Effect of rapamycin, an mTOR inhibitor, on radiation sensitivity of lung cancer cells having different *p53* gene status

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Abstract. Activation to a large extent of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and mutations in the *p53* gene are involved in lung cancer therapeutic resistance. The mammalian target of rapamycin (mTOR) acts as a downstream effector for Akt. Activation of the Akt/mTOR signal is a contributing factor to decreased radiation sensitivity. The purpose of this study was to examine whether the effect of rapamycin on radiation sensitivity is affected by cellular *p53* gene status. Cellular radiation sensitivity was evaluated by using two human non-small cell lung cancer (NSCLC) cell lines with the same genetic background except for their *p53* gene status (H1299/wt*p53* and H1299/m*p53*). The cells were treated with rapamycin and/or radiation. Cell viability, cell proliferation, apoptosis, cell cycle and Akt/mTOR signaling activity were explored. Rapamycin synergistically enhanced the cytotoxicity of radiation, promoting the induction of apoptosis. Moreover, the combined treatment augmented the cytostatic effects of radiation regardless of cellular *p53* gene status. Rapamycin in combination with radiation increased G_1 arrest and suppressed progression to S phase in both cell lines. Furthermore, the combined treatment conduced to a prominent *p53*-independent down-regulation of the mTOR signal and pro-survival molecule, cyclin D1. Rapamycin can enhance the effect of radiation through the repression of pro-survival signals and the reduction in the apoptotic threshold. Taken together, inhibition of the mTOR signal may be a promising strategy for radiosensitization with no relevance to *p53* gene status from the aspects of cell lethality and cell growth depression.

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1). High mortality rates seen in lung cancer reflects its invasive nature and its resistance to current treatment modalities. Most patients with lung cancer are diagnosed at advanced stages and are not cured with a 5-year relative survival rate of 15% (1). Systemic chemotherapy and radiotherapy are the two mainstays of treatment for inoperable advanced disease. Although radiotherapy is applied for local disease control, the beneficial effect of current radiotherapy seems to be restricted to initial responders alone. Most patients will relapse due to the development of radiation resistance and lack of an adequate salvage therapy. This dismal prognosis has prompted a search for new therapeutic strategies.

Radiation resistance in tumors is associated with the activation of cell survival pathways, aberrations in tumor suppressor genes, and DNA damage repair mechanisms (2,3). The PI3K/Akt pathway represents a major cell survival pathway and plays a critical role in oncogenesis and tumor cell growth (4). Recent studies have reported that Akt activation contributes to resistance to radiation, chemotherapy and tyrosine kinase inhibitors by promoting survival signals which protect cancer cells from undergoing apoptosis (5-8). It has been shown that inhibition of PI3k/Akt through pharmacologic or genetic means induces antiproliferative effects on certain NSCLC cell lines *in vitro* and *in vivo* (9,10). Similarly, it has been demonstrated that inhibition of Akt activity, by pharmacologic or genetic approaches, greatly improves the cellular response to radiation in NSCLC cells (11). Although inhibition of the PI3K/Akt signal may be a promising strategy to enhance the effect of radiation, there are concerns about the possible side-effects of inhibiting these upstream proteins.

The mammalian target of rapamycin, mTOR, is a 289-kDa serine-threonine kinase which acts as a downstream effector for Akt (12). It regulates key processes such as cell growth and proliferation, cell cycle progression and protein translation through two distinct pathways: one involving the ribosomal p70S6 kinase (p70S6K), and one involving eukaryotic translation initiation factor 4E (eIF4E) binding proteins

(4E-BPs) (13). A recent study has reported that frequent Akt activation and mTOR phosphorylation occurs in NSCLC, and Akt activation is closely associated with the up-regulation of mTOR activity (14). It has been suggested that dysregulation of mTOR contributes to lung cancer progression (15,16). Alterations in protein synthesis, aberrant cell cycle signaling and inhibition of apoptosis mediated by mTOR may play a critical role (13). Therefore, mTOR may be a potential therapeutic target to inhibit or block the PI3K/Akt pathway.

Several mTOR inhibitors are currently under development: rapamycin and its derivatives CCI-770, AP23573 and RAD001. Antiproliferative effects of mTOR inhibitors have been shown to occur in various tumor cells *in vitro* and *in vivo* (17-19). These mTOR inhibitors are generally regarded as cytostatic agents, because they induce G_1 cell cycle arrest, but not apoptosis (17). Previous studies have reported that cellular sensitivity to mTOR inhibitors has been linked to mutations of *PTEN* or to Akt activation (20-23). It has been suggested that high levels of Akt activity result in hypersensitivity to mTOR inhibition in several tumors (19,24). Recent studies have shown that activation of the Akt/mTOR signal is a contributing factor to decreased radiation sensitivity, and that mTOR inhibitors were able to sensitize some tumor cell lines to radiation (25,26).

The *p53* tumor suppressor gene is activated by DNA-damaging agents such as ionizing radiation (27). This gene regulates proliferation and survival in cells with damaged DNA by modulating the transcription of *p53*-target genes, which can lead to G_1 arrest or apoptosis. Mutations in the *p53* gene are the most common genetic change found in human lung cancer (in ~70% of small cell lung cancer and in 50% of NSCLC, respectively) (28) and these mutations are frequently associated with drug resistance and radiation resistance (2). As previously reported, H1299 (NSCLC) cells with a mutated *p53* gene (m*p53* gene) were more resistant to radiation than wild-type cells (29,30). Recent reports have demonstrated that mTOR inhibitors can enhance the cytotoxic effects of chemotherapeutic agents and radiation in many human cancers (24-26). There are several reports which discuss the dependence of chemosensitization by mTOR inhibitors on cellular *p53* gene status. However, whether mTOR-mediated radiosensitization depends on *p53* status is still uncertain. In examining the relation of mTOR-mediated chemosensitization to *p53* status, some conflicting results in various tumor cells have been reported. Rapamycin was found to sensitize lung cancer cells with a wild-type *p53* gene (wt*p53*) to cisplatin-induced apoptosis by inhibiting p21 translation (17). In contrast, RAD001 enhanced the sensitivity of ovarian cancer cells to cisplatin in a *p53*-independent manner (24). Moreover, rapamycin chemosensitization of tumor cells with a mutant *p53* was reported in ovarian and breast cancer cells (31,32). In particular, a recent study has shown that RAD001 was able to enhance the sensitivity of hepatocellular carcinoma (HCC) cells to cisplatin in both, a *p53*-dependent and *p53*-independent manner (33). It seems possible that understanding the mechanism through which rapamycin functions could lead to improved therapeutic effects in the treatment of radioresistant tumors. Although the effects of *p53* status on chemosensitization by mTOR inhibitors may be tumor type-specific, it seems likely that such factors as a genetically heterogeneous background may have affected previous work in this field.

