Radioresistant Cancer Cells Can Be Conditioned to Enter Senescence by mTOR Inhibition

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Abstract

Autophagy is frequently activated in radioresistant cancer cells where it provides a cell survival strategy. The mTOR inhibitor rapamycin activates autophagy but paradoxically it also enhances radiosensitivity. In this study, we investigated the mechanisms of these opposing actions in radiation-resistant glioma or parotid carcinoma cells. Radiation treatment transiently enhanced autophagic flux for a period of 72 hours in these cells and treatment with rapamycin or the mTOR inhibitor PP242 potentiated this effect. However, these treatments also increased heterochromatin formation, irreversible growth arrest, and premature senescence, as defined by expression of senescence-associated β-galactosidase activity. This augmentation in radiosensitivity seemed to result from a restoration in the activity of the tumor suppressor RB and a suppression of RB-mediated E2F target genes. In tumor xenografts, we showed that administering rapamycin delayed tumor regrowth after irradiation and increased senescence-associated β-galactosidase staining in the tumor. Our findings suggest that a potent and persistent activation of autophagy by mTOR inhibitors, even in cancer cells where autophagy is occurring, can trigger premature senescence as a method to restore radiosensitivity. Cancer Res; 73(14); 1–11. ©2013 AACR.

Introduction

Radiotherapy is a well-established therapeutic modality used in treatment of many cancers. However, the presence of radioresistant cells is one of the major obstacles to successful treatment with radiotherapy. Autophagy is a process in which intracellular double membranes sequester long-lived proteins and organelles for degradation and turnover (1, 2). While autophagy can lead to cell death in some circumstances, it can also serve as a survival mechanism providing metabolic support in times of cellular stress such as exposure to radiation. Thus, activation of autophagic pathways can limit the efficacy of radiotherapy, serving as a resistance mechanism.

Recent studies have suggested that inhibitors targeting the mTOR can sensitize cells to the effects of radiation (3, 4). mTOR plays a critical role in tumor development, invasion, and metastasis, acting as a signal integrator that modulates permissive signals from various pathways and a diversity of growth-promoting processes (5). The mTOR inhibitor rapamycin and its analogs have been shown to induce autophagy, which somewhat paradoxically is associated with its radiosensitizing effects (6–8). The mechanisms by which autophagy can mediate both radiation resistance and radiosensitivity when radiation and mTOR inhibitor are combined remain to be elucidated.

Cellular senescence is an irreversible state of cell-cycle arrest, which was originally described by the replicative exhaustion of human diploid fibroblasts in culture (9). Senescence can be induced prematurely in cells under cytotoxic stresses such as chemotherapeutic drugs, irradiation, and UV (10, 11), and upon oncogenic stress by overexpression of Ras oncogene (12, 13). It is speculated that premature senescence can act as a tumor suppressor mechanism that removes cells harboring DNA damage from the proliferative state (14). Consistent with its role in suppressing cancer, cellular senescence can accompany autophagy induced by cellular stress and starvation. Recent reports suggest that autophagy might serve as a switch to shift the cell fate from apoptosis to senescence (15) and provide protection as a prosenescence mechanism against the tumorigenic activity of the oncogene (16). Although functional links between these two stress response mechanisms remain elusive, senescence and autophagy are implicated in cancer and aging.
We report that combining mTOR inhibitor with radiotherapy induces cellular senescence in in vitro radioresistant cancer cells and in xenograft model. To the best of our knowledge, this study provides the first evidence that promoting cellular senescence via inhibition of the mTOR pathway may enhance efficiency of radiotherapy. Knowledge of how a long-lasting autophagic flux through inhibition of mTOR activity is integrated into the development of cellular senescence will help our understanding of the role of senescence in the response to anticancer treatments.

