Human lung fibroblasts prematurely senescent after exposure to ionizing radiation enhance the growth of malignant lung epithelial cells *in vitro* and *in vivo*

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Abstract. Cellular senescence, being the result of serial subculturing or of exogenous stresses, is considered to be a potent anticancer mechanism. However, it has been proposed that senescent cells may enhance the growth of adjacent malignant epithelial cells. On the other hand, exposure of tumors to repeated low doses of γ-irradiation is a common treatment regime. Nevertheless, γ-irradiation also affects the neighboring stromal cells and the interaction of the latter with cancer cells. Accordingly, in this study, we have exposed confluent cultures of human lung fibroblasts to repeated subcytotoxic doses of 4 Gy of γ-irradiation. We found that a single dose immediately activates a DNA damage response, leading to an intense, but reversible, cell cycle arrest. After a series of doses (total dose approximately 50 Gy) cellular senescence was accelerated, as shown by permanent growth arrest and the upregulation of specific biochemical and morphological senescence-associated markers. This process was found to be p53-dependent. Next, we studied the effect of these prematurely senescent cells on the growth of human malignant lung cell lines (A549 and H1299) and found that the presence of irradiation-mediated senescent cells strongly enhances the growth of these cancer cells *in vitro* and in immunocompromised (SCID) mice *in vivo*. This effect seems not to be related to an induction of epithelial-to-mesenchymal transdifferentiation but, to a significant extent, to the increased expression of matrix metalloproteases (MMPs), as a specific MMP inhibitor significantly restrains the growth of cancer in the presence of senescent fibroblasts. These findings indicate that lung fibroblasts that become senescent after ionizing radiation may contribute to lung cancer progression.

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Introduction

Although most studies in carcinogenesis focus on the neoplastic cell *per se*, there is an increasing interest in the role of tumor stroma in cancer development. Indeed, numerous studies suggest that a normal microenvironment can constrain tumor development, indicating that it can be dominant over the malignant phenotype, while an activated stroma can promote tumor growth (1-4). Several factors are responsible for these cell-cell or cell-matrix interactions, such as matrix metalloproteases (MMPs). Lack of MMPs can suppress tumorigenesis (5-8), whereas an environment ectopically overexpressing stromelysin-1 can produce carcinomas derived from adjacent epithelial cells (9).

Among the stromal cellular components, fibroblasts play a key role in tumor development, as they are responsible for the deposition and remodeling of extracellular matrix components, as well as for the release of cytokines and growth factors acting in a paracrine manner on cancer cells (10-13). Normal fibroblasts have a limited lifespan, i.e., they can undergo only a certain number of doublings, after which they are unable to proliferate. This process, termed replicative senescence, is the consequence of telomeric DNA shortening after each cell doubling (14-16). This shortening after a certain point can trigger a DNA damage response (DDR), activating the tumor suppressor p53 and subsequently the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1}$, leading to pRb hypophosphorylation and consequently to growth arrest. Senescent cells overexpress also another CDK inhibitor, i.e., $p16^{INK4a}$, which also inhibits pRb phosphorylation. Both pathways can alone or in synergy establish and maintain the arrest of senescent cells (16). Beyond serial subculturing, cells exposed to a number of genotoxic insults, such as oxidative stress, UV radiation, or various chemicals, can enter a state of permanent growth arrest termed 'stress-induced premature senescence' (SIPS) (16,17). SIPS can also be provoked by the expression of certain proto-oncogenes (18), indicating that senescence is a powerful anticancer mechanism. Interestingly, oncogene-induced senescence is induced by the DNA damage checkpoints in response to DNA replication stress (19). All the above clearly show that (replicative or stress-induced) cellular senescence can be the outcome of a DNA damage response.

Ionizing radiation represents a major tool in anticancer treatment. It effectively produces DNA double strand breaks and restrains the growth of tumors by inducing cell cycle arrest and/or death of cancer cells. However, radiotherapy inevitably affects stromal cells (20) and it has been shown that ionizing radiation can provoke a senescence-like state in fibroblasts (21). Accordingly, we have irradiated confluent cultures of primary lung human fibroblasts with subsequent curative doses of 4 Gy up to a cumulative dose of approximately 50 Gy and have studied the immediate and long-term effect on cell cycle arrest and the induction of senescence. Furthermore, we have investigated the effect of radiation-treated fibroblasts on the growth of human lung cancer cells *in vitro* and in immunocompromised mice *in vivo*. Our data indicate that irradiation of stromal fibroblasts may support the growth of adjacent cancer cells.

Materials and methods

Materials. Antibodies against p38, phospho-Chk2 (Thr68), phospho-p53 (Ser15), phospho-ATM (Ser1981), total ERK, phospho-p38 (Thr180/Tyr182) and PARP were purchased from Cell Signaling Technology (Hertfordshire, UK). SB431542 was from Tocris (Ellisville, MO, USA). The anti-$p21^{CIP1/WAF1}$, anti-$p16^{INK4a}$, anti-Rb antibodies and E-cadherin were from BD Pharmingen (Bedford, MA); the PanActin antibody was from Neomarkers, Lab Vision Corporation (Fremont, CA, USA). The monoclonal antibody DO1 against p53 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the anti-phospho-γH2AX (Ser139) antibody was from Upstate Biotechnology. GM6001 was from Calbiochem (Scwallbach, Germany). The anti-BrdU antibody was from Roche Diagnostics GmbH (Mannheim, Germany). KU-55933 was a generous gift from Dr Graeme Smith (KuDOS Pharmaceuticals, Cambridge, UK). NtBHA and anti-vimentin antibody, horseradish peroxidase-conjugated secondary antibodies and all other chemicals were from Sigma (St. Louis, MO, USA).

Cells and culture conditions. In this study we have used CCD19Lu human lung fibroblasts, purchased from ECACC (Salisbury, UK) and primary human lung fibroblast cultures that were established from normal lung areas of consenting patients undergoing surgery. A549 human lung adenocarcinoma cells were obtained from American Type Culture Collection (ATCC, Rockville, USA) and ATM(-/-) GM05823 cells were from Corriell Cell Repository (Camden, NJ, USA). Human non-small cell lung carcinoma (H1299) and MDAH041 fibroblasts from a Li-Fraumeni patient were generous gifts from Drs D. Meek and M. Agarwal, respectively. Cells were grown in DMEM supplemented with sodium pyruvate, sodium bicarbonate, penicillin-streptomycin, glutamine (all from Biochrom AG, Berlin, Germany) and 10% FBS (from Gibco BRL, Invitrogen, Paisley, UK). When indicated, cells grown in 100-mm culture dishes were exposed to γ-radiation in a ^{60}Co gamma source (Gamma Chamber 4000A, Isotope Group, Bhadha Atomic Research Company, Trombay, Bombay, India) at a rate of 8 Gy/min.