The aim of this study was to learn if the effect of rapamycin on radiation sensitivity is affected by cellular *p53* gene status in two human NSCLC cell lines with the same genetic background except for their *p53* gene status. These cell lines exhibit different radiation sensitivities which presumably derive from their differing *p53* status (29). In addition, molecular mechanisms underlying radiosensitization by mTOR inhibition were also to be investigated.

Materials and methods

Cell lines and cell culture. Human H1299 cells, a non-small cell lung cancer cell line with a deleted *p53* gene (provided by Dr Moshe Oren, Weizmann Institute of Science, Rehovot, Israel) were stably transfected with either a wt*p53* gene or an m*p53* gene [in which codon 248 is altered to code for Trp (TGG) rather than Arg (CGG)]. The cell lines with a wt*p53* or an m*p53* gene are designated H1299/wt*p53* or H1299/m*p53* cells, respectively (34). These resulting H1299/wt*p53* and H1299/m*p53* cell lines were kindly provided by Dr Hideki Matsumoto (Fukui University). H1299/m*p53* cells have lost *p53* functions such as the induction of apoptosis and *p53*-regulated gene products after X-irradiation. All cells were cultured in Dulbecco's modified Eagle's medium (MP Biomedicals Inc., Illkirch, France) containing 10% (v/v) fetal bovine serum (MP Biomedicals Inc.), 20 μmol/ml 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (Nacalai Tesque, Kyoto, Japan), 50 U/ml penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), 50 μg/ml streptomycin (Meiji Seika Kaisha Ltd.), and 50 μg/ml kanamycin (Nacalai Tesque) (DMEM-10). The doubling time of these cell lines was ~24 h. Exponentially growing cells grown to a density of ~80% of confluency were used for each experiment, and were cultured at 37˚C in a conventional humidified CO_2 incubator.

Drug preparation. Rapamycin was purchased from Calbiochem (San Diego, CA). Rapamycin was dissolved in DMSO at a concentration of 1 mM and stored at -20˚C. A stock solution was diluted to the appropriate concentration in serum containing culture medium just before addition to cell cultures. The drug was used at a final concentration of 0.01% of the vehicle.

Treatment with rapamycin and/or radiation. Exponentially growing cells were seeded in 60-mm dishes or in 25-cm^2 flasks containing DMEM-10 and cultured overnight. The day after plating, cell cultures were prepared for individual experiments as follows: DMSO alone as a control, a single treatment (rapamycin or radiation alone), or a combined treatment with rapamycin and radiation. Cells used for each treatment were pretreated with either DMSO (0.01%) or rapamycin (100 nM) for 1 h at 37˚C in a CO_2 incubator. Cells were irradiated with 0-9 Gy as indicated, at a dose rate of 1.2 Gy/min with a 150-kVp X-ray generator (Model MBR-1520R; Hitachi, Tokyo, Japan). Following X-irradiation, culture medium was replaced with fresh medium and the cells were returned to a 37˚C incubator for further growth.

Colony formation assay. Exponentially growing cells were treated at 12 h after plating in 60-mm dishes. The surviving

cell fraction was determined using colony formation assays. Three replicate dishes were used per experiment, and two or more independent experiments were performed for each survival point. Eight days after X-irradiation, colonies were fixed with methanol and stained with a 2% Giemsa solution. Microscopic colonies containing >50 cells were counted as having arisen from single surviving cells. Points indicate mean values. Error bars indicate standard deviations.

Analysis of apoptosis. After a 100-nM rapamycin exposure and/or 6 Gy of radiation exposure, attached and floating cells were collected with trypsin, fixed with 1% glutaraldehyde (Nacalai Tesque) in phosphate-buffered saline (PBS) at 4˚C, washed with PBS, stained with 0.2 mM Hoechst 33342 (Nacalai Tesque), and then observed under a fluorescence microscope. Induction of apoptosis was scored by the detection of nuclear and cytoplasmic condensation and the formation and release of apoptic bodies. A minimum of 300 cells was counted in every sample, and the percentage of apoptotic cells was determined. Values are the means of two independent experiments. Error bars indicate standard deviations.

Analysis of cell proliferation. Using colony formation assays as described above, the size of all of the colonies was also measured for analysis of cell proliferation. Ten days after X-irradiation, colonies were fixed with methanol and stained with a 2% Giemsa solution. Photographs of the colonies were taken under a microscope (Olympus BX51, Olympus Optical, Tokyo, Japan). The diameter of each colony was measured from scanning profiles using a Windows computer with the Scion image program (Scion Co., Frederick, MD). The area of each colony (*Ax*) in mm^2 was calculated from the diameter of the colony. On the basis of the mean value of the colony area, *Am*, for each cell line control treated with DMSO only, colonies were sorted into three groups as follows; $Ax \leq 1/2xAm$, where *Ax* was defined as a small-sized colony; $1/2xAm < Ax \leq Am$, where *Ax* was defined as a medium-sized colony; $Am < Ax$ was defined as a large-sized colony. The number of colonies in each colony group classification was determined and calculated as a percentage of the entire colony population. Values are the means of at least two independent experiments. Error bars indicate standard deviations.