**Materials and Methods**

**Cell culture**

The AMC-HN-9 cells used in the study were established from undifferentiated primary head and neck cancer isolated from a patient treated at the Asan Medical Center (Seoul, Korea; ref. 17) and have been authenticated as a result of sequencing. To evaluate the response of AMC-HN-9 cells to radiation, we selected multiple cancer cells that obtained from American Type Culture Collection according to their clonal radiosensitivity (18, 19). These included the radioresistant U-87 and U251 (human glioma-derived), A549 (lung carcinoma), and HT-29 (colorectal adenocarcinoma) cell lines, and the relatively radiosensitive MDA-MB-231 (breast cancer) cell line. AMC-HN-9 and U251 cells were cultured in Minimum Essential Medium (MEM-α; Invitrogen) and RPMI-1640 (Invitrogen), respectively. U-87, A549, HT-29, and MDA-MB-231 cells were cultured in Dulbecco’s Modified Eagle Medium (Invitrogen). In all cases, media contained 10% FBS (Invitrogen) and 100 μg/mL of penicillin/streptomycin. Cells were cultured at 37°C in a humidified 5% CO2 atmosphere. Cells were grown to approximately 70% confluency and were irradiated using 6-MV photon beam generated by a linear accelerator (CLINAC 600C; Varian) at a dose rate of 2 Gy/min. All reagents were purchased from Sigma, unless otherwise specified. Cells were treated with autophagy regulators for 1 hour and then exposed to the indicated dose of radiation.

**Cellular proliferation and real-time monitoring of cell growth**

Cell viability was determined using a Cell Counting Kit-8 (Dojindo Molecular Technology, Inc.) or by measuring 5-bromo-2’-deoxyuridine (BrdUrd) incorporation (Roche Applied Science), according to the manufacturers’ instructions. Cell survival, expressed as a percentage, was calculated relative to that of control cells.

Time-dependent cell response profiling was conducted using the xCELLigence RT-CA DP System (ACEA Biosciences) as described by the manufacturer. Briefly, cells exposed to radiation were harvested after 18 hours and transferred to a 96-well E-Plate (6,000 cells/well). The cells were monitored by measuring electrical impedance every 10 minutes for 2 hours and then every hour for 72 to 96 hours. Continuous recording of impedance in cells was represented by the cell index (CI) value, which is correlated with dynamic changes in the number, morphology, and physiology of cells attached to the bottom of the plate (20, 21). Each treatment was conducted in quadruplicate to obtain an average value, and the SD was obtained using software provided with the instrument. The growth rate of treated cells was determined by measuring the slope of the maximal CI value for cells.

**Xenograft model**

To determine antitumoral activities in combination of rapamycin and irradiation, flank xenografts were established in 5- to 6-week-old male athymic nude mice (nu/nu; Harlan-Sprague-Dawley) by subcutaneous injection of 1 × 107 AMC-HN-9 cells. When tumors reached approximately 50 mm3, animals with established xenografts were stratified by tumor volume and randomized into 4 treatment groups: (i) control, vehicle; (ii) radiation only; (iii) 1 mg/kg rapamycin only intraperitoneally daily for 7 days; and (iv) rapamycin + radiation. All mice were treated for 1 week with 5 to 6 mice per group and sacrificed after approximately 6 weeks of treatment. For irradiation, unanesthetized animals were immobilized in a lead jig that shielded the head, thorax, and upper abdomen. Radiation was delivered with 4 or 8 Gy, using a 6-MV therapeutic linear accelerator (CLINAC EX; Varian) at a dose rate of 2 Gy/min; 1.0 cm bolus was used for radiation dose buildup. For rapamycin injections, stock rapamycin was prepared first in 10 mg/mL dimethyl sulfoxide (DMSO) and then diluted in sterile 0.9% saline before use. To ensure that mTOR would be fully inhibited at the time of irradiation, rapamycin was administered once a day for two days before starting the course of radiotherapy. All animal experiments were approved by and carried out according to the Laboratory of Animal Research, Asan Institute for life Sciences Institutional Animal Care and Use Committee guidelines.

**Statistical analysis**

Statistical analyses were conducted using unpaired, two-tailed Student t tests (n = 3). Differences with P values less than 0.05 were considered statistically significant and the P value of tests was provided in the figure legends. Values were expressed as means ± SDs.