Immunofluorescence. Cells cultured on glass-coverslips were fixed with 4% formaldehyde in PBS for 15 min, were permeabilized with 0.2% Triton X-100 in PBS on ice, blocked with bovine serum albumin (BSA) (Sigma) 1% (w/v) for 1 h and labeled with an antibody against phospho γH2AX (Ser139) for 2 h. After a wash in PBS, the cells were probed with a FITC-conjugated IgG (Santa Cruz Biotechnology) for 1 h (22), and the cells were visualized using a BioRad MRC 1024 scaning confocal microscope.

Conditioned medium. Lung fibroblasts were grown in 100-mm culture dishes in DMEM containing 10% FBS until confluence. The cultures were washed repeatedly with PBS and were incubated in serum-free medium for 48 h. Subsequently, the conditioned medium was harvested, clarified from cell debris by centrifugation, aliquoted while on ice and stored at -80˚C until use.

DNA synthesis assay. DNA synthesis in young and senescent fibroblasts was estimated after dual labeling with 5-bromo-2'-deoxyuridine (BrdU) and 4',6-diamino-2-phenylindole (DAPI) dihydrochoride, as previously described (23). Briefly, cells were plated sparsely on glass coverslips in DMEM containing 10% FBS. For measuring the immediate effect of irradiation, BrdU (50 μM) was added 24 h after treatment and after an additional incubation of 24 h the cells were fixed with freshly prepared 4% formaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, treated with 2 N HCl, incubated with anti-BrdU FITC-conjugated mAb (1 h), and then stained with 2.5 mg/ml DAPI in PBS (10 min). Cells were washed three times with PBS at each step. DAPI- and BrdU-positive nuclei were observed on a Zeiss Axioplan 2 fluorescent microscope. For the estimation of the percentage of proliferating cells in senescent cultures, BrdU incorporation after a 48-h incubation was assessed. For measuring the effect of conditioned media on cancer cell growth, A549 or H1299 cells were plated at a density of $8x10^3$ cells/cm^2, in DMEM containing 10% FBS. After 6 h the medium was changed to DMEM containing 1% FBS for another 24 h. Then, the conditioned media to be tested were added to the sparse cultures, diluted 1:1 with fresh DMEM containing 2% FBS along with [methyl-^{3}H]-thymidine (0.2 μCi/ml, 25 Ci/mmol) and after 24 h of thymidine incorporation was measured by scintillation counting, as previously described (23).

Cell cycle analysis. Cell cycle analysis was performed by flow cytometry, as previously described (22): cells were plated at a density of 10^4 cells/cm^2 in DMEM containing 10% FBS and after a 24- and 48-h period they were trypsinized, washed with PBS, fixed in 70% (v/v) ice-cold ethanol, and stained with propidium iodide (50 μg/ml), in the presence of $MgCl_2$ (5 mM) and RNAse A (10 μg/ml) in Tris-HCl pH 7.5 (10 mM). DNA content was analyzed on a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA) using the Modfit (Verity Software House, Topsham, ME) software.

Western immunoblot analysis. Cells were washed with ice-cold tris buffered saline (TBS: 10 mM Tris-HCl pH 7.4, 150 mM NaCl) and scraped immediately in hot 2X SDS-PAGE sample buffer [125 mM Tris-HCl pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 125 mM β-mercaptoethanol, 0.02% (w/v) bromophenol blue] supplemented with protease- and phosphatase-inhibitor cocktails (Sigma). Cell lysates

were boiled for 3 min, sonicated for 15 sec, clarified by centrifugation, aliquoted and stored at -80˚C until use. The samples were separated on SDS-PAGE and the proteins were transferred to PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with 5% (w/v) non-fat milk in 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20 (TBS-T) buffer and incubated overnight at 4˚C with the appropriate primary antibodies. After washing with 5% non-fat milk, the membranes were incubated with the respective second antibody for 1 h, washed again with 5% non-fat milk and TBS-T, and the immunoreactive bands were visualized on Kodak-X-OMAT AR film by chemiluminescence (ECL kit), according to the manufacturer's (Amersham Biosciences) instructions.

SA-β-gal staining. Senescence-associated β-gal (SA-β-gal) staining in cells was performed as previously described (24): Sparse cultures (1-5x10^3 cells/cm^2) were washed in PBS, fixed for 3-5 min (at room temperature) in 3% (v/v) formaldehyde, washed again in PBS and incubated for 12-48 h at 37˚C with fresh senescence-associated β-gal (SA-β-Gal) staining solution (1 mg/ml X-Gal in a buffer containing 40 mM citric acid/sodium phosphate pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride and 2 mM magnesium chloride). Cells were visualized by using a Zeis Axioplan 2 microscope.

Co-cultures. A549 or H1299 cells were layered on top of confluent cultures of young or senescent fibroblasts (50 cells/35-mm dish). The co-cultures were further incubated for a 15-day period with medium changes every 4 days. At the end of this time period co-cultures were washed with PBS (3X), fixed with 4% formaldehyde for 10 min at room temperature, and stained with 1% (w/v) Rhodanile blue, which preferentially stains epithelial cell colonies (25).

Tumorigenesis assays. A549 cells alone or in the presence of an equal number of lung fibroblasts were injected (150 μl/injection) in the back of SCID mice. One month later, the animals were sacrificed and the tumors were removed from the animals and their weight was measured. All animal studies were conducted according to the institutional guidelines conforming to international standards and the protocols were approved by the relevant committee of the Veterinary Direction, Ministry of Rural Development and Food.

Quantitative RT-PCR. Quantitative RT-PCR was performed as previously described (26). Total RNA was isolated by the RNeasy mini kit (Qiagen). First strand cDNA was synthesized from 1 μg of total RNA, using oligo-dT12-18 and M-MLV reverse transcriptase following standard procedures indicated by the manufacturer (Invitrogen, Paisley, UK). For the real-time PCR reaction a mastermix of the following reaction components was prepared: 10 μl KAPA SYBR Fast qPCR Master Mix (2X), 0.4 μl KAPA SYBR Fast Rox low (50X) (KAPA Biosystems), 0.2 μl forward primer (100 μM), 0.2 μl reverse primer (100 μM) and 8.2 μl H_2O. Optical PCR tubes were filled with 19 μl of the above mastermix and 1 μl of cDNA (1:100) was added as PCR template. Reactions were run on an Mx 3000P QPCR Systems Cycler (Stratagene, La Jolla, USA) with the following conditions: denaturation program (95˚C for 3 min), amplification and quantification program repeated 40 times (95˚C for 5 sec, 58˚C for 20 sec, 72˚C for 10 sec), melting curve program (60-95˚C with a heating rate of 0.1˚C per second and a continuous fluorescence measurement) and finally a cooling step to 55˚C. RT-PCR data analysis was performed with MxPro QPCR Software. Highly purified salt-free primers for *MMP-1* (forward primer, 5'-CCT TCT ACC CGG AAG TTG AG-3'; reverse primer, 5'-TCC GTG TAG CAC ATT CTG TC-3'), *MMP-2* (forward primer, 5'-AAG AAC CAG ATC ACA TAC AGG ATC A-3'; reverse primer, 5'-GTA TCC ATC GCC ATG CTC C-3'), *MMP-3* (forward primer, 5'-TTT TGG CCA TCT CTT CCT TCA-3'; reverse primer, 5'-TGT GGA TGC CTC TTG GGT ATC-3') and for the reference gene *actin* (forward primer, 5'-TTG GCA ATG AGC GCT TCC-3'; reverse primer, 5'-AGC ACT GTG TTG GCG TAC-3') were generated by VBC Biotech.

