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Original Article

VEGF functions as a key modulator in the radioresistance formation of A549 cell lines via regulation of Notch1 expression

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Abstract: In this study, we generated a radioresistant subclone of non-small cell lung cancer A549 cells by treating them with fractionated X-rays. A clonogenic assay showed that 6 Gy per fraction resulted in the selection of a radioresistant population within five fractions. We further found that ionizing radiation not only increased the expression of vascular endothelial growth factor (VEGF), but also induced Notch1 expression, both in a radiation dose-dependent manner. Inhibition of VEGF or Notch1 by antibodies or siRNA demonstrated that VEGF acted upstream of Notch1, and together they were responsible for formation of the radioresistant A549 cell subclone. The cell cycle checkpoint of the radioresistant subclone differed from the non-irradiated parental population and the isolated non-radioresistant subclones. In addition, we found that the extent of apoptosis was an important factor in determining radioresistance of A549 cells. Taken together, these findings suggest that VEGF is responsible for the formation of radioresistant A549 cells by inducing the expression of Notch1 to regulate the cell cycle checkpointand to decrease apoptosis.

Keywords: VEGF, Notch1, NSCLC, cell cycle, radioresistance

Introduction

Radiation therapy is a cornerstone of NSCLC treating non-small cell lung cancer. Radiation therapy is used either alone or in combination with chemotherapeutic drugs [1]. Unfortunately, series of evidences show that local cancer radioresistance remains a major obstacle to recovery in many lung cancer cases [2, 3]. Establishment of radioresistant model system is the accepted measure to study radioresistance in vitro. Presently, there are conflicting views as what dose and fraction is feasible to isolate radioresistant population in vitro tumor cells. Pearce et al described an in vitro system that used 2-6 Gy per fraction and resulted in the rapid selection of radioresistant populations, within three to five fractions [4, 5]. However, there are conflicting views as to what radiation dose and fraction are feasible to isolate a radioresistant population of tumor cells in vitro. Studies in vitro assay reveal that tumor tissues with high expression of vascular endothelial growth factor (VEGF) are more resistant to the cytotoxic effects of ionizing radiation than those with low or null VEGF expression, and combining antiangiogenic therapy with radiation may enhance tumor response. Irradiation of lung carcinoma cells with carbon ions induced VEGF mRNA expression and increased protein levels. Induction of VEGF by ionizing radiation is proposed to favor tumor survival by increasing the radioresistance of tumor vascular endothelium [6-10]. Although data regarding the role of the Notch pathway in cancer cell radiosensitivity are still limited, studies suggest that Notch signaling plays a critical role in the development of non-small cell lung cancer [11]. In addition, new evidence shows that Notch signaling from tumor cells is able to trigger tumor angiogenesis in vitro and in a xenograft mouse tumor modle [12, 13]. Overall, interaction of

Notch with conventional VEGF pathways is important in tumor angiogenesis and cancer development. Recently, it has been demonstrated that the proposed VEGF-Notch feedback loop is sufficient to dynamically select tip and stalk cells under VEGF stimulation. VEGF is able to induce the expression of DLL4, Notch1, and Notch4 in human umbilical vein endothelial cells, and Notch1, Notch4, and Hey1 can downregulate VEGFR expression [14-16]. Moreover, series of previous studies have indicated that Notch activation can induces tumor and endothelial cell cycle arrest in lung cancer [17-19].

The radioresistant phenotype is often correlated with some factors, such as alterations of the cell cycle checkpoints, decreased apoptosis and slow growth [20-23]. It is well known that large numbers of tumor cells are killed after irradiation by a form of apoptosis. Radiation can induce apoptosis through damage to the DNA or the plasma membrane [20, 21]. In response to irradiation, cancer cells can temporarily arrest their progression through the cell cycle to repair the DNA damage [22]. These cell cycle checkpoint control mechanisms are thought to be significant way for cell survival [22].

In the current study, we show that the formation of radioresistance subclone is due at least in part to the upregulation of VEGF, and over expression of VEGF could induce expression of Notch1. Finally, the cell cycle distribution adapted correspondingly to promote the repair of DNA damage so that the cell apoptosis induced by irradiation was decreased. Thus, we provide direct evidence for the hypothesis that VEGF acts upstream of the Notch1 receptor and cooperation between VEGF and Notch1 is responsible for the formation of the radioresistant A549 subclone.