**Results**

**AMC-HN-9 cells are resistant to irradiation-induced effects on survival, proliferation, and cell cycle**

AMC-HN-9 cells used in this study were derived from a highly aggressive head and neck cancer that was resistant to radiotherapy and chemotherapy, as evidenced by a lack of apoptotic cell death and an increase in DNA damage repair proteins and antioxidants after radiation. (22, 23). To verify the response of AMC-HN-9 cells to irradiation, we compared their clonogenic survival following irradiation with that of several radioresistant (A549, HT-29, U251, and U-87) and radiosensitive (MDA-MB-231) cancer cell lines. As expected, AMC-HN-9 cells were as resistant to radiation as were other known resistant cell lines (A549, U251, and U-87; ref. 19), but were much more resistant than some other cell lines (MDA-MB-231, and HT-29; Fig. 1A and Supplementary Fig. S1A).

To examine whether radiation exposure induces apoptotic changes, apoptotic cell death was evaluated by flow cytometry.
The level of Annexin V-positive apoptotic cells gradually increased with increase in radiation dose, but most cells survived 48 hours (82% ± 3.74) after exposure to high doses of radiation (16 Gy, Fig. 1B). Cell-cycle analysis revealed that 4 and 8 Gy induced a G_1-phase arrest 48 hours after irradiation and accumulation of a G_2-M phase cell population was induced as doses of radiation increased (Fig. 1C). However, irradiation (4–16 Gy) did not increase the sub-G_1 fraction corresponding to apoptotic cells with hypodiploid DNA content (data not shown).

To continuously monitor the cellular response to increasing doses of ionizing radiation (4–16 Gy), we used the impedance-based xCELLigence System, which converts cellular proliferation and cytotoxic effects to CI values. As the radiation dose increased, the growth slope of irradiated AMC-HN-9 cells increased more rapidly compared with control cells approximately 40 hours after plating, and CI values also reached earlier maximal levels in proportion to radiation dose. After reaching a peak, CI values of irradiated cells decreased in a dose-dependent manner at 72 hours (Fig. 1D). This growth profile suggests that radioresistant AMC-HN-9 cells undergo robust changes in cellular morphology and physiology, including changes in gene expression and metabolic profiles, at early periods of irradiation. Thereafter, the cells showed a dose-dependent cytotoxic effect.

Figure 1. Characteristic resistance responses in AMC-HN-9 cells exposed to radiation. AMC-HN-9 cells were exposed to graded doses of radiation. Survival curves, apoptotic cell death, cell-cycle distribution, and cellular proliferation were analyzed. A, the survival curve for AMC-HN-9 cells was compared with those from other tumor cell lines defined as resistant (U-87, U251, A549, and HT-29) or radiosensitive (MDA-MB-231). Values in curves are presented as mean values. Experiments were carried out at least 3 times. B and C, cells exposed to increasing doses of radiation were harvested at 48 hours and the cells positive for Annexin V staining and cell-cycle distribution were assessed by flow cytometric analyses. D, irradiated cells were transferred to an E-plate and cellular proliferation was monitored for 72 hours in real-time using an xCELLigence system. Cell Index (CI) value obtained from each well represents the level of growth, proliferation, and cytotoxic effects of cells to radiation. All data are representative of at least 3 experiments. E, changes in cellular morphology were examined by transmission electron microscopy in AMC-HN-9 cells irradiated for 48 hours. Arrowheads in images indicate autophagic vacuoles. Scale bar, 500 nm. F, cells exposed to radiation (4 Gy) were harvested at the indicated time points and analyzed for activation of autophagy and the p53 pathway as a function of time by immunoblotting.
Irradiation-induced autophagy represents a survival mode that protects AMC-HN-9 cells against the consequences of radiation exposure

We next examined the morphology of AMC-HN-9 cells with transmission electron microscopy. As reported in previous studies, autophagosomes were frequently found at a basal level in untreated AMC-HN-9 cells. Irradiation with 4 Gy induced a dramatic increase in the number of autolysosomes containing cytoplasmic structures, lamellar structures, or undigested material (Fig. 1E). In cells treated with a higher dose of irradiation (12 Gy), large degradation vacuoles were often present and almost all mitochondria were small and swollen. Immunolabeling for LC3 protein also revealed an increase in cytosolic LC3 as well as an increase in LC3 localized to autophagosomal membranes (Supplementary Fig. S2A).

The increase in autophagy structures correlated with dynamic changes in the autophagy regulatory proteins, ATG5, p62, and LC3 II; LC3 II is a lipidated form of LC3 I bound to the autophagosome membrane. As shown in Fig. 1F, the level of ATG5 expression began to increase from 12 hours after irradiation and maintained up to 72 hours. However, the levels of LC3 II and p62 were decreased at 2 to 24 hours after irradiation, indicating active protein turnover within the autolysosome. After transiently increasing at 48 hours, the level of LC3 II returned to that observed in control cells.