Results

Repeated low doses of ionizing radiation provoke premature senescence in human lung fibroblasts. First, we tested the effect of a single dose of 4 Gy of γ-irradiation on the growth of human lung fibroblasts. As can be seen in Fig. 1A, this dose immediately provokes a DNA damage response, shown by the sequential activation of ATM-Chk2-p53-$p21^{WAF1}$ axis. In contrast, there was no significant alteration of the levels of the cdk inhibitor $p16^{INK4a}$ and of the total and phosphorylated levels of $p38^{MAPK}$. The upregulation of $p21^{WAF1}$ is followed by a dephosphorylation of pRb and a cell cycle arrest, as can be seen by the dramatic decrease of BrdU incorporation (Fig. 1A and B). Higher doses of irradiation provoke even more intense inhibition of DNA synthesis (not shown). Cell cycle analysis indicates that ionizing radiation initially arrest cells at the G2/M phase (Fig. 1C). Twenty-four hours later an additional block in the G0/G1 phase is observed, which is more intense at 48 h post-irradiation. The percentage of cells in S phase is significantly down-regulated in the time interval tested. However, and in contrast to previous reports (21,27), a single dose of 4 Gy is unable to permanently arrest human lung fibroblasts by provoking premature senescence, as after the initial arrest the cells are again able to proliferate (Fig. 1D), while no senescence-associated β-galactosidase (SA-β-gal)-positive cells were observed after this treatment.

Trying to mimic the conditions prevailing in the stroma during radiotherapy, we repeatedly irradiated confluent non-proliferating fibroblasts cultures with low doses of ionizing radiation (4 Gy) up to a cumulative dose of ~50 Gy. In preliminary experiments, by using an MTT assay, we found that repeated low doses or single doses up to 50 Gy were not cytotoxic for these cells (data not shown). In addition, no signs of cell death were observed microscopically, with DAPI staining and observations under a UV microscope or with FACS analysis (not shown), in contrast to previously reported data (28). The treated cells were collected either one day after the course of irradiations (IR cells) or they were subcultured and were collected two weeks later (IS cells), as to avoid the immediate effect of irradiation. For comparison, young proliferating human lung fibroblasts (Y cells) and fibroblasts that became senescent after serial subculturing (replicative

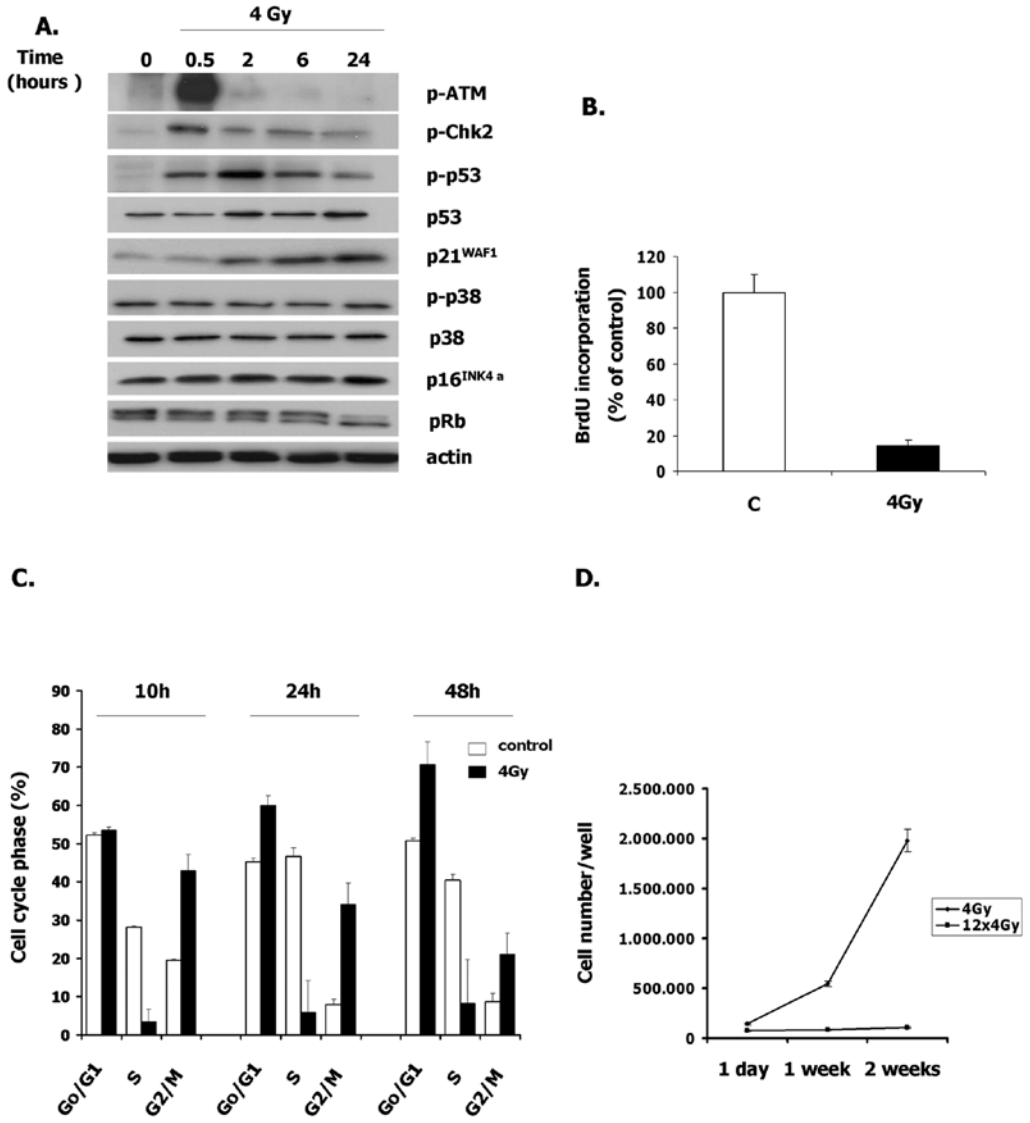

Figure 1. Effect of a single 4-Gy dose of ionizing radiation on primary human lung fibroblasts. (A) Cells were irradiated and cell lysates, collected at the indicated time-points, were subjected to Western blot analysis by using specific antibodies. (B) Subconfluent fibroblast cultures were irradiated or not and DNA synthesis was estimated after a 24-h incubation, by measuring BrdU incorporation, as described in Materials and methods. (C) Exponentially growing fibroblasts were irradiated and the cells were collected at 10, 24 and 48 h later and subjected to FACS analysis. (D) Fibroblast cultures were irradiated once with 4 Gy of ionizing radiation or with 12x4 Gy within a period of six days. Cell numbers were measured one day after the end of irradiation(s), as well as after one and two weeks. (B-D) Mean of triplicate dishes, as well as standard deviations are presented from a representative experiment out of three similar ones.

