total of 10^6 fibroblasts and 2.5×10^5 MDA-MB-231 cells were cocultured. MDA-MB-231 cells were separated from the cocultures by magnetic cell sorting and counted with a Coulter counter. Results are means ± SE of three independent experiments. $*P < 0.05$ compared to cell number at day 1; $\dagger P < 0.05$ compared to coculture with normally growing fibroblasts. Panel C: Cell doublings per week for MDA-MB-231 cells in different cocultures relative to monotypic cultures. $*P < 0.05$ compared to monotypic culture; $\dagger P < 0.05$ compared to coculture with normally growing fibroblasts, as determined by Mann-Whitney *U* test.

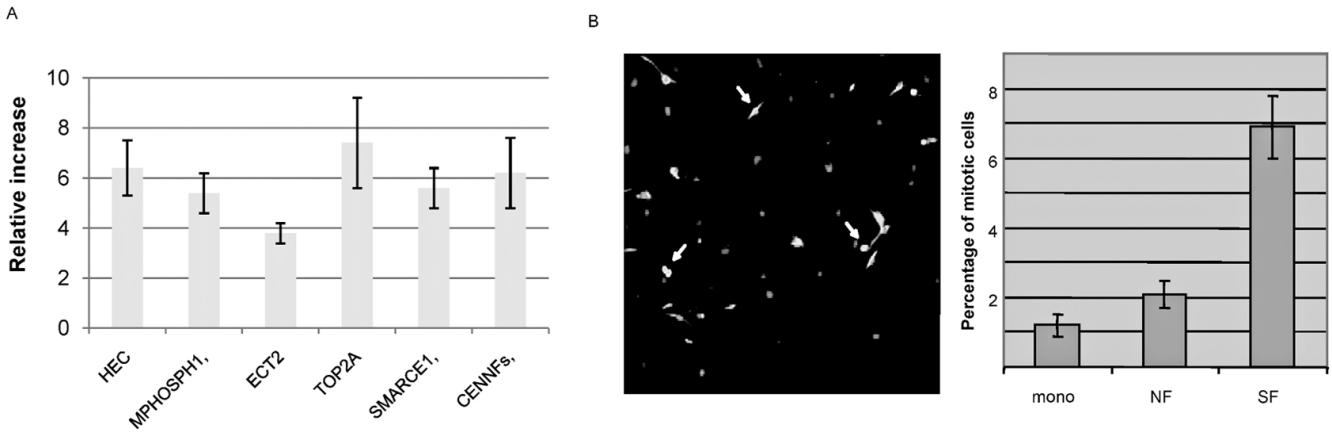

FIG. 2.

Senescent-like fibroblasts stimulate mitosis of MDA-MB-231 cells in 2D cocultures. Panel A: The relative increase represents the mRNA level of MDA-MB-231 cultured with senescent-like fibroblasts normalized to that with normally growing fibroblasts. Data are means ± SE of three independent experiments. Panel B: A representative immunofluorescence image (100×) showing mitotic MDA-MB-231 cells (phospho-histone H3-positive, arrowhead) in coculture with senescent-like fibroblasts. MDA-MB-231 cells were labeled with cytokeratin. The nuclei of senescent-like fibroblasts and MDA-MB-231 cells were counterstained with DAPI. Panel C: Percentage of mitotic MDA-MB-231 cells in homotypic culture or different cocultures. Data are means ± SE of three independent experiments.