Materials and methods

Reagents and cell lines

The non-small cell lung cancer A549 cell line was purchased from the Cancer Hospital of Shanghai Fudan University (Shanghai, China). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and a combination of penicillin (50 U/mL) and streptomycin (50 μg/mL). Cells were cultured at 37°C in a 5% CO_2 atmosphere humidified to 95-100%.

The VEGF monoclonal antibody (Mab) and purified anti-Notch1 Mab A6 were purchased from Novus-Biologicals (Littleton, COUSA). Antibodies for western blotting against Notch1, VEGF, caspase-3, and tubulin were obtained from eBiosciences (San Diego, CA, USA). Mouse anti-poly-ADP-ribose polymerase was purchased from Trevigen (Gaithersburg, MD, USA). The secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG and rabbit anti-goat IgG) were obtained from Thermo Scientific (Rockford, IL, USA).

Irradiation

A high-energy linear accelerator (Precise 1120, Elekta Instrument AB, Stockholm, Sweden), was used to provide 6 MV X-ray exposures to two groups. A549 cells in the first group were irradiated five times with a 6 Gy fractionated dose. The second group was irradiated 15 times with a 2 Gy fractionated dose. After irradiation, surviving cells yielded two monoclones: subclones A549-S1 (from group 1) and A549-S2 (from group 2).

After determination of the protocol that induced radioresistant subclones, six flasks of A549 cells were divided equally into 3 groups: Irradiation alone, irradiation combined with the anti-VEGF Mab, and irradiation combined with the anti-Notch1 Mab. The final concentrations of the anti-VEGF and anti-Notch1 Mabs were 0.1 and 0.2 μg/mL, respectively. Expression of Notch1 and VEGF proteins was detected by western blot 24 h after each time of irradiation. The remaining flasks of cells were trypsinized and subcultured into two new flasks when they became confluent or the medium was depleted of nutrients. The cells were then irradiated again until the total dose reached 30 Gy. Three monoclones were obtained from surviving cells: subclones A549-S1, -S3, and -S4.

siRNA

Scrambled RNAi oligonucleotides and siRNAs targeting VEGF and Notch1 (ON-TARGETplus SMARTpool RNAi reagents) were obtained from Dharmacon (Lafayette, CO, USA). All siRNAs were transfected into cells using the DharmaFECT 4 transfection reagent (Dharmacon)