senescent - RS cells) were also used in these studies. IR cells do not express major morphological alterations compared to Y cells. In contrast, two weeks after subculture IS cells are growth arrested (Fig. 2B), are nearly all SA-β-gal-positive (Fig. 2C) and they express a phenotype similar to that of RS cells in terms of expression of cell cycle regulatory proteins, i.e., intense upregulation of phosphorylated $p38^{MAPK}$ and of the cdk inhibitors $p21^{WAF1}$ and $p16^{INK4a}$ (Fig. 2A). Of note, p53 was intensely upregulated one day after irradiations and was highly phosphorylated in Ser15, while two weeks later (in IS cells), as in RS cells, its levels have returned to baseline and p-p53(Ser15) expression is below the detection level. Interestingly, it has been reported that other DNA damaging agents, e.g., bleomycin or actinomycin D, enhance intensely p53 expression which returns to baseline levels 8 days after treatment (29).

Finally, having in mind that γ-irradiation provokes double strand breaks in DNA, we examined control and treated cells for the formation of γH2AX foci, representing sites of DNA double strand breaks. Such foci were infrequently detected in proliferating Y cells. However, 30 min after irradiation numerous tiny foci were observed in the cell nuclei (Fig. 2D), that disappeared after a few hours (not shown). In contrast, in IS and RS cells a limited number of highly enlarged foci was found (2-3 foci per nucleus) (Fig. 2D), in agreement with previous publications (27,30). In summary, it can be seen from the above that the profile of IS cells is very similar to that of RS cells, indicating that repeated low doses of γ-irradiation provoke prematurely a senescent phenotype in normal human lung stromal fibroblasts. This phenomenon is irreversible after 2 weeks (Fig. 1D) or even after 6 months (not shown).

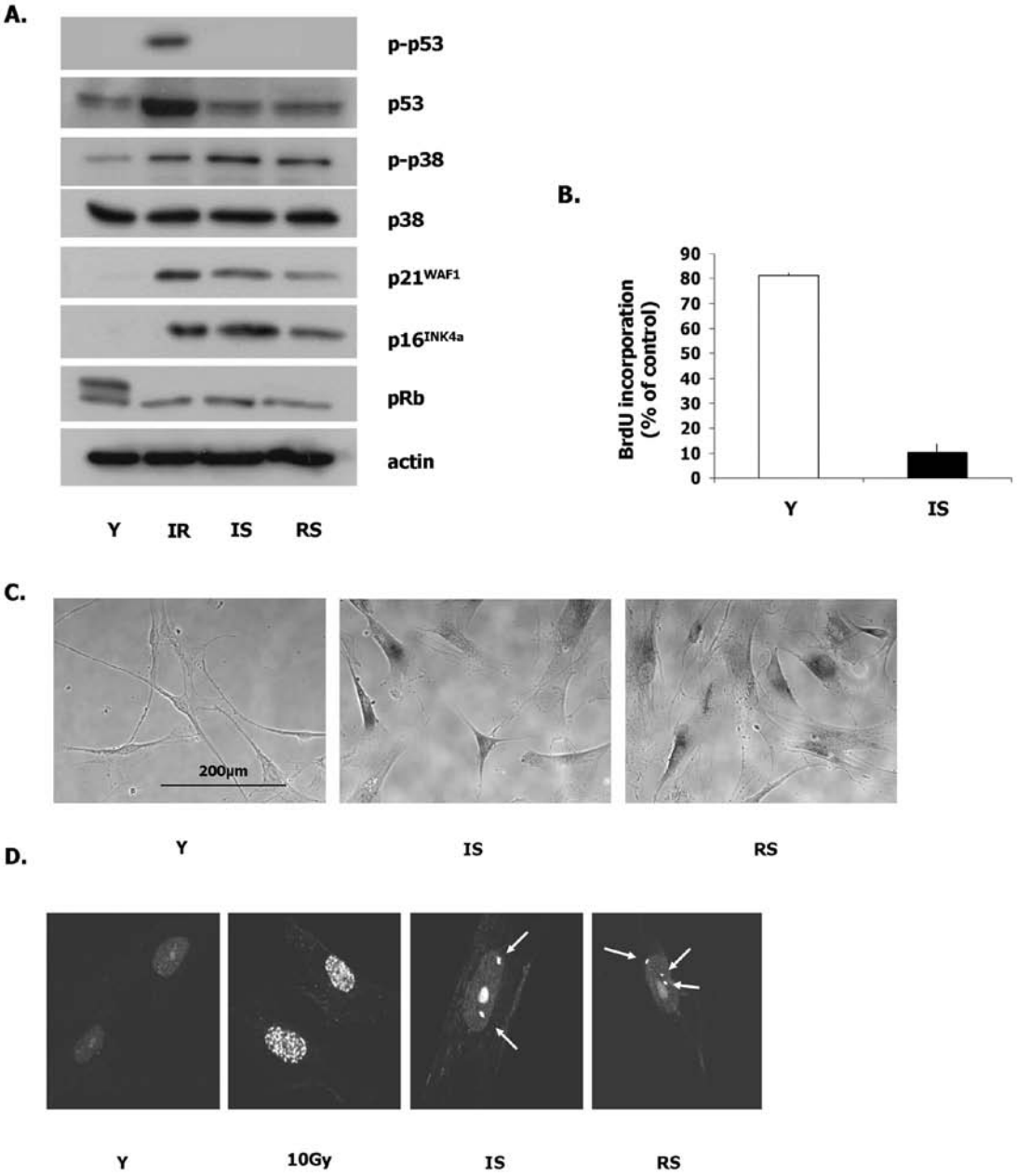

Figure 2. Characterization of fibroblast senescent by ionizing radiation. (A) Lysates were collected from early-passage cells (Y), one day after the course of 12x4 Gy irradiations (IR), two weeks after the course of irradiations (IS) and from replicative-senescent cells (RS) (all cultured in the presence of 10% FBS), and were subjected to Western blot analysis by using specific antibodies. (B) In subconfluent cultures of Y and IS cells BrdU was added and novel DNA synthesis was measured after 48 h of incubation. A representative experiment, performed in triplicates, is shown here. (C) Fibroblasts (Y, IS and RS) were stained with SA-β-gal staining and photographed under a phase contrast microscope. (D) Cells (Y, IS and RS) were fixed and stained with γH2Ax and were visualized under a confocal microscope. Y cells treated with 10 Gy of ionizing radiation and fixed 30 min later, were used as a positive control.