Cell cycle analysis. Cell cycle distributions were assayed by determining DNA content. After treatment with 100 nM rapamycin and/or 9 Gy of radiation, cells were fixed with cold 70% methanol and stored at 4˚C for 24 h before analysis. For cell cycle analysis, the cells were incubated for 30 min at room temperature with 1 mg/ml RNase and 50 μg/ml propidium iodide (PI). Before flow cytometric analysis, samples were filtered through a 35-μm nylon mesh. Samples were analyzed using a flow cytometer (Becton-Dickinson, San Jose, CA). Three independent experiments were performed for each data point. Values are the means of three independent experiments. Error bars indicate standard deviations.

Western blot analysis. Cells were treated with rapamycin alone (100 nM, 1 h), radiation alone (6 Gy), or a combination in individual experiments. After X-irradiation, the medium was replaced with fresh medium. At indicated time points, cells were harvested and then washed with ice-cold PBS twice. Cells were suspended in RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.05% SDS). Whole cell protein lysates were then subjected to freezing and thawing three times. The protein levels in the whole cell protein lysate supernatants obtained after centrifugation (15,000 rpm x 10 min) were quantified with a Bio-Rad protein assay kit (Bio-Rad Labs, Richmond, CA). Aliquots containing 20 μg of protein were electrophoresed through 7, 10, or 15% polyacrylamide gels containing 0.1% SDS. The separated proteins were transferred electrophoretically onto Poly Screen PVDF membranes (Dupont/Biotechnology Systems, NEN Research Products, Boston, MA). Membranes were blocked with 1% (w/v) non-fat dry milk in PBS-T for 1 h at room temperature. The membranes were then incubated overnight at 4˚C with primary antibodies. Rabbit polyclonal anti-phospho-mTOR (Ser^{2448}), anti-phospho-Akt (Ser^{473}) antibodies, and rabbit monoclonal anti-phospho-S6 ($Ser^{240/244}$) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal anti-cyclin D1 (Ab-3), and anti-$p21^{WAF1}$ (Ab-1) antibodies were acquired from Oncogene Sciences, Inc. (Uniondale, NY) and Calbiochem (San Diego, CA), respectively. Goat polyclonal anti-actin antibody (I-19) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Appropriate horseradish peroxidase-conjugated secondary antibodies [anti-mouse IgG antibody (Zymed Laboratories Inc., San Francisco, CA), anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Inc., Piscataway, NJ)] were incubated for 1 h at room temperature. For visualization of the bands, an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) was used according to the manufacturer's protocol. The amounts of the proteins in the samples were quantified by scanning profiles using the Scion imaging program.

Statistical analysis. Significance levels were calculated using the Student's t-test. Values of $P<0.05$ were considered statistically significant.

Results

Effect of rapamycin on radiation sensitivity. To clarify the effect of rapamycin on radiation sensitivity in H1299 cells with a wt*p53* or m*p53* gene, cell survival was estimated with colony formation assays. The inhibitory effect of rapamycin alone on cell survival was not striking at concentrations ranging from 0.01 to 1,000 nM in either cell line (data not shown). Using a non-toxic concentration of the drug, cells were treated with or without 100 nM rapamycin for 1 h and/or 0-9 Gy of radiation. Previously, it was shown that 1 h provides sufficient time for mTOR inhibition to be induced without significant cytotoxicity (35). In agreement with a previous report (36), H1299/m*p53* cells were more resistant to radiation than H1299/wt*p53* cells (Fig. 1). In the presence of 100 nM rapamycin, decreased cell survival levels were observed in a dose-dependent manner in both cell lines. At a dose of 6 Gy, there was a statistically significant difference in the inhibition of cell survival between the combined

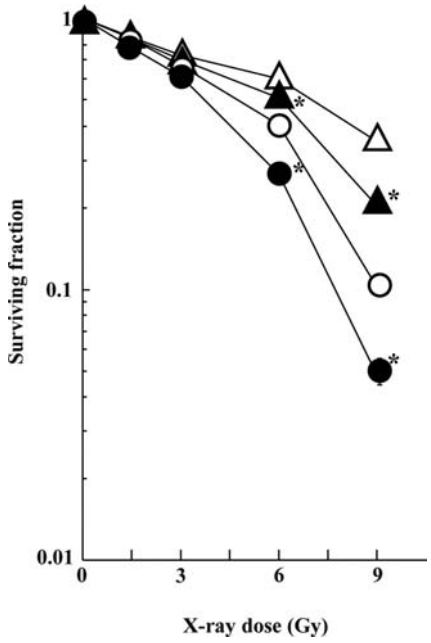

Figure 1. Effect of rapamycin on radiation sensitivity. Cells were treated with or without 100 nM rapamycin for 1 h and/or X-rays. After X-irradiation, the cells were cultured with fresh medium for eight days to assay cell survival with colony formation assays. Circles, H1299/wt*p53* cells; triangles, H1299/m*p53* cells. Open symbols, radiation alone; closed symbols, rapamycin plus radiation. Points, mean values; bars, SD. *P<0.01 versus radiation alone at the same dose.

treatment and radiation alone. These results showed that the cytotoxic effect of radiation is synergistically enhanced by rapamycin, independently of cellular *p53* gene status.

Effect of rapamycin on radiation-induced apoptosis. To examine whether the cytotoxic effect of rapamycin depends on the induction of apoptosis, H1299 cells were analyzed at 48 h after X-irradiation with or without 100 nM rapamycin, using Hoechst 33342 staining. As shown in Fig. 2, a single treatment with rapamycin did not alter the proportion of apoptotic cells when compared to controls in either H1299-derived cell line. Radiation-induced apoptosis to a significant extent, and the proportion of apoptotic cells in H1299/wt*p53* cells was almost three times as high as in H1299/m*p53* cells (8.0% in H1299/wt*p53* cells and 2.6% in H1299/m*p53* cells). Moreover, in the presence of rapamycin, the incidence of radiation-induced apoptosis was increased 1.6-fold in the wild-type cells and 2.2-fold in the mutated cells. These results indicated that the combined treatment promotes radiation-induced apoptosis regardless of *p53* gene status.