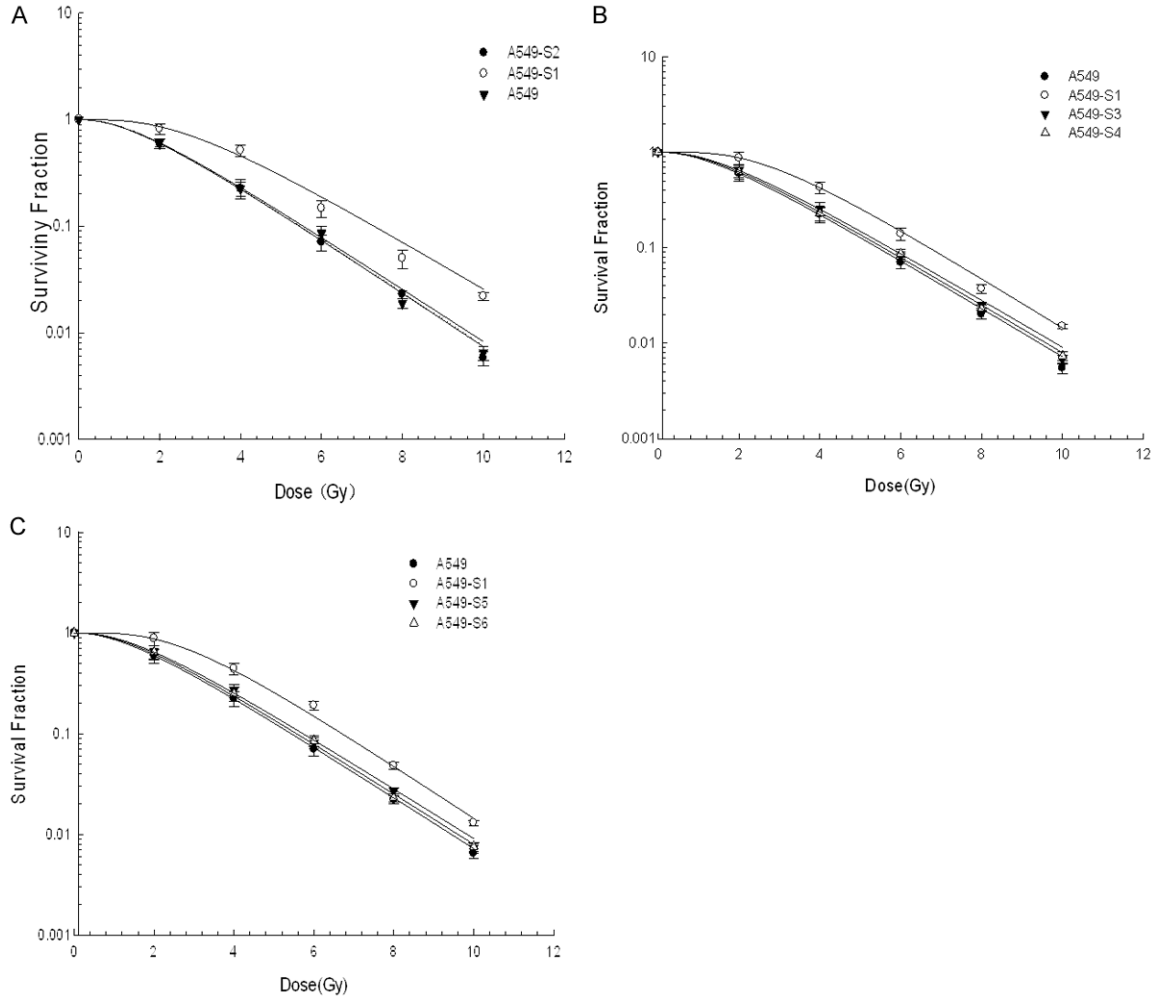

Figure 1. Comparison of survival curves of subclone cells A549-S1, A549-S2, A549-S3, A549-S4, A549-S5, A549-S6 and parental A549 cells. Survival curves were obtained and radiosensitivity parameters were calculated using the multi-target single-hit model. The surviving fractions of all cells at different radiation doses were determined by using the clonogenic formation assay to construct survival curves and calculate D0 (mean lethal dose), Dq (quasi-threshold dose), and N (target number) values. A. A549-S1 cells were derived by irradiating A549 cells five times with a 6 Gy fractionated dose. These cells were more radioresistant than the parental A549 cells or A549-S2 cells derived by irradiating A549 cells 15 times with a 2 Gy fractionated dose. A549-S1 cells had higher D0, Dq, and N values and a broader initial shoulder than those of the parental A549 or A549-S2 cells. There was a 1.38-fold increase in radioresistance with SF2 (cell survival fraction at 2 Gy). B, C. The sensitivity of subclones derived by exposure of A549 cells to 6 Gy X-ray irradiation 5 time in which VEGF or Notch1 was inhibited by a monoclonal antibody (A549-S3 and -S4, respectively) or siRNA knockdown (A549-S5 and -S6, respectively). There were no marked changes compared with parental A549 cells.

according to the manufacturer's protocol. siRNA effectiveness was validated by western blotting.

Western blot

After each designated treatment, cells were lysed in M2 lysis buffer (20 mM Tris, pH 7.0, containing 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM glycerol phosphate, 1 mM sodium vanadate, and proteinase inhibitor cocktail). Equal amounts of protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat milk, the membrane was probed with primary and secondary antibodies, developed with the enhanced chemiluminescence method (Thermo Scientific), and visualized with the Kodak Image Station 440CF (Kodak, Rochester, NY, USA).