Following irradiation at 4 Gy, Ser-15–phosphorylated p53 levels increased rapidly (2 hours) and decreased slightly at 72 hours, whereas p53-induced p21 was maintained for up to 72 hours (Fig. 1F). Consistent with the observation that a sub-G1 fraction was rarely detected in irradiated cells, active caspase-3, cleaved PARP, and apoptotic morphologic signs, such as nuclear fragmentation and plasma membrane blebbing, were also absent (Supplementary Fig. S2B). Collectively, these results show that the AMC-HN-9 cells activate the autophagy process as an early protective mechanism against radiation damage, in which the cells go through rapid and dose-dependent restorative responses, such as a transient arrest of the cell cycle and a decrease of cellular proliferation.

mTOR inhibition improves the efficacy of radiation treatment by augmenting autophagic flux in AMC-HN-9 cells

Cancer cells dynamically regulate autophagic flux to protect themselves against external stress. During anticancer therapy, this response may be cytoprotective or harmful to cancer cells, depending on the cellular context or the extent of stress. We assessed the effect of further enhancement of autophagy induction on the response to subsequent irradiation exposure. A greater degree of autophagy activation was achieved by targeting mTOR with two mechanistically different inhibitors, rapamycin and PP242 (24, 25). The regulatory effects of mTOR inhibitors were assessed by microscopic examination, measurement of BrdUrd incorporation, and analysis of cell growth profiles and cell survival. Rapamycin and PP242 decreased cellular proliferation with distinguishable changes in cellular morphology. Cells appeared flattened and enlarged or lean shaped for rapamycin or PP242, respectively (Fig. 2A). When combined with irradiation, the drugs induced inhibitory effects on cellular proliferation by 63% for rapamycin and 73% for PP242, respectively, compared with nontreated control cells at maximally effective concentrations (Fig. 2B). To further assess the effect of mTOR inhibition with time, we monitored cell proliferation in real time. As shown in Fig. 2C, combined treatment with mTOR inhibitor and irradiation induced persistent growth inhibition in the all observed periods, which was evidenced by dramatically decreased CI values (rapamycin, 57%; PP242, 30%) at 72 hours. When used alone, neither mTOR inhibitor decreased CI values compared with untreated control cells. The inhibitory effect on cellular proliferation and growth caused by rapamycin or PP242 was confirmed by using clonogenic survival assays as an endpoint (Fig. 2D and Supplementary Fig. S1B).

To determine the cellular mechanism by which mTOR inhibitors increase the radiosensitivity of AMC-HN-9 cells, we investigated the effects of mTOR inhibitor treatment and irradiation on the mTOR downstream pathway. Both drugs effectively suppressed the phosphorylation of established mTOR targets (p70S6K, 4E-BP, and AKT) and induced activation of autophagy. A relatively low dose of radiation treatment (4 Gy) had no clear regulatory effect on mTOR downstream proteins (Supplementary Fig. S3A–S3C). However, when combined with mTOR inhibitor, high level of LC3 II and long-lastingly decreased p62 was observed to 72 hours after radiation indicating the maintenance of enhanced autophagic flux (Fig. 2E). Interestingly, while a single treatment of rapamycin or radiation increased the number of autophagic vacuoles for an early period (from 8 to 48 hours) and declined at 72 hours, combinational treatment maintained a slightly increased autophagic flux to 72 hours after radiation (Supplementary Fig. S4D). Taken together, these results suggest that radiation-induced autophagy occurs independently of the mTOR pathway, but inactivation of the pathway by mTOR inhibitor augments long-term autophagic flux in irradiated AMC-HN-9 cells, ultimately sensitizing the cells to irradiation. This may trigger another mechanism that enables cells to suppress the emergence of radioresistance.