p53 is necessary for irradiation-induced premature senescence. Next, we tried to investigate the mechanisms responsible for the induction of premature senescence by repeated doses of ionizing radiation. It has been shown that ionizing radiation generates reactive oxygen species and activates TGF-β, two well known inducers of premature senescence (31,32). In addition, it also provokes the activation of $p38^{MAPK}$ (Fig. 2A), also involved in premature senescence (33). Accordingly, we have incubated the cells during the whole course of irradiations and the period of two weeks until cell collection with two antioxidants (NAC and NtBHA), an inhibitor of TGF-β receptor type II (SB431542), or an inhibitor of $p38^{MAPK}$ (SB203580) (34-36). However, all these inhibitors used were unable to prevent the senescent phenotype, as judged by the expression of the cdk inhibitors $p21^{WAF1}$ and $p16^{INK4a}$, by the phosphorylation status of pRb (Fig. 3A), as well as by morphological criteria and SA-β-gal staining (not shown). Following that, we studied also the involvement of ATM in the observed phenomenon. As can be seen in Fig. 3A, the presence of an ATM inhibitor (KU-55933) (37) cannot rescue normal fibroblasts from γ-irradiation-mediated premature senescence. Similar results were obtained with fibroblasts deficient in ATM (GM05823 cells) (not shown here). In addition, we found that the ATM/ATR inhibitor caffeine is also unable to inhibit the senescence

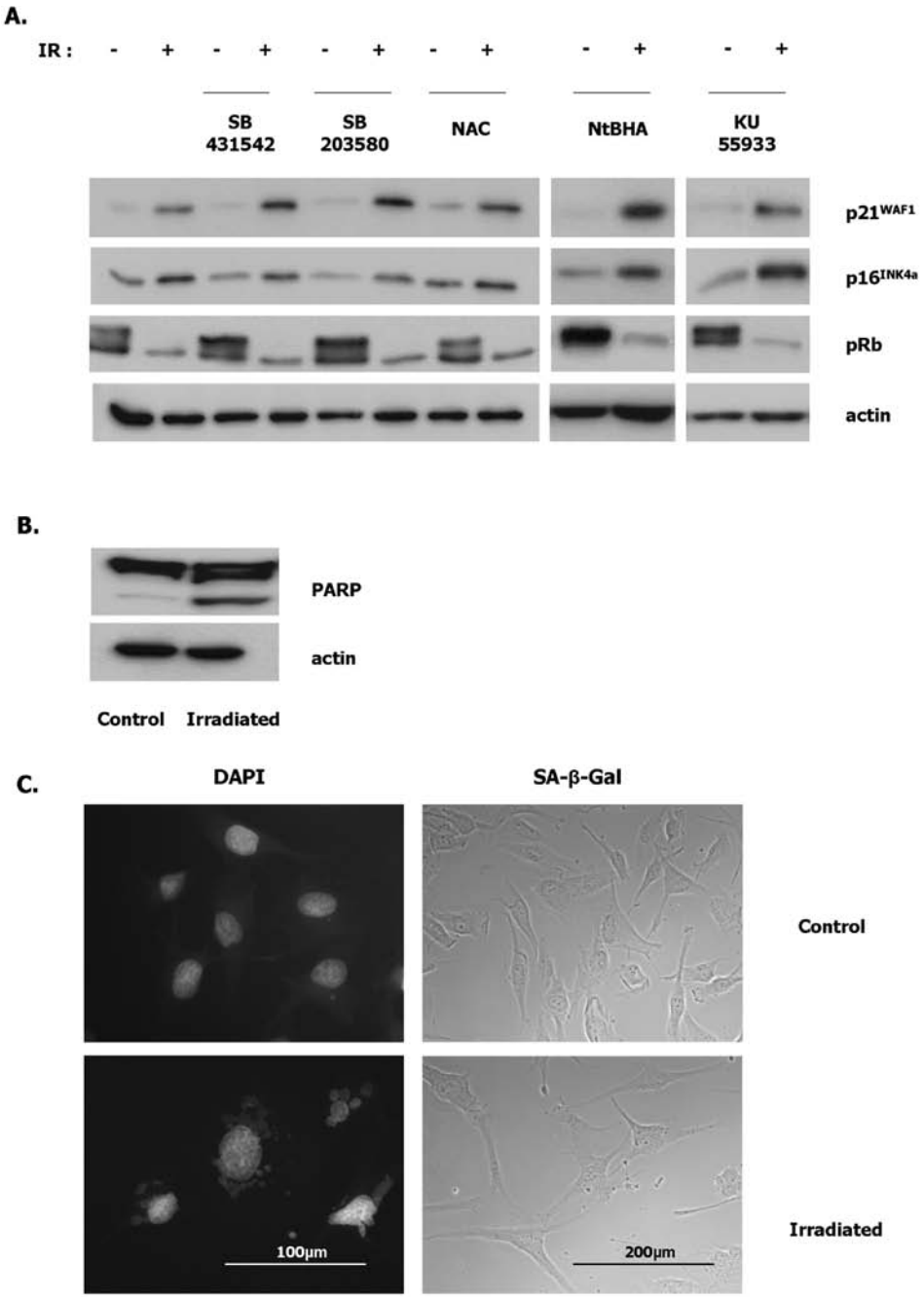

Figure 3. p53 is required for ionizing radiation-induced premature senescence. (A) Early-passage human lung fibroblasts were exposed to 4-Gy doses of ionizing radiation up to a cumulative dose of ~50 Gy and one day later they were subcultured. Cell lysates were collected after two weeks and subjected to Western blot analysis. In all this period they were treated or not with the SB431542 (10 μM), SB203580 (10 μM), NAC (10 mM), NtBHA (100 μM) or KU-55933 (10 μM). The medium in the presence or absence of the above mentioned compounds was changed every two days. (B) MDHA041 cells were exposed to 4-Gy doses of ionizing radiation as mentioned above and 7 days after the course of irradiations cell lysates were collected and subjected to Western blot analysis by using an antibody recognizing the intact and fragmented PARP. (C) MDHA041 cells were exposed to radiation as above and after 7 days were fixed and stained with DAPI or SA-β-gal and visualized under a UV- or an inverted light-microscope, respectively.

process (data not shown). Finally, we have used fibroblasts from a patient with Li-Fraumeni syndrome (MDHA041). These cells are deficient in p53, as there is a frameshift mutation of one p53 allele at codon 184 and the normal p53 allele has been lost (38). Irradiation of these cells does not lead to premature senescence, judged also by the absence of SA-β-gal staining (Fig. 3C), but rather a massive apoptosis-like cell death is observed, as can be seen by the abrupt decrease in cell number (not shown), the appearance of fragmented PARP (Fig. 3B) and of apoptotic-like nuclei (Fig. 3C). Cumulatively the above data indicate that γ-radiation-induced premature senescence is a p53-dependent process.

Senescent fibroblasts enhance the growth of tumor cells in vitro and in vivo. Previous studies on cells from several tissues revealed that stromal fibroblasts that became senescent by several treatments can support the growth of tumor cells by soluble and extracellular matrix associated factors (25,39,40).