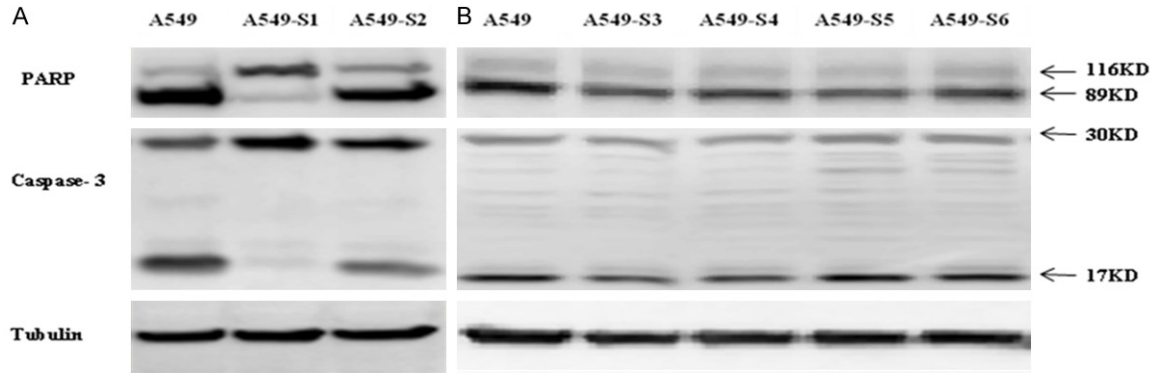

Figure 2. Apoptosis is induced by X-ray irradiation in the radioresistant subclone, A549-S1, but not in the non-radioresistant subclones or the parental A549 cells. A549-S1 cells were derived by irradiating A549 cells five times with a 6 Gy fractionated dose. A549-S2 cells were derived by irradiating A549 cells 15 times with a 2 Gy fractionated dose. A549-S3, -S4, -S5, and -S6 subclones were treated with the VEGF Mab, Notch1 Mab, VEGF siRNA, and Notch1 siRNA, respectively. Poly-ADP-ribose polymerase and caspase-3 cleavage occurred in A549 and A549-S2, -S3, -S4, -S5, and -S6 cells but not in A549-S1 cells 24 h after exposure to 6 Gy X-ray irradiation. Cell lysates were prepared and analyzed by western blot with the indicated antibodies.

Clonogenic assay

Cells were trypsinized and counted using a hemacytometer. In a typical experiment, 50 to 5000 cells were plated per well in a six-well plate for irradiation with dosages of 0, 2, 4, 6, 8, and 10 Gy. The plates were then incubated at 37°C for 10 to 14 days. The cells were stained with Giemsa and colonies with more than 50 cells were scored under a light microscope. The surviving fraction at each dose was calculated based on the number of colonies. Using the simple multi-target model, survival curves were constructed by plotting colony-forming ability on a logarithmic scale as a function of the radiation dose administered on a linear scale. The radiation biology values D0 (mean lethal dose), Dq (quasi-threshold dose), N (target number), and SF2 (cell survival fraction at 2 Gy) were thencalculated. All experiments were repeated three times.

Flow cytometry assay

Before or after all irradiation exposures, cells were trypsinized, made into a single-cell suspension in PBS and pelleted by centrifugation. Pelleted cells were washed twice in cold phosphate-buffered saline and fixed in 70% ethanol for at least 24 h at -20°C. Ethanol was removed by centrifugation and the cells (5×10^5 cells/mL) were resuspended in 0.5 mL propidium iodide staining solution (0.1% sodium citrate, 0.3% Triton X-100, 100 mg/mL RNase A, 100 mg/mL propidium iodide) at 37°C for 30 min. Cellular fluorescence was measured by FACSort flow cytometry (Becton Dickinson, USA). Data were analyzed by CellQuest software (BD Bioscience, San Jose, CA, USA). Experiments were repeated three times.

Statistical analysis

All experiments were done in triplicate. Data are presented as means ± standard deviation (SD). Data were analyzed using the one-way analysis of variance and least significant difference tests with SPSS 22 software (SPSS, Inc., Chicago, IL, USA) to determine statistical significance. $P<0.05$ was considered to indicate a statistically significant difference between values.

Result

The A549-S1 cell population surviving after irradiation 5 times with 6 Gy per fraction was significantly more radioresistant than the parental A549 cell population

Subclone A549-S1, derived from irradiation 5 times with 6 Gy per fraction, was 1.38-fold more radioresistant (SF2) than the parental A549 cells. However, the sensitivity of A549-S2 cells, derived from irradiation 15 times with 2 Gy, was unchanged; the SF2 value of these cells was similar to the A549 parental cells (**Figure 1A**).

Radioresistant and radiosensitive cell populations exhibited different cellular responses to ionizing radiation

The effect of ionizing radiation on cell death and the cell cycle was studied by western blot