The combination of mTOR inhibition and irradiation induces a cell-cycle block and an irreversible proliferation arrest

To understand how the combined mTOR inhibition and irradiation induce a decrease in cell growth and survival, and to determine whether the growth-arrested state can be reversed, we examined cell-cycle kinetics in irradiated and/or mTOR inhibitor-treated cells. As depicted in Fig. 3A (top), AMC-HN-9 cells that had been cultured with an mTOR inhibitor and/or exposed to radiation for 3 days were harvested, replated with fresh complete media without mTOR inhibitor, and then maintained for another 3 days. This strategy was designed to avoid cell-cycle arrest and the resulting growth inhibition caused by a high degree of cell confluence. Cells treated with a combination of mTOR inhibitor and radiation were in a state arrested in G1 phase (rapamycin, 58%) or G1–G2 phase (PP242, 54%/18%) at 6 days (Fig. 3A, bottom). In contrast, cells treated with mTOR inhibitor alone, which had accumulated in the G1 phase at 3 days before replating, reentered S and G2–M phases.
the cells were freed from G1 arrest and were restored to a cell-cycle pattern similar to that of control cells. The arrested state of cells was confirmed by measuring population doubling time (PDT), which was remarkably increased in irradiated cells that did not change growth with cells treated with radiation alone (2-fold; Fig. 3B).

Next, we determined whether the arrested cell population was responsive to mitogenic stimuli, such as serum or growth factors. Insulin was added to the treated cells, and cell proliferation was continuously monitored for 3 days using the xCELLigence system. As shown in Fig. 3C, insulin treatment promoted the growth of irradiated cells and cells treated with rapamycin or PP242 alone. However, it did not change growth curves of cells exposed to combined treatment of mTOR inhibitor and irradiation, indicating that the arrested cells are strictly limited in their response to a mitogenic stimulus. These results show that the combination of mTOR inhibition and irradiation induces persistent cell-cycle arrest and renders the cells unresponsive to growth stimuli.

### Downregulation of the mTOR pathway in cooperation with radiotherapy induces cellular senescence and heterochromatin formation

Microscopic examination showed that combination-treated cells exhibited a senescence-like morphology (Fig. 2A). To investigate whether mTOR inhibition is associated with the induction of senescence in irradiated cells, we measured SA-β-gal activity, which is the most widely used marker of senescence (26). Very few SA-β-gal–positive cells were found among cells treated with rapamycin (1.4 ± 0.15-fold) or PP242 (1.6 ± 0.55-fold) alone. However, as shown in Fig. 4A, pretreatment with rapamycin (3.5 ± 0.36-fold, P < 0.004) or PP242 (2.7 ± 0.45-fold, P < 0.05) increased the number of senescent cells compared with that induced by irradiation alone (2.1 ± 0.23-fold).
To identify possible changes in chromatin structure that are known to contribute to cellular senescence, we examined heterochromatin organization in cells. The number of cells positive for senescence-associated heterochromatin foci (SAHF) increased more in cells treated with an mTOR inhibitor (rapamycin or PP242) alone than in cells exposed to radiation alone. However, these differences were not statistically significant. Cells exposed to combined treatment with mTOR inhibitor and radiation showed a clear difference when compared with nontreated control cells \((P < 0.05)\) or \((P < 0.01)\); Fig. 4B. Furthermore, these morphologic changes were associated with increases in chromatin-bound HMGA2 protein and H3(K9)met, as shown in Fig. 4C (left). mTOR inhibitors increased the levels of HMGA2 and H3(K9) met expression in cells subjected to subsequent irradiation, even though the only drug treatment was insufficient to increase expression of the proteins. We next determined the subcellular localization of H3(K9)met using immunofluorescence. H3(K9)met staining was diffuse throughout the nucleus in control cells. In contrast, as senescence was induced, cells showed an increase in H3(K9)met localized to the condensed regions of DNA, exhibiting an intense yellow fluorescence on merged images (Fig. 4C, right). The changes in these phenotypes confirm that cellular senescence accompanied by gross changes in chromatin composition was induced by inhibition of the mTOR pathway and irradiation.

**mTOR inhibitor-induced senescence is associated with the RB tumor suppression pathway**

The insensitivity of senescent cells to mitogenic signals, noted above, might be due to the combined action of p53 and p16-RB tumor suppressor networks. To test this possibility, we further examined the molecular characteristics of senescence induced in AMC-HN-9 cells. As shown in Fig. 4D, cells treated with mTOR inhibitor alone exhibited no noticeable change in phosphorylated p53 or induction of the p53 target, p21. mTOR inhibitor rather induced slight decrease in the p53 pathway, implying suppression at the translational levels due to downregulation of the mTOR pathway. These results were in contrast with the results from cells exposed to radiation, which showed high levels of p53 and p21 expression during the experimental time. This indicates that the activated p53-21 is a damage response against irradiation.