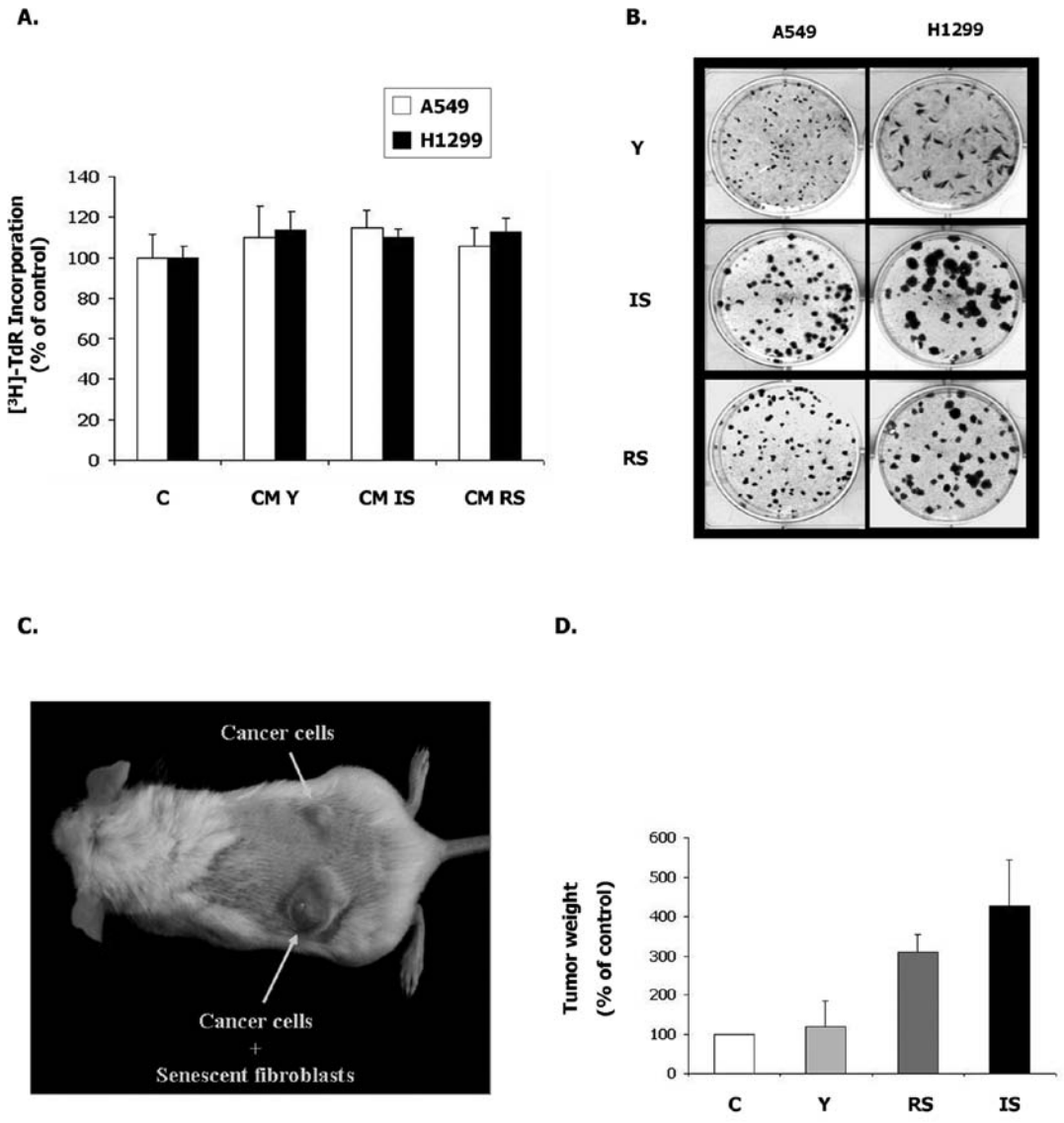

Figure 4. Irradiated fibroblast-cancer cell interactions. Conditioned media were collected from Y (CM Y), IS (CM IS) and RS (CM RS) fibroblasts after an incubation of 48 h and were added to cultures of human lung cancer cells (A549 or H1299) grown in 1% FBS, along with tritiated thymidine. DNA synthesis was measured after 24 h of incubation. Serum-free medium, incubated for the same period in the absence of cells, was used as control. A representative experiment out of three similar ones performed in triplicates, is presented here. (B) A549 or H1299 cells were plated on top of Y, IS or RS cells and after 15 days the cultures were fixed and stained with Rhodanile blue. (C) A549 ($5x10^5$) cells were injected in the dermis of SCID mice in the absence or presence of the same number of Y (n=6), IS (n=4) or RS (n=4) cells. A representative picture of an animal with tumors of A549 cells and of A549 in the presence of IS cells is presented. (D) Thirty days after injection the animals were sacrificed and tumor weight was estimated. The mean (± SD) is presented here, estimated (%) on the basis of the tumor formed by the A549 cells alone in the same animal.

Accordingly, we collected conditioned media (CM) from Y, IS and RS cells and tested their effect on the proliferation of two human lung tumor cell lines (A549 and H1299). As can be seen in Fig. 4A, these CM were unable to enhance significantly the proliferation of these tumor cell lines. In order to exclude the possible loss of biological activity of secreted factors during collection and storage of conditioned media, we also performed experiments in two-chambered dishes. Also in this case, cancer cell proliferation was unaffected by the influence of senescent cell-secreted factors (not shown). Then we tested the direct interaction of senescent stromal fibroblasts and tumor cells. The latter were sparsely plated on top of confluent fibroblast cultures and after a 15-day incubation the co-cultures were stained with Rhodanile blue, as to identify epithelial cell colonies. In Fig. 4B is clearly depicted that tumor cells on top of RS, and especially on IS, cells are able to form much larger colonies compared to those plated on Y cells. This indicates that senescent cell-derived secreted matrix components and possible matrix-associated growth factors enhance the growth of tumor cells in a paracrine manner. Interestingly, stimulation of tumor cell growth was observed also when we incubated them on sheets in a combination of Y and IS cells (80% and 20%, respectively), although to a lesser extent (not shown). Finally, we injected tumor lung cells (A549 cells) in the skin on the one side of the back of immunocompromised (SCID) mice and in the other side the same number of A549 cells with stromal lung fibroblasts. A month later the animals were sacrificed and the growth of tumors of A549 with or without normal cells were expressed as a percentage of the growth of A549 alone. Fig. 4C and D, show that Y cells do not enhance significantly the