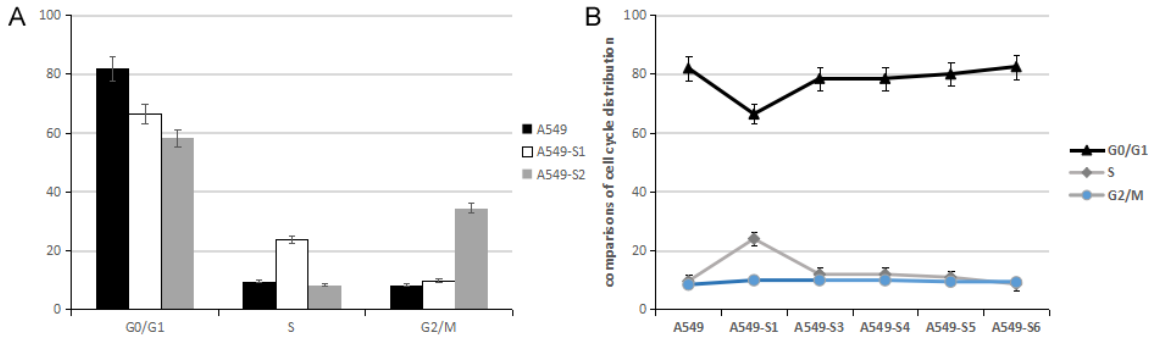

Figure 3. Comparisons between the cell cycle distribution of A549 parental and subclone cells. A549-S1 cells were derived by irradiating A549 cells five times with a 6 Gy fractionated dose. A549-S2 cells were derived by irradiating A549 cells 15 times with a 2 Gy fractionated dose. A549-S3, -S4, -S5, and -S6 subclones were treated with the VEGF Mab, Notch1 Mab, VEGF siRNA, and Notch1 siRNA, respectively. A. The radioresistant A549-S1 subclone showed more S-phase cells in response to ionizing radiation. The non-irradiated parental A549 cell population and non-radioresistant subclones showed more cells in G1- and G2-phase. These cells were irradiated with 6 Gy and incubated for 24 h before being fixed in ethanol and stained with propidium iodide. The DNA content was analyzed using flow cytometer. B. Parental A549 cells and subclones A549-S3, -S4, -S5, and -S6 had a similar cell cycle distribution after irradiation with 6 Gy. This distribution differed from radioresistant A549-S1 cells. All results are expressed as means ± SD of three independent experiments.

and flow cytometry. Cells were confirmed to be apoptotic by analysis of poly-ADP-ribose polymerase, a known caspase substrate cleaved in apoptosis, and caspase-3 cleavage. These results showed that A549 parental and A549-S2 subclone cells underwent apoptosis in response to 6 Gy X-ray irradiation whereas the radioresistant A549-S1 subclone did not (**Figure 2A**). Additionally, the cell cycle distribution of the radioresistant subclone differed from the non-irradiated parental population and the isolated non-radioresistant subclone. **Figure 3** shows the cell cycle distribution 24 h after 6 Gy irradiation. For exponentially growing A549-S1 cells, the fractions of cells in the G0/G1, S, and G2/M phases were 7.73 ± 2.78%, 22.8 ± 1.54%, and 10.0 ± 0.79% respectively. Corresponding fractions for A549 and A549-S2 cells were, respectively, 82.5 ± 3.74%, 10.5 ± 1.19%, 7.11 ± 0.81%, and 56.6 ± 2.53%, 7.92 ± 0.87%, and 35.8 ± 2.04%. Overall, A549-S1 cells showed a higher fraction of cells in the S phase and a lower fraction in the G1 phase than parental A549 cells (**Figure 3A** and **3B**).

The expression of Notch1 and VEGF proteins was upregulated with increasing radiation dose

The western blot results showed that the expression of VEGF and Notch1 proteins was elevated during the course of irradiation and that the extent of expression was dependent on the cumulative X-ray irradiation dose (**Figure 4A**). To examine the whether VEGF induced Notch1, Notch1 induced VEGF, or their induction was independent of each other, VEGF and Notch1 were knocked down separately with siRNA and changes in the other assessed. Knockdown of VEGF blocked the increased expression of Notch1 induced by irradiation. However, the expression of VEGF was not influenced by the knockdown of Notch1 (**Figure 4B**). These results indicate that X-ray irradiation can induce the expression of VEGF, which then promotes the expression of Notch1. In other words, VEGF acts upstream of Notch1 in the signaling pathway during the formation of radioresistant A549 subclones.