Effect of rapamycin on cell proliferation. To investigate the effect of rapamycin on cell proliferation in H1299 cells containing differing *p53* gene status, colony formation assays were used. Cells were treated with or without 100 nM rapamycin for 1 h and/or 6 Gy of radiation. The area of each resulting cell colony was measured and colonies were classified into three groups; small, medium and large-sized colonies. As shown in Fig. 3, the number of each group of colonies was calculated as a percentage of the entire population of colonies. For H1299/wt*p53* cells, each single treatment increased the proportion of small colonies (55.5% for rapamycin alone and 80.7% for radiation alone, versus 20.3% for controls). For H1299/m*p53*, the cytostatic effect of each single treatment was similar (57.6% for rapamycin alone and 80.1% for radiation alone, versus 29.0% for controls, respectively). Moreover, the combined treatment produced a greater cytostatic effect than either single treatment did in both of the cell lines (H1299/wt*p53*, 97.4% and H1299/m*p53*, 91.7%, respectively). These results showed that the combined treatment results in an augmented cytostatic effect of radiation in a *p53*-independent manner.

Effect of rapamycin combined with radiation on the cell cycle. To explore the effect of radiosensitization by rapamycin

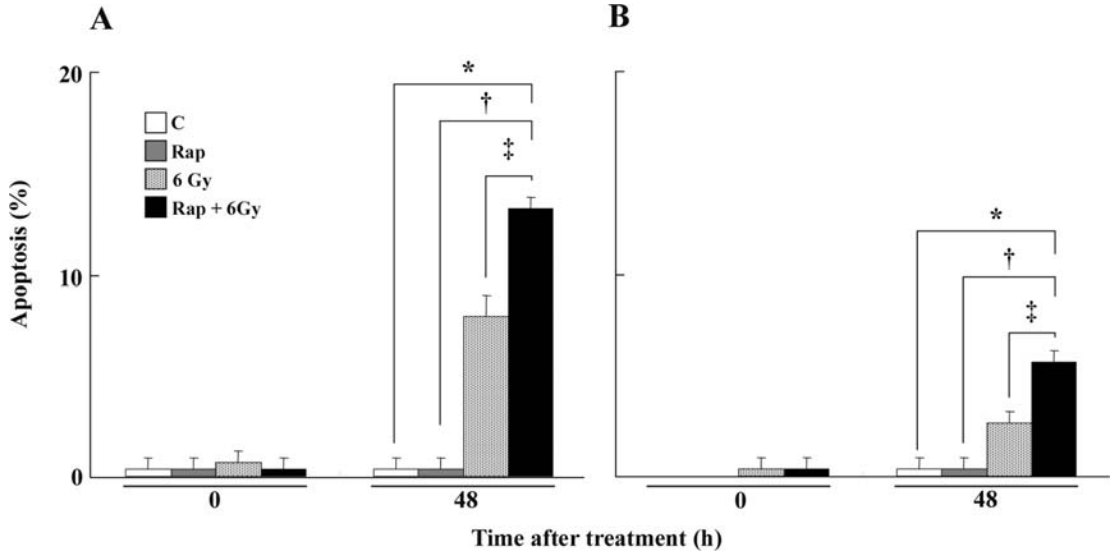

Figure 2. Effect of rapamycin on radiation-induced apoptosis; (A) H1299/wt*p53*, (B) H1299/m*p53*. Cells were treated with or without 100 nM rapamycin (Rap) for 1 h and/or 6 Gy of radiation. At 48 h after X-irradiation, cells were analyzed with Hoechst 33342 staining. Open columns, controls; shaded columns, rapamycin alone; dotted columns, radiation alone; solid columns, combined treatment. Columns, mean values; bars, SD. *P<0.01 versus control; †P<0.01 versus rapamycin alone; ‡P<0.01 versus radiation alone.

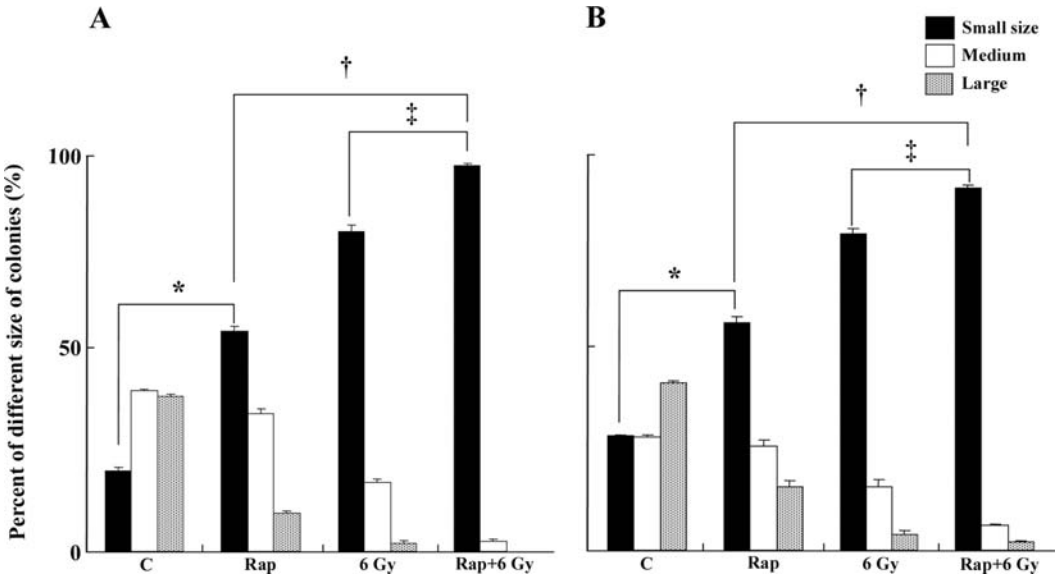

Figure 3. Effect of rapamycin on cell proliferation; (A) H1299/wt*p53*, (B) H1299/m*p53*. An increase in the number of small-sized colonies after exposure to rapamycin and/or radiation is illustrated. Cells were treated with or without 100 nM rapamycin (Rap) for 1 h and/or 6 Gy of radiation. The cells were cultured with fresh medium for ten days to assay cell proliferation with colony formation assays. The diameter of each colony was measured using the Scion image program, and the images were used to estimate the area of each colony. Solid columns indicate small-sized colonies; open columns, medium sized colonies; dotted columns, large-sized colonies. Columns, mean values; bars, SD. *P<0.01 versus control; †P<0.01 versus rapamycin alone; ‡P<0.01 versus radiation alone.