As a tumor suppressor, RB is another important regulator of senescence programs, in addition to its ability to halt...
cell-cycle transitions through p21-CDK/cyclin activity (28). The senescent cells that were treated together with mTOR inhibitor and radiation for 6 days irreversibly maintained a decreased state of RB phosphorylation, whereas phosphorylation of RB was restored to control levels in the cells treated with mTOR inhibitor or radiation alone (Fig. 4D). Furthermore, the expression of RB-bound E2F-responsive genes, MCM3, cyclin A2, and DHFR, significantly decreased in senescent cells compared with nontreated control cells at 6 days of treatment (Fig. 4E). This implies that activation of RB and the stable repression of E2F target genes may be involved in maintenance of senescence in cells. However, it was difficult to detect activation of the p38 kinase pathway, although many reports have noted that p38 MAPK activation occurs independently of critical shortening of telomeres, and therefore represents stress-induced senescence (29). These results collectively indicate that mTOR inhibitor-induced senescence is associated with the RB tumor suppression pathway rather than with activation of the p53 pathway.

Figure 4. Radiation treatment combined with mTOR inhibitor causes cellular senescence depending on activation of RB pathway. A and B, AMC-HN-9 cells were irradiated (4 Gy) in the presence or absence of mTOR inhibitor [rapamycin (RAPA), 20 nmol/L; PP242, 200 nmol/L]. After 6 days, cells were assessed for SA-β-gal activity and SAHF formation. The level of SA-β-gal activity and the percentage of SAHF-positive cells are indicated. *+, significantly different versus each nonirradiated cell; **, significantly different versus control cells without mTOR inhibitor and irradiation; ###, significantly different versus cells treated with irradiation alone without mTOR inhibitor. * and ++, P < 0.05; ** and ++, P < 0.01; ###, P < 0.005. C, the levels of HMGA2 and H3(K9)met expression were measured by immunoblot analysis. β-actin was used as a loading control (left). H3(K9)met was also detected by immunofluorescence, and 4',6-diamidino-2-phenylindole (DAPI) was used to visualize DNA. H3(K9)met and DAPI staining are displayed as pseudo colors of green and red, respectively, and their colocalizations are shown in yellow (right). The percentages of positive cells are indicated in the merged image. All images show representative data from at least 3 experiments. Scale bar, 10 μm. D and E, after 6 days of treatment, AMC-HN-9 cells were harvested and analyzed for p53-p21 activation and RB phosphorylation by immunoblotting (D) and for the levels of RB-bound E2F target genes, MCM3, cyclin A2, and DHFR by real-time PCR (E). **, significantly different P values versus each nonirradiated cells; ###, significantly different P values versus control cells without mTOR inhibitor and irradiation. #, P < 0.01; **, P < 0.005; *** and ###, P < 0.0001.
mTOR inhibition increases the sensitivity to irradiation via senescence induction in AMC-HN-9 xenografts

Having established the in vitro effects of combined mTOR inhibitor and irradiation on sensitivity of radioresistant cancer cells, the biologic efficacy of combined treatment was tested in tumor regrowth delay using AMC-HN-9 flank xenografts established in nude mice. As shown in Fig. 5A, control tumors grew rapidly with a volume doubling time of approximately 7 days. High doses of radiation (8 Gy) did not affect tumor growth rate compared with 4 Gy radiation although it decreased clonogenic survival of AMC-HN-9 cells in vitro (Fig. 1A). However, rapamycin enhanced the efficacy of radiation: combination therapy of rapamycin and radiation resulted in a noticeable tumor growth delay, 18 days and 30 days, compared with irradiation of 4 Gy and 8 Gy, respectively. Mouse body weight monitoring suggested that all treatments were relatively well tolerated (Fig. 5B). Furthermore, combination of radiation (8 Gy) and rapamycin showed stronger β-gal staining in tumor sections than those of other treatment groups (Fig. 5C).