The functional effects of VEGF and Notch1 were responsible for the formation of A549 radioresistant subclones and changing the cell cycle checkpoint

We showed that the elevated expression of VEGF and Notch1 proteins depended on the cumulative dose of ionizing radiationduring the formation of radioresistant subclones. To determine whether VEGF and Notch1 were responsible for the radioresistance, VEGF and Notch1 were inhibited by Mabs or siRNA knockdown and changes in the radiosensitivity of each subclone were determined. A549-S3, -S4, -S5, and -S6 subclones were treated with the VEGF Mab,

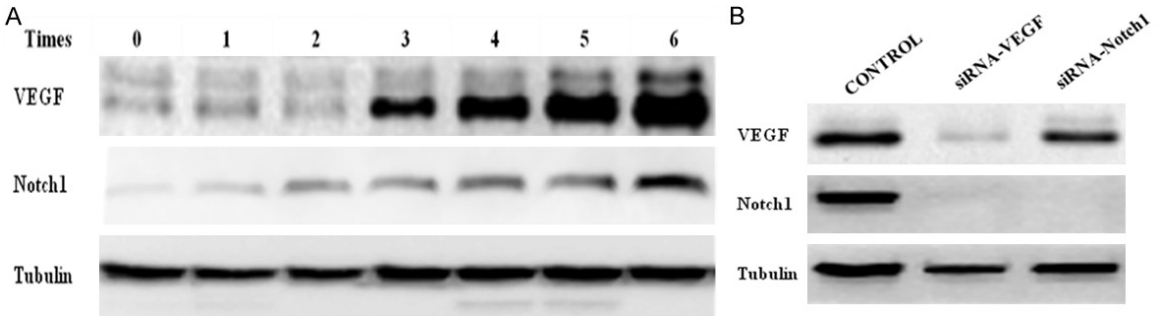

Figure 4. Expression of VEGF and Notch1 during the development of radioresistance. A. The expression of VEGF and Notch1 was increased during the process of radioresistant A549 cell subclone formation. Cells were irradiated with 6 Gy X-rays then incubated for 24 h. Cell lysates were prepared and analyzed by western blots with the indicated antibodies. B. VEGF promotes the expression of Notch1 in response to X-ray irradiation. After transfection with VEGF or Notch1 siRNA for 48 h, cells were treated with 6 Gy X-ray irradiation. After incubation for 24 h, cells lysed and proteins detected by western blots. Knockdown VEGF blocked the expression of Notch1 while knockdown of Notch1 had no effect on the expression of VEGF.

Notch1 Mab, VEGF siRNA, and Notch1 siRNA, respectively. After inhibiting the expression of VEGF and Notch1 by anti-VEGF or anti-Notch1 Mabs, respectively, 5 treatments with 6 Gy X-ray irradiation could not generate a radioresistant A549 subclone. The radiosensitivity of A549-S3 and -S4 was similar to the A549 parental cells. The results derived from knockdown of VEGF or Notch1 with siRNA were similar (**Figure 1B** and **1C**). Correspondingly, apoptosis induced by irradiation of these radiosensitive subclone cells was much more apparent than with the radioresistant A549-S1 cells (**Figure 2B**). Moreover, the cell cycle checkpoint of subclones A549-S3, -S4, -S5, and -S6 was different after irradiation compared to the radioresistant A549-S1 subclone. These non-radioresistant subclones did not exhibit the same cell cycle distribution change as the radioresistant A549-S1 subclone that showed a higher fraction of cells in the S phase and a lower fraction in the G1 phase compared to parental A549 cells (**Figure 3C**).

Discussion

The current study was conducted with the goal of determining whether fractioned radiation could select radioresistant subclones and, if so, what mechanisms would allow these cells to survive after irradiation. The radioresistant subclone, A549-S1, was obtained initially by exposing parental A549 cells 5 times to 6 Gy. The clonogenic assay showed that the A549-S1 subclone obtained through this process differed from the parental A549 cells with an increased ability to survive exposure to ionizing radiation; there was a higher and broader initial shoulder on the survival curve. In terms of SF2, A549-S1 cells were 1.38-fold more radioresistant than the parental cells. The characteristics of A549-S1 cells suggest that radioresistant subclones might be selected by repeated high dose irradiation. Some recent papers have reported similar findings [4, 23]. However, at present, the mechanism underlying the radioresistance induced by ionizing radiation is unclear.

There are two different types of cell death induced by ionizing radiation: Necrosis and apoptosis. Apoptosis is usually significant in determining cellular radiosensitivity [20, 21]. In the present study, radioresistant A549-S1 subclone cells exhibited much less apoptosis when exposed to ionizing radiation than the parental A549 cells and other non-radioresistant subclones. Therefore, differences in radiosensitivity between radioresistant and non-radioresistant cells can, at least in part, be explained by differences in the extent of apoptosis.