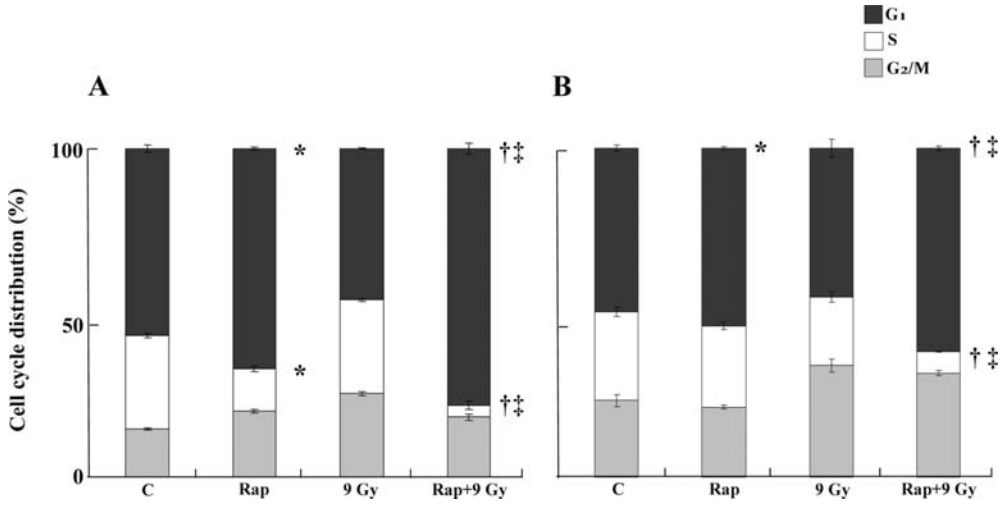

Figure 4. Effect of rapamycin combined with radiation on the cell cycle; (A) H1299/wt*p53*, (B) H1299/m*p53*. Changes in the cell cycle distribution 24 h after exposure to rapamycin (Rap) and/or 9 Gy of radiation. Cells were treated with or without 100 nM rapamycin for 1 h and/or radiation. The proportion of cells in each phase of the cell cycle was analyzed by flow cytometry. Solid columns, G_1 phase cells; open columns, S phase cells; shaded columns, G_2/M phase cells. Columns indicate mean values; bars indicate the SD. *P<0.01 versus control; †P<0.01 versus rapamycin alone; ‡P< 0.01 versus radiation alone.

on the cell cycle in H1299 cells, the proportion of cells in each phase of the cell cycle was analyzed with flow cytometry using propidium iodide staining. In addition, the question of whether the cell cycle response to the combined treatment was altered by *p53* gene status was examined. At 12 h after a 100-nM rapamycin exposure, there was a slight increase in G_1 phase cells in both cell lines, whereas following 9 Gy of radiation there was a transient arrest in G_2/M phase even in the presence of 100 nM rapamycin (data not shown). At 24 h, rapamycin showed larger effects on the cell cycle distribution, whereas there was no significant difference in the cell cycle distribution in cells treated with radiation alone or in controls in both H1299 cell lines (Fig. 4). A significant accumulation of cells in G_1 phase was induced by rapamycin (67% in H1299/wt*p53* cells versus 46.1% for radiation alone, and 56.9% for control cells, 54.1% in H1299/m*p53* cells versus 45.3% for radiation alone, and 49.7% in controls, respectively). Furthermore, a marked decrease in S phase cells was observed in H1299/wt*p53* cells after exposure to rapamycin (13.0% versus 28.4% for radiation alone, and 28.4%

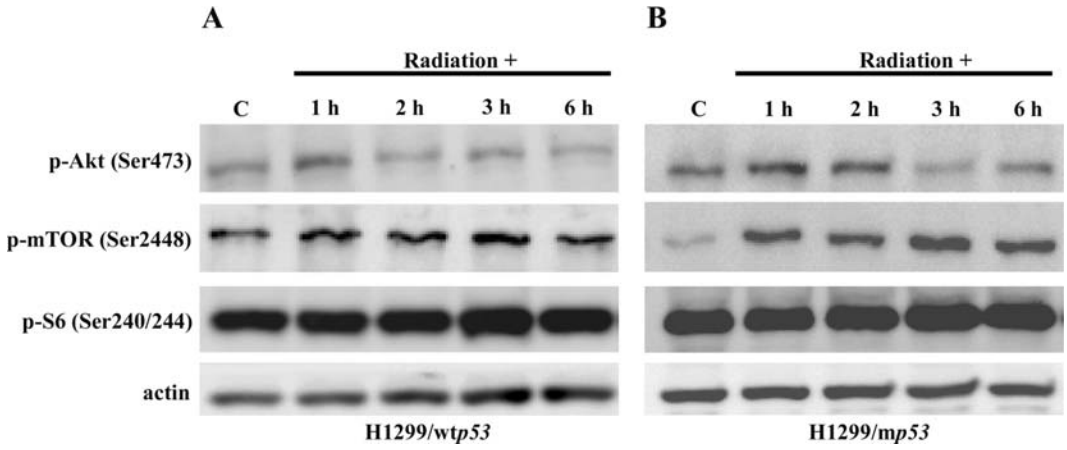

Figure 5. Radiation-induced Akt/mTOR signal; (A) H1299/wt*p53*, (B) H1299/m*p53*. Cells were exposed to 6 Gy of radiation in medium containing 10% serum. At the indicated time points, cells were harvested and whole cell protein lysates were extracted. The levels of phospho-Akt (Ser^{473}), p-mTOR (Ser^{2448}), p-S6 ($Ser^{240/244}$) were estimated with Western blotting. Equal protein loading was confirmed by blotting for actin (42 kDa) protein.