Rapamycin injection increased the levels of HMGA2 and H3 (K9)met expression in xenograft subjected to subsequent irradiation (Fig. 5D). However, we investigated that only a subset and not the majority of AMC-HN-9 cells seem to respond that way, which indicated significant cellular heterogeneity with the tumor cells. These data suggest that combination therapy with rapamycin and radiation was more effective than either radiation alone or rapamycin alone and that the antitumoral effect has relevance to senescence induced in irradiated AMC-HN-9 xenografts.

In conclusion, we report that maximum efficacy of radiotherapy could be achieved by triggering autophagy induction and premature senescence through concomitant inhibition of the mTOR pathway.

Discussion

The purpose of this study was to explore the mechanism by which therapeutic strategies targeting mTOR augment the radiosensitivity of cancer cells. Our findings revealed that...
inhibition of the mTOR pathway maintains a growth-arrested state in response to radiation by inducing a senescence phenotype through cell-cycle blockage and heterochromatin formation.

To better understand the radiosensitizing effects of mTOR inhibitor in radiosensitive AMC-HN-9 cells bearing wild-type p53, we evaluated the biologic characteristics of cells after treatment with radiation or mTOR inhibitor. Exposure to radiation rapidly activated the p53–p21 signaling pathway, resulting in arrest of cells at the G1 or G2–M phases of the cell cycle and growth inhibition in a dose-responsive manner. The cells were highly sensitive to mTOR inhibitors (rapamycin and PP242), showing the distinct downregulation signatures of mTOR activity. Furthermore, the cells possessed intact autophagy machinery that was dynamically activated by exposure to radiation or the anticancer drug, doxorubicin (Supplementary Fig. S2B) and that was effectively controlled by autophagy inhibitors or by genetic abrogation of the autophagy-related gene, ATG7 (Supplementary Fig. S4B and S4D). We postulate that transiently increased autophagic flux and the corresponding temporary growth arrest might be a survival mode that protects cells against radiation, increases stress tolerance, and delays the onset of apoptosis.

Recent evidence links autophagy to another stress response, senescence. It is shown that autophagy mediates the transition to a senescent phenotype by promoting highly efficient protein turnover and the translation of proteins necessary for senescence establishment (16, 30). Recently, Narita and colleagues (2011) showed the spatial coupling compartment of mTOR autophagy during Ras-induced senescence (31). The cellular localization could augment their respective functions and contribute to establishment of secretory phenotypes (31). On the other hands, another study reported that autophagy and senescence responses may occur independently without inexorable link, because senescence can develop when autophagy is abrogated (32). In principle, combining radiation with inhibition of the mTOR pathway might induce a distinctive autophagy type, resulting in autophagic cell death. Alternatively, a certain threshold could be attained at which cells select another salvage pathway that avoids autophagy and averts cell death. Interestingly, the combination of mTOR inhibitor and irradiation did not induce a noticeable additive or synergic effect on autophagy activation, but consolidated the autophagic flux and growth-arrested state in the in vitro model of autophagic cell survival. Taken together, AMC-HN-9 cells are likely to use autophagy as an effector mechanism to provide nutrients and energy needed for the establishment of senescence.

Several inhibitors targeting the mTOR-AKT pathway have been developed as attractive therapeutic strategies for cancer treatment (24, 33). However, how mTOR inhibitors exert an acceptable therapeutic effect, and more importantly, what potential benefits such inhibitors might provide in combination therapy with radiation, remain to be established. We observed that downregulation of the mTOR pathway inhibited G1–S progression. This indicates that the proliferative potential of cells is not only decreased, but the cells become more sensitive to the following irradiation (34, 35). Another possibility, based on evidence that both radiation and mTOR inhibitors induce the autophagy process, is that radiation increases autophagic flux as part of a cytoprotective response mediated by p53, but via the mTOR-independent pathway. Boosting the autophagic flux at the level of mTOR signaling using inhibitor may dominantly lead to a transition of irradiated cancer cells to senescent status. Herein, we showed that inhibition of the mTOR pathway could create a precondition of premature senescence as cancer therapeutic mechanism. More studies are required to determine a possible link between autophagy and senescence, and to distinguish a radiation-responsive, mTOR-independent autophagy from autophagy induced by combinational treatment with mTOR inhibitor. However, it was technically difficult to sort autophagy at the level of autophagic processes.