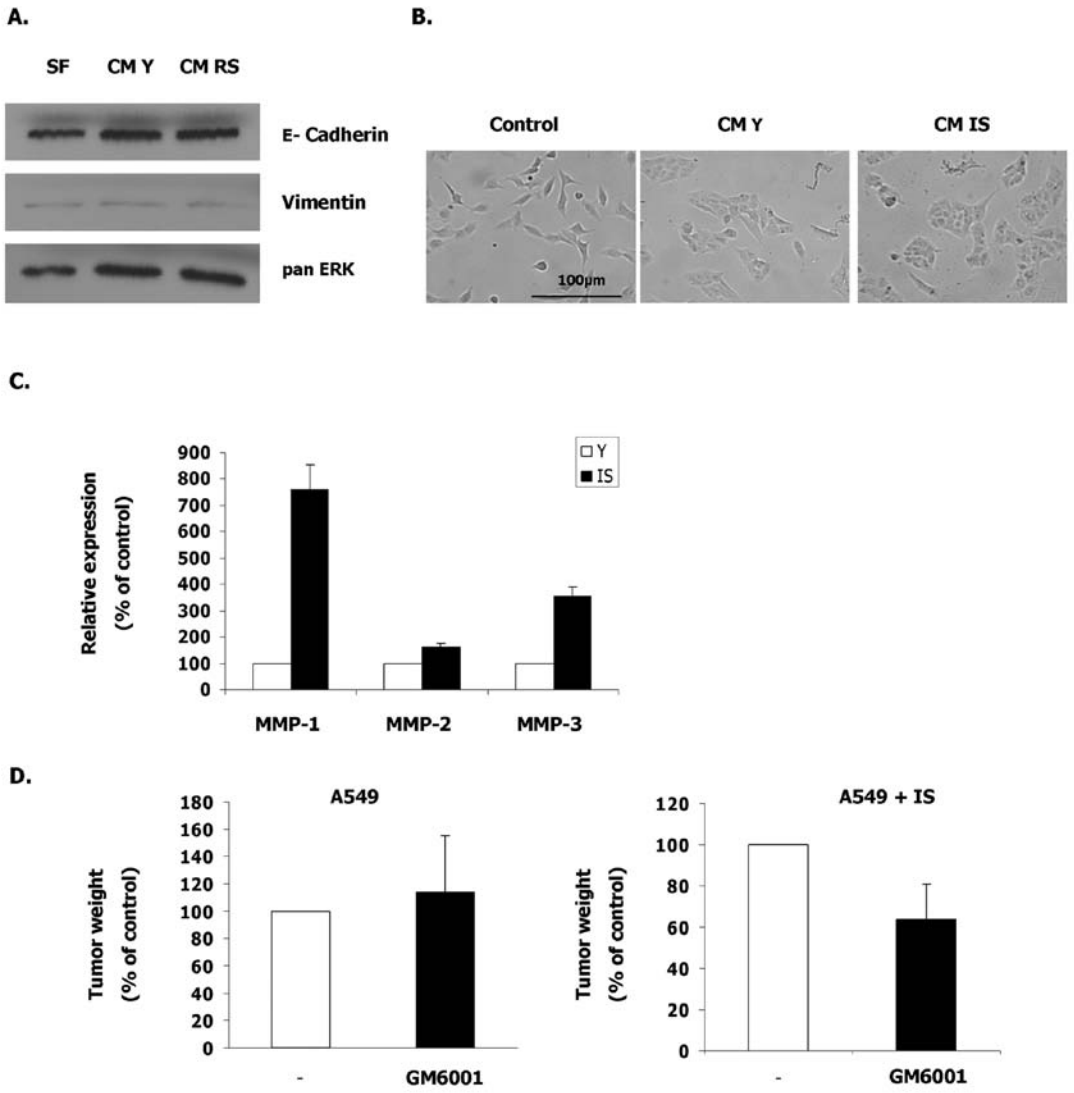

Figure 5. Biological activities of factors secreted from senescent lung fibroblasts on lung cancer cells. (A) A549 cells were incubated for three days with conditioned media from young fibroblasts (CM Y) or fibroblasts senescent after irradiation (CM IS). Serum-free medium incubated in the absence of cells has been used as control. Three days later cell lysates were collected and the expression of E-cadherin and vimentin was estimated by Western blot analysis. Pan ERK was used as a loading control. (B) The cells, treated as above, were photographed under an inverted microscope. (C) Expression of MMP-1, MMP-2 and MMP-3 in young (Y) and irradiation-mediated senescent (IS) fibroblasts by quantitative RT-PCR analysis. A representative experiment out of three similar ones performed in duplicates, is presented here. (D) SCID mice were injected with A549 cells or with A549 cells plus IS fibroblasts (10^6 cells each), in the presence or absence of the MMP inhibitor GM6001 (100 μM). Two weeks later the animals were sacrificed and tumor weight has been measured. The mean (± SD) (n=4) is presented here, estimated (%) on the basis of the tumor formed by the A549 cells or A549 plus IS cells in the same animal.

growth of tumor cells. On the other hand, RS and even more IS cells increased dramatically tumor growth, supporting the idea that senescent cells can promote cancer progression.

The role of secreted matrix metalloproteases in the support of tumor growth by senescent fibroblasts. Subsequently, we studied the mechanisms underlying the support of tumor growth by senescent stromal fibroblasts. Epithelial-to-mesenchymal transition (EMT) is an important event in cancer progression and as it has recently shown that factors secreted from senescent fibroblasts provoke EMT in breast cancer cells (41), we studied the effect of media conditioned from Y and IS lung fibroblasts on A549 concerning similar alterations. Our data indicate that conditioned media from IS cells are unable to alter the expression of the mesenchymal marker vimentin or the epithelial marker E-cadherin (Fig. 5A). In addition, microscopic observations indicate no change of A549 cells towards a mesenchymal phenotype in the presence of IS conditioned medium (Fig. 5B). Cumulatively, these data indicate that it is rather unlikely that radiation-induced senescent lung fibroblasts provoke an EMT response in the lung cancer cells examined. Next, we focused on the effect of factors overexpressed in senescent cells that has been reported to affect tumor growth of adjacent cancer cells, namely matrix metalloproteases (MMPs) (16). By quantitative real-time PCR analysis we have found that IS fibroblasts overexpress (compared to Y cells) MMP-2, -3 and especially MMP-1 (Fig. 5C). Accordingly, we injected in SCID mice A549 cells plus IS fibroblasts, in the presence or absence of GM6001, a general inhibitor that blocks these MMPs. As can be seen in Fig. 5D, in the presence of this inhibitor tumor growth was significantly diminished, while having no effect on the

growth of tumors provoked by A549 cells alone. The above indicate that IS fibroblast-secreted MMPs are in part responsible for the synergy between senescent and cancer cells in tumor growth.

Discussion

Cellular senescence represents a potent anticancer mechanism (16,19,42). However, evidence is accumulating supporting the concept that senescent cells once formed are able to promote the growth of cancer cells (43). Senescence can be the outcome of serial cell duplication or of repeated subcytotoxic doses of various exogenous stresses. Interestingly, a major motif in both types of senescence is the establishment of a DNA damage response (16,19). Ionizing radiation is a major DNA damaging agent and is largely used in radiotherapy to prevent tumor growth. On the other hand, radiation can induce carcinogenesis and numerous studies indicate that this is the combined outcome of targeted effects, i.e., mutations, and non-targeted effects, such as alterations in the stromal microenvironment (44). However, as radiotherapy can affect also the adjacent stroma, we aimed at investigating the mechanisms underlying premature senescence in human lung fibroblasts by serial low doses of γ-irradiations and the role of these cells on the growth of human lung cancer cells *in vitro* and *in vivo*.