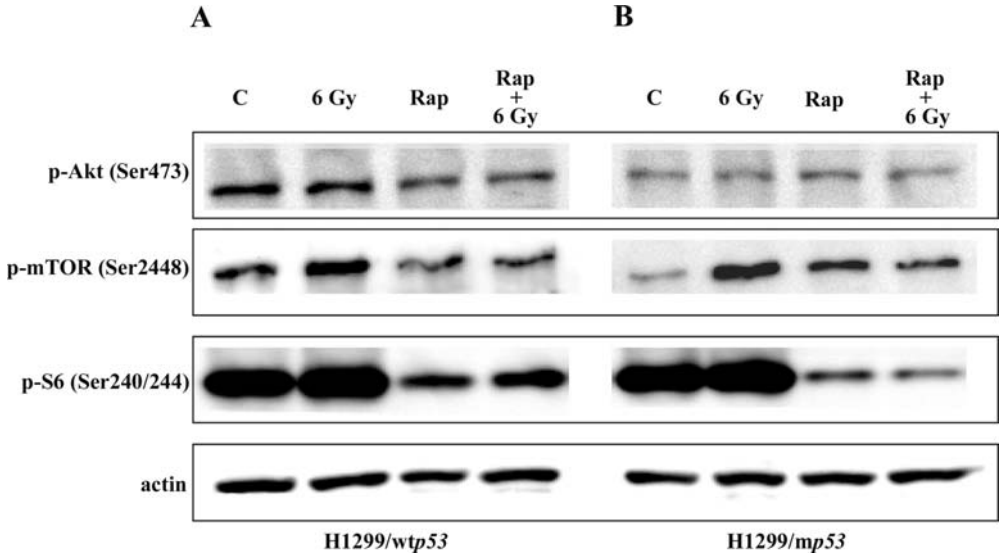

Figure 6. Inhibition of the mTOR signal by rapamycin; (A) H1299/wt*p53*, (B) H1299/m*p53*. Cells were treated with or without 100 nM rapamycin (Rap) for 1 h and/or 6 Gy of radiation. Cells were grown in medium containing 10% serum. At 3 h after X-irradiation, cells were harvested and whole cell protein lysates were extracted. Expression of phospho-Akt (Ser^{473}), p-mTOR (Ser^{2448}), p-S6 ($Ser^{240/244}$), relative to actin (loading control) was determined with Western blotting.

for controls, respectively). In particular, regardless of *p53* gene status, the combined treatment showed a greater increase in G_1 arrest (78.1% in H1299/wt*p53* cells, and 61.9% in H1299/m*p53* cells), and a reduction of S phase cells (3.6% in H1299/wt*p53* cells, and 6.5% in H1299/m*p53* cells versus 24.7% for rapamycin alone, 20.8% for radiation alone and 27.0% for controls, respectively).

Radiation-induced Akt/mTOR signal. To investigate dynamic molecular changes in the Akt/mTOR signal in response to radiation, the activation states of Akt and mTOR were first examined in the two H1299 cell lines. Secondly, to evaluate whether radiation activates the Akt/mTOR signal in H1299 cells, Western blotting was used to examine the time course of alterations in the expression levels of phospho-Akt (Ser^{473}), p-mTOR (Ser^{2448}) and p-S6 ($Ser^{240/244}$) ribosomal protein, which is a downstream effector of mTOR action. Cells were irradiated with 6 Gy of radiation in medium containing 10% serum. In both H1299 cell lines, Akt and mTOR were both phosphorylated, indicating the constitutive activation of the Akt/mTOR signal pathway (Fig. 5). After X-irradiation, the phosphorylation level in both of these proteins was increased in both H1299 cell lines. The p-Akt (Ser^{473}) levels increased soon after X-irradiation, and this increase was sustained for up to 1 h in H1299/wt*p53* cells and 2 h in H1299/m*p53* cells. In both H1299 cell lines, the increase of p-mTOR (Ser^{2448}) was evident at 1 h after X-irradiation. The elevated level of p-mTOR (Ser^{2448}) in the *p53*-mutated cells was higher than in the wild-type *p53* cells. The levels of p-mTOR (Ser^{2448}) remained elevated up to 6 h after X-irradiation in both cell lines. A delayed increase in p-S6 ($Ser^{240/244}$) levels was observed at 3 h after X-irradiation in both cell lines. The level of p-S6

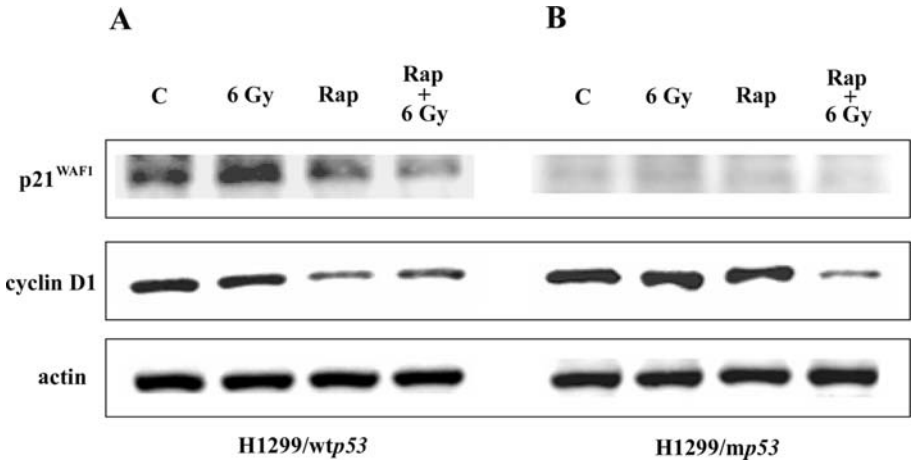

Figure 7. Rapamycin-mediated repression of pro-survival signals; (A) H1299/wt*p53*, (B) H1299/m*p53*. Cells were treated with or without 100 nM rapamycin (Rap) for 1 h and/or 6 Gy of radiation. At 6 h after X-irradiation, cells were harvested and whole cell protein lysates were extracted. The expression of cyclin D1 and p21^{WAF1}, relative to actin (loading control) were assessed with Western blotting.