The phase of the cell cycle is another factor that affects the radiosensitivity of cells [22-25]. Generally, cells in the G2-M phase tend to be more sensitive than cells in the S and G1 phases, with cells in the S phase being the most resistant. Our data show that the radioresistant A549 subclone has more cells in the S phase than the parental A549 cells and non-radioresistant subclones. This supports the concept

that a larger S phase population might contribute to the radioresistant phenotype. Previous results indicated that Notch controlled neither G1 nor G2 arrest directly by the induction of target genes such as cyclins A and B, string, Wg, and cyclin-dependent kinase inhibitors [17-19]. In contrast to these reports, we observed that over-expression of Notch induced an increase of S-stage cells in the radioresistant clone. When Notch1 and VEGF were inhibited by either mAbs or siRNAs, the S-stage distribution of subclones induced by irradiation disappeared. It is probable that Notch1 and VEGF regulate distribution of the cell cycle to the S stage as an adaptation to the damage caused by ionizing radiation.

It has been suggested that VEGF may play an important role in the increased radioresistance seen after exposure to ionizing radiation [4, 26]. Our results confirmed that the level of VEGF expression correlated with the extent of radiation exposure during the process of radioresistant subclone formation and, more importantly, there was a similar trend with Notch1 expression. These results demonstrated that VEGF and Notch1 may protect cells from radiation-induced death and they may have a relationship with each other. When VEGF or Notch1 were blocked, the subclones derived from parental A549 cells by irradiation were not radioresistant compared with the parental cells. This finding informs us of the key role these two genes have in the formation of radioresistance. Importantly, inhibition of VEGF expression blocked the expression of Notch1, which is consistent with a review of previous literature that the Notch signaling pathway is closely correlated with VEGF expression, and interaction with VEGF induces the expression of DLL4, Notch1, and Notch4 in human umbilical vein endothelial cells [14-16]. Therefore, we infer that the activation of Notch1 is caused by irradiation through the upregulation of VEGF expression.

Together, these results indicate that activation of VEGF induced by irradiation leads to an increased production of Notch1, which then adjusts the cell cycle distribution. The importance of enhanced Notch1 expression by VEGF in the formation of radioresistant cells was not studied previously. Thus, this is the first time that we can confirm the important functional relationship between Notch1 and VEGF to modulate cell death and the cell cycle distribution to regulate the radiosensitivity of A549 cells. Further research is neededin a mouse tumor model to investigate the mechanism by which enhanced Notch1 expression regulates the cell cycle to decrease apoptosis of radioresistant cells in vivo, and confirm the relationship between and functions of VEGF and Notch1.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

[1] Feld R, Ginsberg RJ, Payne DG, et al. Lung. In: Abeloft MD, Armitage JO, Lichter AS, et al. editors. Clinical oncology. Harcourt: Churchill Livingstone; 2001. pp. 1398-1477.

[2] Zimmermann FB, Molls M, Jeremic B. Treatment of recurrent disease in lung cancer. Semin Surg Oncol 2003; 21: 122-127.

[3] Wu KL, Jiang GL, Qian H, Wang LJ, Yang HJ, Fu XL, Zhao S. Three-dimensional conformal radiotherapy for locoregionally recurrent lung carcinoma after external beam irradiation: a prospective phase I-II clinical trial. Int J Radiat Oncol Biol Phys 2003; 57: 1345-1350.

[4] Pearce AG, Segura TM, Rintala AC, Rintala-Maki ND, Lee H. The generation and characterization of a radiation-resistant model system to study radioresistance in human breast cancer cells. Radiat Res 2001; 156: 739-750.

[5] Tyrsina EG, Kalendo GS and Slanina SV. The appearance of a fraction of radioresistant cells in a fibroblast population radiated at high doses. Radiats Biol Radioecol 1997; 37: 207-212.

[6] Ando S, Nojima K, Ishihara H, Suzuki M, Ando M, Majima H, Ando K, Kuriyama T. Induction by carbonion irradiation of the expression of vascular endothelial growth factor in lung carcinoma cells. Int J Radiat Biol 2000; 76: 1121-1127.

[7] Hess C, Vuong V, Hegyi I, Riesterer O, Wood J, Fabbro D, Glanzmann C, Bodis S and Pruschy M. Effect of VEGF receptor inhibitor PTK787/ZK222584 combined with ionizing radiation on endothelial cells and tumor growth. Br J Cancer 2001; 85: 2010-2016.