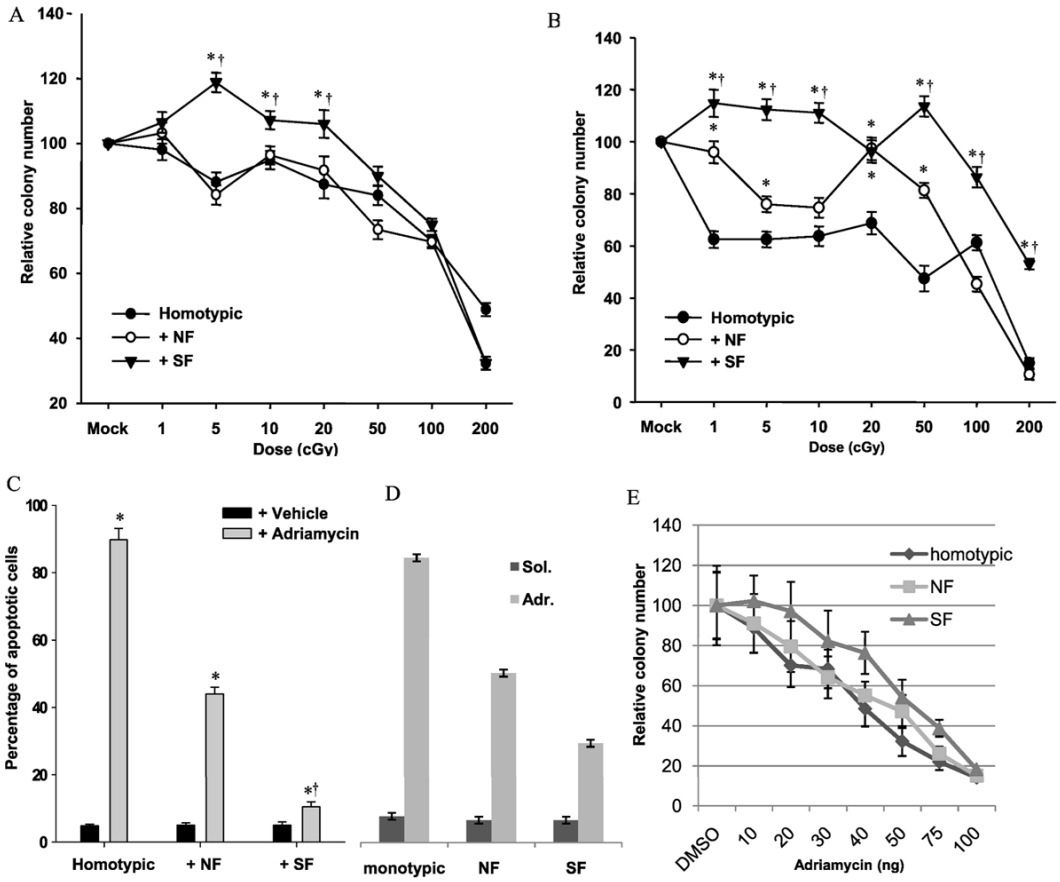

FIG. 3.

Senescent-like fibroblasts render MDA-MB-231 cells more radioresistant in 2D cocultures. MDA-MB-231 (panel A) or MCF-7 (panel B) cells were cultured alone (homotypic culture) or with either normally growing or senescent-like fibroblasts in LSGM for 24 h, irradiated, isolated from the coculture 24 h later, and assayed for colony formation. Numbers were normalized to the mock-treated sample. Values are means ± SE from triplicate experiments; *$P < 0.05$ compared to homotypic culture; †$P < 0.05$ compared to coculture with normally growing fibroblasts, as determined by Mann-Whitney *U* test. Panel C: MDA-MB-231 cells were grown in homotypic culture or coculture with normally growing or senescent-like fibroblasts for 3 days and then treated with 0.1 μg/ml adriamycin for 72 h. The culture was then fixed and stained using an Annexin V-FITC Apoptosis Detection Kit, and the percentage of MDA-MB-231 cells that were Annexin V-positive was determined. Values are means ± SE from triplicate experiments; *$P < 0.05$ compared to vehicle controls; †$P < 0.05$ compared to coculture with normally growing fibroblasts, as determined by Mann-Whitney *U* test. Panel D: MDA-MB-231 cells were cultured with conditioned medium from normally growing or senescent-like fibroblasts for 12 h before adriamycin was added. Cell viability was analyzed as described for panel C.