(Ser$^{240/244}$) in both H1299 cell lines still remained high up to 12 h after X-irradiation (data not shown). The level of the Akt/mTOR signal in H1299/m*p53* cells was higher than in H1299/wt*p53* cells. From this finding, it would appear that H1299 cells with elevated Akt/mTOR activity are good candidates for mTOR inhibition combined with radiation.

Inhibition of the mTOR signal by rapamycin. To investigate the molecular mechanism underlying the effect of mTOR inhibition on the Akt/mTOR pathway, cells were treated with or without 100 nM rapamycin for 1 h and/or 6 Gy of radiation. The changes in the phosphorylation status of Akt (Ser^{473}), mTOR (Ser^{2448}) and S6 (Ser$^{240/244}$) were estimated. At 3 h after exposure to rapamycin, significant decrease in p-S6 (Ser$^{240/244}$) was observed in H1299/wt*p53* and in H1299/m*p53* cells (Fig. 6), and this decrease persisted for 12 h (data not shown). In particular, the level of this protein was much lower than its basal level, and the levels of p-Akt (Ser*473*) and p-mTOR (Ser*2448*) were attenuated in the wild-type cells. Furthermore, despite activation of the Akt/mTOR signal induced by radiation, the combined treatment led to a dramatic suppression of p-mTOR (Ser^{2448}) and p-S6 (Ser$^{240/244}$) *p53* independently. As seen after a single treatment with rapamycin, the level of p-S6 (Ser$^{240/244}$) was much lower than its basal level in both types of cells. The combined treatment also resulted in a small decrease in p-Akt (Ser^{473}) in H1299/wt*p53* cells, whereas there was no difference in p-Akt (Ser^{473}) levels in H1299/m*p53* cells. In addition, the expression of the pro-survival molecules cyclin D1 and p21^{WAF1} were analyzed. At 6 h after a single treatment with rapamycin, repression of cyclin D1 was detected only in the wild-type cells (Fig. 7). At the same time point, regardless of *p53* gene status, significant down-regulation of cyclin D1 was detected after the combined treatment. Induction of p21^{WAF1} was apparent following X-irradiation in H1299/wt*p53* cells and rapamycin repressed p21^{WAF1} expression through blockade of mTOR. However, no significant induction of p21^{WAF1} was detected in H1299/m*p53* cells after any treatment, confirming an impaired p53/p21^{WAF1} signal pathway.

Discussion

It was observed that the Akt/mTOR signal is constitutively activated in H1299 cells harboring either a wt*p53* or m*p53* gene (Fig. 5). It was also seen that dynamic molecular changes in the Akt/mTOR signal in response to X-irradiation occurred in H1299 cells, regardless of *p53* gene status. Radiation activated Akt, stimulating downstream signal transduction molecules such as mTOR and S6. However, rapamycin at a non-toxic concentration (100 nM) caused a pronounced *p53*-independent down-regulation of p-S6 (Ser$^{240/244}$) and cyclin D1 after the combined treatment (Figs. 6 and 7). The sensitivity of cancer cells to radiation-induced cytotoxicity depends on the balance between pro-survival and pro-apoptotic signals. Therefore, the effective induction of apoptosis by a specific blockade of pro-survival signals can be a significant event leading to radiosensitization. It was found that rapamycin sensitizes both types of H1299 cells (containing either wt*p53* or m*p53*) to radiation by promoting apoptosis, whereas a single treatment of rapamycin did not induce apoptosis in either H1299 cell line (Fig. 2). In comparison to H1299/m*p53* cells, a 3-fold higher incidence of apoptosis was seen in H1299/wt*p53* cells after the combined treatment. This result suggests that rapamycin can enhance radiation-induced apoptosis through a *p53*-dependent pathway. Although in the presence of rapamycin, there was still a small increase in radiation-induced apoptosis in m*p53* cells, this result indicates that the combined treatment may promote radiation-induced apoptosis in a *p53*-independent manner by down-regulation of pro-survival signals. It has been shown that Akt deactivation acts as a causal mediator of cell death in several types of cancer cells in response to diverse stimuli including DNA-damaging agents and UV-B irradiation (37). In particular, Akt activation is closely associated with the up-regulation of mTOR in NSCLC (14). Therefore, in this study, mTOR inhibition by rapamycin combined with radiation might reduce the level of the Akt/mTOR pro-survival signal below a threshold level which maintains cell survival. If so, an apoptotic signal may be promoted in both cell lines. The

p53-independent enhanced cytotoxicity of radiation produced by rapamycin was confirmed by colony formation assays (Fig. 1). The cytotoxic effect of radiation was synergistically enhanced by rapamycin at a non-toxic concentration regardless of cellular *p53* gene status.