It is known that inhibition of mTOR by rapamycin converts senescent into quiescent cells in human fibroblasts (36), and results in antiaging effects that can increase the lifespan of mice (37). However, this effect of rapamycin is fundamentally different from that modeled in this study in an anticancer therapeutic context. In our study, mTOR inhibitors improved the efficacy of the therapeutic modality against radioresistant cancer cells by triggering a senescence-like growth arrest program.

In tumor cells, chemotherapeutic drugs or radiation accelerates cellular senescence, by which entering a state of permanent cell-cycle arrest can constitute a potent tumor-suppressive mechanism. However, it is not clear whether senescence is indefinite, transient, or transient but with the potential for proliferative recovery. Under certain conditions, senescent cells may turn into a dormant phenotype capable of causing tumor resurgence (38, 39). Moreover, it is well established that senescent cells can promote various facets of cancer progression, which create a permissive tumor microenvironment allowing nonsenescence tumor cells to thrive (40). Indeed, our results showing that not all cells with combinational modality exhibited senescence features indicate the possibility for presence of cells escaped from senescence.

Alternatively, due to the heterogeneity of the tumor cell population, even if exposed to same anticancer therapeutics, some tumor cells may not come under a relationship between autophagy and senescence established in our study. Thus, clinically relevant concept that at least a small subpopulation of tumor cells regains the ability to reenter the cell cycle or incurs unexpected responses to chemotherapeutic modalities would be considered in implicating the responses of autophagy and senescence in patients with cancer.

Cellular senescence is an irreversible proliferation arrested state, but the reasons for which have only partially been elucidated. Consistent with the role in suppressing cancer, two major tumor suppressors, p53 and RB, govern senescence program in human cells (41). Skinner and colleagues (2012) reported that p53 activity, and in some cases protein levels, increases radiation-induced senescence with radiosensitivity in head and neck squamous cell carcinoma (42). We observed that combination of mTOR inhibitor and radiation caused ATM and CHK2 activation in AMC-HN-9 cells (data not shown), which could trigger entry into ATM/p53-mediated senescence.
senescence by imposing a cell-cycle block via activation of p53 and the cyclin-dependent kinase inhibitor p21 (11, 43). Thus, we regarded that the constant activation of p53 and p21 in senescent cells can contribute to the maintenance of prematurity senescence by inhibiting the mTOR pathway or locking cell-cycle arrest (44, 45).

Besides the p53 pathway, RB has been suggested as another senescence regulator. The active nonphosphorylated form of RB is an initiator of SAHF formation that are believed to contribute to stable repression of genes such as cyclin A and stabilization of senescence-associated cell-cycle arrest (46–48). Furthermore, RB can induce autophagy by repressing E2F1 activity (49), suggesting that RB/E2F1 pathway has a dual role in cancer suppression and resistance to cancer therapy. Given the critical role that RB plays during cellular senescence and in the response to cancer therapeutics such as CDK inhibitors (50), there might be a link between RB-mediated autophagy and senescence and in consequential tumor suppression. We suggest a model in which RB activation triggered by radiation and mTOR inhibitor induces autophagy and reinforces a growth-arrested state via heterochromatin formation and permanent cell-cycle arrest (Supplementary Fig. S5).

To address concerns about the cell line used, we determined whether combining mTOR inhibitor with radiation would induce premature senescence in radiosensitive U-87 cells that undergo autophagy-dependent survival after radiation. Similar to results observed for AMC-HN-9 cells, mTOR inhibitors induced the formation of heterochromatin foci and increased the expression of HMG2A and H3K9(met). The following irradiation increased the senescent phenotypes, such as SA-β-gal activity, heterochromatin formation, and decreased cell growth. In addition, it prevented inactivation of hypophosphorylated RB and decreased the level of cyclin A expression (Supplementary Fig. S6A–S6C).

In summary, this study indicates that radiation treatment combined with mTOR inhibitor induces an irreversible growth-arrested state via p53 and RB-E2F pathways with a persistent autophagic flux. Our results provide a source for integrative understanding the senescence program on radiosensitivity of cancer and for new insight into mTOR targeting as an adjuvant method of radiotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

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