It has been previously reported that a single 4-Gy dose of ionizing radiation can provoke premature senescence in normal human fibroblasts (21,27). In contrast, our observations indicate that under these conditions lung fibroblasts do not express a senescent phenotype and their growth arrest is temporary. The controversy with the above mentioned studies probably has to do with the culture conditions, the period of observation or the criteria used. However, when fibroblasts were treated with repeated 4-Gy doses of ionizing radiation up to a cumulative dose of 50 Gy and then subcultured a full senescent phenotype was observed. The profile of these cells is similar to that of replicative senescent cells. Interestingly, some of the hallmarks of senescence, e.g., the activation of $p38^{MAPK}$ and the upregulation of $p16^{INK4a}$ are detected only several days after irradiation, in accordance with previous reports (29,45,46), indicating that the development of a full senescent phenotype is a delayed phenomenon, probably requiring the accumulation of a series of molecular alterations. In accordance, a few, but highly enlarged, foci of γH2AX are detected in IS cells, in agreement to previous reports (27,30,47), probably representing sites of unrepairable damage, triggering a persisting DNA damage response leading to the development of the senescent phenotype.

Ionizing radiation induces the production of reactive oxygen species and of TGF-β1, and these events may be mechanistically linked (31). In addition, it has been shown that oxidative stress and stress-induced TGF-β1 overexpression can induce premature senescence that can be blocked by antioxidants or antibodies against TGF-β1 or its respective receptor (32,35,48). However, in our experiments the presence of antioxidants or an inhibitor of TGF-β receptor, during the whole period of irradiation and the time needed for the appearance of the senescent phenotype, was unable to prevent this phenomenon. As $p38^{MAPK}$ is activated in radiation-induced senescent fibroblasts, an alteration that may be crucial for the induction of premature senescence (33), we used its specific inhibitor SB203580, in an effort to prevent radiation-induced SIPS, but with no result, in agreement with previous reports on osteosarcoma cells (49). Subsequently, we evaluated the role of p53 in radiation-induced senescence by using fibroblasts from Li-Fraumeni patients lacking the expression of this tumor-suppressor, and after irradiations we have not observed the appearance of a senescent phenotype but rather a massive apoptotic-like cell death, indicating the decisive role of p53 in the induction of premature senescence. Interestingly, also in cancer cells, the presence of p53 drives cells to senescence after irradiation, while its absence can lead to apoptosis (46,50). It must be stressed here that, in contrast to the above, the presence of p53 is dispensable in ras-induced premature senescence in human fibroblasts (18), showing that different stimuli activate alternative mechanisms towards senescence. Finally, we have found that KU-55333, an inhibitor of the upstream kinase of the ATM-Chk2-p53-$p21^{WAF1}$ axis, is also unable to inhibit the radiation-induced senescence. This has been confirmed by using an ataxia telagienctasia (AT) fibroblast cell line lacking ATM (data not shown), in agreement with previous data showing that a single high dose (55 Gy) of ionizing radiation can drive AT fibroblasts to senescence (45). The above collectively indicate that ionizing radiation-mediated premature senescence of human fibroblasts is a p53-mediated process.

Tumor growth is significantly influenced by its microenvironment that can play a negative or a positive role in this process (6,7,11,43,51). It has already been shown that perturbation of breast tissue microenvironment by total-body irradiation in mice promotes the neoplastic behaviour of mammary epithelial cells (39). Especially fibroblasts are involved in this host-tumor interaction by secreting paracrine growth factors, as well as by the synthesis, deposition and remodeling of extracellular matrix components. Irradiated breast fibroblasts can affect mammary ductal morphogenesis and promote the growth and invasiveness of mammary epithelial cancer cells in three-dimensional cultures (52,53), while certain pancreatic cancer cells express accelerated invasiveness when co-cultured with irradiated stromal fibroblasts (40). In addition, it has been reported that human fibroblasts senescent by replicative exhaustion, expression of oncogenic RAS or after exposure to hydrogen peroxide can promote epithelial cell growth and tumorigenesis (25). Here, we studied the effect of human lung fibroblasts senescent after exposure to ionizing radiation on the growth of human lung cancer cells (A549 and H1299). Soluble factors secreted by senescent cells were unable to stimulate further the growth of these cancer cell lines. However, both cancer cell types formed much larger colonies when plated on sheets of senescent cells, indicating that the matrix formed by the latter support the growth of these cancer cells. However, this cannot rule out the possibility that increased quantities of paracrine growth factors and cytokines can be secreted by senescent cells (41) and be entrapped in this matrix, thus contributing to this effect. Furthermore, in the presence of senescent cells the tumors formed in SCID mice were greatly enlarged, showing that stromal lung fibroblasts senescent by irradiation can promote tumorigenesis.

Recently, it has been proposed that the sum of factors secreted by senescent cells can affect the growth and

invasiveness of two breast cancer cell lines by inducing an epithelial-to-mesenchymal transition (EMT) (41), the latter being important for cancer progression. However, it seems that this is not the case in lung cells, as we found that the lung tumor cell lines used in this study do not express morphological or biochemical markers of EMT when exposed to conditioned medium from senescent lung fibroblasts. This probably indicates that this phenomenon observed in breast cells is probably tissue- or cell line-specific. On the other hand, it is known from previous studies that matrix metalloprotease (MMP) overproduction by stromal fibroblasts can promote tumor growth (10), while many MMPs are overexpressed in replicative senescent cells (54). Here we have found that several MMPs are also overexpressed in fibroblasts senescent after exposure to ionizing radiation. In addition, a general MMP inhibitor was able to reduce significantly the growth of tumors in the presence of senescent fibroblasts, without affecting the growth of cancer cells alone. The above indicate that these proteases represent a major contributor in this senescent stroma-tumor interaction, probably by catabolising extracellular matrix components and thus releasing tumor cell growth from the restriction imposed by the surrounding stroma. Interestingly, similar results have been reported for the interaction of foreskin fibroblasts rendered senescent by bleomycin and their interaction with breast cancer cells (55). However, in the same study the authors reported that irradiation of fibroblasts with a single high dose (i.e., 44 Gy) of ionizing radiation does not lead to a significant increase in the percentage of senescent cells, to an overexpression of MMPs, or to an increase of breast cancer cell xenografts. The observed differences may again be tissue type-specific or due to the different irradiation protocols used. From all the above it seems that major differences exist in the mechanisms underlying the ionizing-radiation induced senescence in fibroblasts originating from different tissues, as well as the interaction of stress-induced senescent fibroblasts with cancer cells.

Several lines of evidence indicate that replicative or stress-induced cellular senescence represents a major anticancer mechanism. However, it is believed that senescent cells, due to their specific phenotype may affect local tissue homeostasis, to contribute to age-related pathologies and even to promote tumor growth. On the other hand, ionizing radiation is an important tool in anticancer treatment but a major concern on its use is its ability to cause mutations in epithelial cells. Here we describe that it can also provoke premature senescence in lung stromal fibroblast that can facilitate tumor growth by altering its microenvironment. The above may also have clinical significance as they suggest that the antitumor effect of radiotherapy can also be compromised by late effects on the surrounding stromal cells.

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