A single treatment of rapamycin suppressed cell proliferation in both cell lines (Fig. 3). In particular, observations of cell cycle distributions with flow cytometric analysis provide additional evidence of the cytostatic effect of rapamycin on H1299 cells. At 24 h after exposure, a single treatment with rapamycin (100 nM) mediated a slight increase in the proportion of G_1 phase in cells containing wt*p53* (10%) and it led to a remarkable reduction in S phase cells (15%) (Fig. 4). In H1299/m*p53* cells, a small increase in G_1 arrest (5%) was induced by rapamycin alone, along with a less pronounced decrease in S phase cells. In agreement with previous reports (38,39), these findings indicate that a G_1 cell cycle arrest or a suppression of progression to S phase may contribute to the cytostatic effect of rapamycin observed in H1299 cells. These results were associated with the down-regulation of cyclin D1 in wt*p53* cells (Fig. 7). However, the proportion of S phase cells in cells containing m*p53* was unaffected by rapamycin which was consistent with no change in cyclin D1 expression. In agreement with a previous study using rhabdomyosarcoma cells with an m*p53* gene (40), these results suggest that rapamycin does not stop the cell cycle, but may retard progression of the cell cycle in H1299/m*p53* cells. Cell cycle regulation in tumor cells is important in order to account for the radiosensitizing effect of this drug. At 12 h after radiation with or without rapamycin, a remarkable cell cycle arrest at G_2/M was induced when compared with control cells (data not shown). After 24 h, no G_2/M delay was seen in cells treated with radiation alone, showing no significant difference in cell cycle distributions when compared with controls (Fig. 4). However, at the same time point, rapamycin showed promising effects on the cell cycle distribution when compared with control cells and radiation alone. Regardless of *p53* gene status, the combination of radiation and rapamycin caused a significant increase in G_1 arrest and a remarkable inhibition of progression to S phase. This result corresponded with the observed decrease in cell proliferation seen with the colony formation assay, and the repression of the mTOR signal and cyclin D1 expression seen with Western blotting (Fig. 7). The activation of mTOR can increase the proliferation rate through the increased translation of cell cycle effectors such as cyclin D1 (41). In thyroid cancer cells, RAD001 treatment reduced cyclin D1 and cyclin D3 protein levels, confirming that mTOR activation contributes to cell proliferation (42). Therefore, these results demonstrated that rapamycin might enhance the cytostatic effect of radiation due to cell cycle arrest. However, it has been demonstrated that growth-arrested cells can be relatively radioresistant when compared to actively cycling cells (43). In glioma spheroid cells, it has been shown that despite the induction of G_1 arrest and the inhibition of tumor cell proliferation, rapamycin does not diminish the efficacy of radiation. Another study has shown that an increase in the frequency of G_2/M cell cycle arrest was seen in breast cancer cells treated with rapamycin and radiation, suggesting that cell cycle arrest may have been a contributing factor in the increased radiosensitization (26). In the data shown here, a time-dependent effect on the cell cycle was seen in both cell lines after a combined treatment although there could conceivably be an alteration in the effect of these treatments in asynchronously growing cell populations. However, the actual response to a treatment may depend on the cellular context, including the cell cycle, even in asynchronous cell populations. Thus, the mechanism underlying the radiosensitization by rapamycin described in this study may be reasonable.

In response to radiation, the p53 protein is stabilized and can both initiate and suppress gene expression. The transactivation of *p53*-target genes mediates apoptosis and cell cycle arrest (2,27,44). *p53*-dependent cell cycle arrest is primarily mediated by the CDK (cyclin-dependent kinase) inhibitor $p21^{WAF1}$. $p21^{WAF1}$ acts as a crucial regulator of the cell cycle checkpoint during the G_1 transition, and enables the potential for DNA damage repair (45). In addition to being an inhibitor of cell proliferation, $p21^{WAF1}$ plays a role as an inhibitor of apoptosis. High levels of $p21^{WAF1}$ expression protect damaged cells from *p53*-dependent apoptosis. Some previous studies suggested that RAD001 sensitizes cancer cells with wild-type *p53* to cisplatin by blocking *p53*-induced $p21^{WAF1}$ expression through the inhibition of mTOR, and enhances cisplatin-induced apoptosis in human lung cancer and HCC (17,33). Treatment with p21 antisense resulted in enhanced radiation sensitivity by converting growth arrest to apoptosis in colon cancer cells (46). Consistent with these previous reports, Western blotting here showed that rapamycin strongly inhibits *p53*-induced $p21^{WAF1}$ expression following X-irradiation and results in induction of apoptosis in H1299 cells with a wt*p53* (Figs. 2 and 7). These findings suggest that rapamycin leads to radiosensitization in a *p53*-dependent manner through the suppression of $p21^{WAF1}$. Although H1299/m*p53* cells lack the ability to induce expression of $p21^{WAF1}$ after X-irradiation, radiation-induced apoptosis was promoted following the combined treatment regardless of *p53* gene status (Fig. 2). In human HCC with a mutated *p53* gene, RAD001 enhanced cisplatin-induced apoptosis through the down-regulation of pro-survival molecules such as Bcl-2, survivin and cyclin D1, suggesting that rapamycin could lower the apoptotic threshold (33). In this study, the combined treatment led to a decrease in cyclin D1 expression and increased the incidence of apoptosis in m*p53* cells (Figs. 2 and 7). There is a possible explanation for these results. The sensitivity of cancer cells to radiation-induced apoptosis depends on the balance between pro-survival and pro-apoptotic signals. It appears that the *p53*-independent signaling pathway leading to the induction of apoptosis might be dominant over the pro-survival signals in the presence of a blockade of the Akt/mTOR signal, and result in an induction of apoptosis in H1299 cells with m*p53*.

In conclusion, this study indicates that rapamycin may enhance radiation sensitivity in a *p53*-dependent and in a *p53*-independent manner. The sensitivity of cancer cells to radiation-induced apoptosis may involve the depression of pro-survival signals. Both, the loss of *p53*-pathway functions and radiation-induced up-regulation of the mTOR signal are responsible for radiation resistance. Therefore, inhibition of the mTOR signal may be a promising strategy to enhance the effect of radiation regardless of *p53* gene status through the

down-regulation of pro-survival signals and a reduction in the apoptotic threshold. Since these results suggest that rapamycin can enhance radiation therapy, not only in wt*p53* cancer patients but also in m*p53* cancer patients, through the two aspects of cell lethality and cell growth depression, it may be possible to hope for a high efficacy in treatments against human lung cancer with a suitable application of these agents.

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