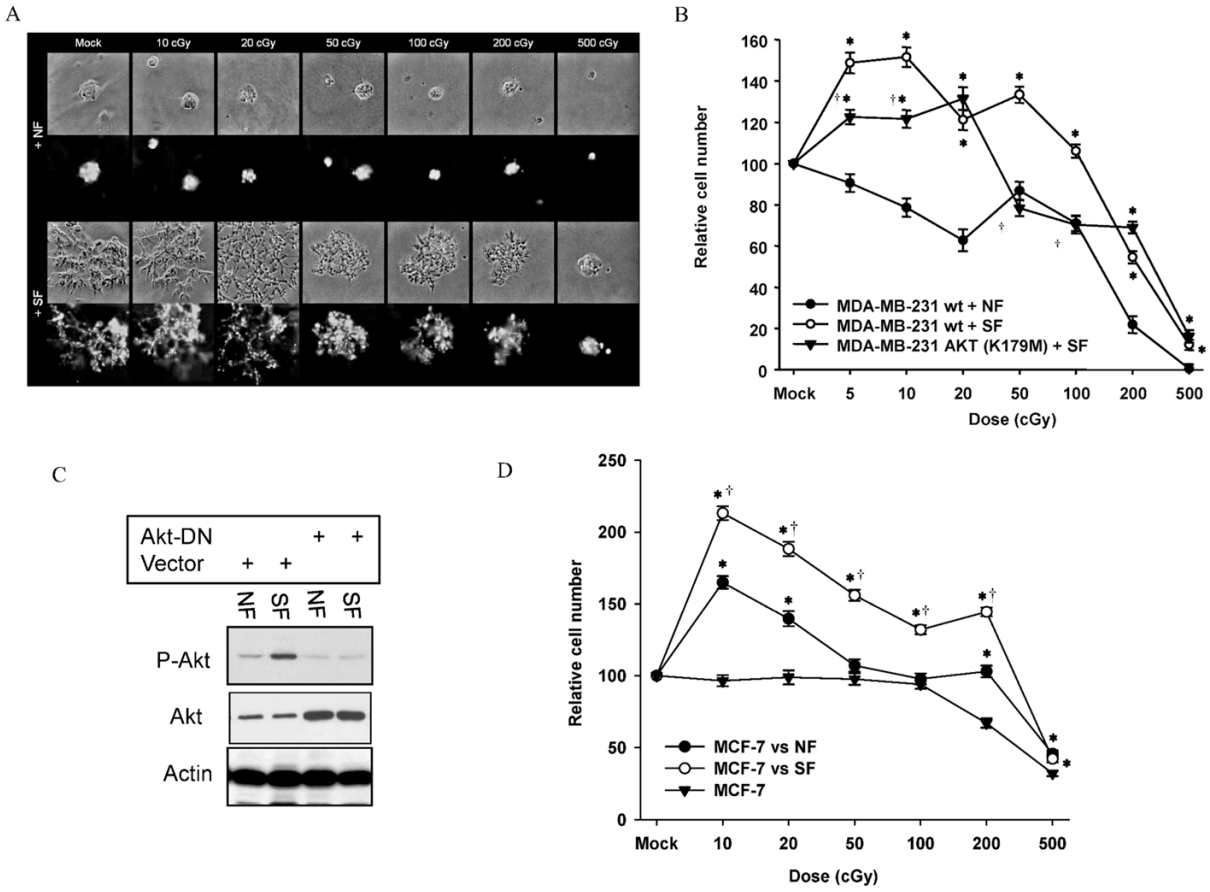

FIG. 4.

Senescent-like fibroblasts render MDA-MB-231 cells more radioresistant in 3D cocultures. Panel A: MDA-MB-231 cells were cocultured with normally growing or senescent-like fibroblasts in LSGM for 4 days, after which the coculture was irradiated. Shown are phase-contrast or immunofluorescence images of representative 3D structures at day 10 after irradiation. MDA-MB-231 cells were labeled by immunostaining with cytokeratin (FITC). Panel B: Wild-type (wt) MDA-MB-231 cells or those expressing a dominant-negative mutant of Akt (K179M) were cocultured with normally growing or senescent-like fibroblasts and irradiated. Single cells were then recovered from the ECM gels after digestion with dispase and trypsin. MDA-MB-231 cells were isolated from HMFs using MACS and counted. Counts are normalized to the mock-treated samples. Data are plotted as means ± SE of three independent experiments. *$P < 0.05$ compared to coculture with normally growing fibroblasts; †$P < 0.05$ compared to MDA-MB-231 Akt (K179M) cells, as determined by Mann-Whitney *U* test. Panel C: Another aliquot of the cells was analyzed by Western blotting using the indicated antibodies. Panel D: MCF-cells were cultured alone or with normally growing or senescent-like fibroblasts in LSGM for 4 days, after which the coculture was irradiated. Single cells were then recovered from the ECM gels after digestion with dispase and trypsin. MCF-7 cells were isolated from HMFs using MACS and counted. Cell numbers were normalized to the mock-treated samples. Data are plotted as means ± SE of three independent experiments. *$P < 0.05$ compared to homotypic MCF-7 culture; †$P < 0.05$ compared to cocultures with normally growing fibroblasts, as determined by Mann-Whitney *U* test.