[8] Huang SM, Li J, Armstrong EA and Larari PM. Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa). Cancer Res 2002; 62: 4300-4306.

[9] Brieger J, Schroeder P, Gosepath J, Mann WJ. Vascular endothelial growth factor and basic fibroblast groth factor are released by squamous cell carcinoma cell after irradiation and increase resistance to subsequent irradiation. Int J Mol Med 2005; 16: 159-164.

[10] Shibuya K, Komaki R, Shintani T, Itasaka S, Ryan A, Jürgensmeier JM, Milas L, Ang K, Herbst RS, O'Reilly MS. Targeted therapy against VEGFR and EGFR signaling with ZD6474 enhances the therapeutic efficacy of irradiation in an orthotopic mouse model of human non-small cell lung cancer. Int J Radiat Oncol Biol Phys 2007; 69: 1534-43.

[11] Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, Pelosi G, Spaggiari L, Mazzarol G, Viale G, Pece S, Di Fiore PP. Alterations of the Notch pathway in lung cancer. Proc Natl Acad Sci U S A 2009; 106: 22293-8.

[12] Li JL, Harris AL. Notch signaling from tumor cells: a new mechanism of angiogenesis. Cancer Cell 2005; 8: 1-3.

[13] Buchler P, Gazdhar A, Schubert M, Giese N, Reber HA, Hines OJ, Giese T, Ceyhan GO, Muller M, Buchler MW, Friess H. The Notch signaling pathway is related to neurovascular progression of pancreatic cancer. Ann Surg 2005; 242: 791-800, discussion 800-801.

[14] Bentley K, Gerhardt H and Bates PA. Agent-based simulation of Notch-mediated tip cell selection in angiogenic sprout initialisation. J Theor Biol 2008; 250: 25-36.

[15] Liu ZJ, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, Fairman RM, Velazquez OC, Herlyn M. Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. Mol Cell Biol 2003; 23: 14-25.

[16] Patel NS, Li JL, Generali D, Poulsom R, Cranston DW, Harris AL. Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. Cancer Res 2005; 65: 8690-8697.

[17] Sriuranpong V, Borges MW, Ravi RK, Arnold DR, Nelkin BD, Baylin SB, Ball DW. Notch signaling induces cell cycle arrest in small cell lung cancer cell. Cancer Res 2001; 61: 3200-3205.

[18] Noseda M, Niessen K, McLean G, Chang L, Karsan A. Notch-dependent cell cycle arrest is associated with downregulation of minichromosome maintenance proteins. Circ Res 2005; 97: 102-104.

[19] Herranz H, Pérez L, Martín FA, Milán M. A wingless and notch double-repression mechanism regulates G1-S transition in the drosophila wing. EMBO J 2008; 27: 1633-45.

[20] Tang JT, Yamazaki H, Inoue T, Koizumi M, Yoshida K, Ozeki S, Inoue T. Mitochondrial DNA influences radiation sensitivity and induction of apoptosis in human fibroblasts. Anticancer Res 1999; 19: 4959-4964.

[21] Maity A, Kao GD, Muschel RJ, McKenna WG. Potential molecular targets for manipulating the radiation response. Int J Radiat Oncol Biol Phys 1997; 3: 639-653.

[22] Coucke PA and Crompton NE. Molecular basis of radioresistance. Eur J Cancer 1995; 31A: 844-6.

[23] Tyrsina EG, Slanina SV, Kakpakova ES, Kalendo GS, Kan NG, Tyrsin OY, Ryskov AP. Isolation and characterization of highly radioresistant malignant hamster fibroblasts that survive acute gamma irradiation with 20 Gy. Radiat Res 2005; 164: 745-54.

[24] Hunakova L, Chorvath M, Duraj J, Bartosová Z, Sevcíková L, Suliková M, Chovancová J, Sedlák J, Chorváth B, Boljesíková E. Radiation induced apoptosis and cell cycle alterations in human carcinoma cell lines with different radiosensitivities. Neoplasma 2000; 47: 25-31.

[25] Humar B, Muller H, Scott RJ. Cell cycle dependent DNA break increase in ataxia telangiectasia lymphoblasts after radiation exposure. Mol Pathol 2001; 54: 347-350.

[26] Ko YH, Jung CK, Lee MA, Byun JH, Kang JH, Lee KY, Jo KH, Wang YP, Hong YS. Clinical significance of vascular endothelial growth factors (vegf)-c and -d in resected non-small cell lung cancer. Cancer Res Treat 2008; 40: 133-40.