Enhanced radiosensitivity of SW480 cells via TRAIL up-regulation mediated by Egr-1 promoter

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Abstract. Radiosensitization of cancer cells to irradiation could improve the efficacy of radiotherapy. The early transcriptional factor (Egr-1) promoter induced expression of downstream genes after irradiation. TNF-related apoptosis-inducing ligand (TRAIL) is known to induce apoptosis in malignant cells, but displayed little or no toxicity on normal cells. In this study, we constructed pcDNA3.1-Egr-1-TRAIL (pEgr.1-TRAIL) recombinant plasmid and evaluated its effect on human colon cancer cell line SW480. pEgr.1-TRAIL transfection combined with radiotherapy caused dramatically elevation of TRAIL expression both in mRNA and protein levels, much lower radiobiological parameters in clonogenic assays, accompanied by remarkably increase in apoptosis ratio. Furthermore, pEgr.1-TRAIL transfected cells displayed higher proportion in G0/G1 phase. Our results suggested that pEgr.1-TRAIL can sensitize SW480 cells to radiation, and the radiosensitization is related to cell cycle changes and apoptosis mediated by up-regulation of TRAIL expression. These findings support the potential future application of genetic radiotherapy against carcinoma.

Introduction

Radiotherapy is a widely used local and regional modality for the treatment of cancer. New developments in radiotherapy technologies, such as 3-D conformal radiotherapy, intensity modulation radiated therapy, make radiotherapy more acceptable. However, one of the main disadvantages

is their complete reliance on dose-escalation, which make it impossible to achieve sufficient dose to eradicate radio-resistant tumors without unacceptable acute and late normal tissue toxicity (1). Radiotherapy remains facing difficulties like recurrence or metastasis after radiation and damage to normal tissues surrounding the tumor (2,3).

Cancer gene therapy (CGT) is a novel treatment of great interest. However, CGT has failed to fulfill its initial promise of improving overall treatment outcome due to tumorigenesis is a multi-step process (4). There is increasing acceptance of the fact that CGT is unlikely to succeed as a single modality (1). However, combining with radiotherapy to improve the overall response rate and the therapeutic index seems to be feasible. In addition, there have been studies showing that radiation improves the efficiency of gene transference (5,6). Combination of radiotherapy with gene therapy potentially provides new modes of targeting toxicity on malignant cells, with minimal effect on normal cells (7).

Egr-1 gene is a member of the immediate early gene family, and its promoter can be rapidly induced by various stimuli like irradiation, mitogens and growth factor (8). The selection of downstream gene is crucial to the efficacy of gene therapy. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) belongs to the TNF super family, which plays important roles in regulating cell death, immune response and inflammation (9,10). The specificity of TRAIL, compared with other members of the TNF family, is its ability to induce apoptosis in a variety of malignant cells, while displaying minimal toxicity on normal cells and tissues (11). Moreover, combine TRAIL gene therapy with radiotherapy has been reported to induce a synergistic effect on apoptosis in cancer cells (12), which may be attributed to the up-regulation of TRAIL receptors by radiation (12,13). To study the feasibility of radiation inducible gene therapy, we constructed a combined plasmid containing the Egr-1 promoter and cDNA of the gene encoding TRAIL (pcDNA3.1-Egr-1-TRAIL), and investigated whether this genetic radiotherapy could enhance the radiosensitivity in SW480 cells.

Materials and methods

Plasmid construction. Dr Wu Cong-Mei (Medical College of Shantou University, Guangdong, China) kindly provided the

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plasmid pMD18T-Egr-1 and Dr J.Y. Chang (The University of Texas M.D. Anderson Cancer Center, TX, USA) gave the plasmid PAAV-TRAIL. pcDNA3.1+ vector was purchased from Invitrogen Life Technologies. The Method of TRAIL expression vector construction was described in our previous study (14).

Cell lines and culture conditions. The human colon cancer cell line SW480 was obtained from the China Center for Type Culture Collection (CCTCC) in Wuhan University. Cells were cultured in RPMI-1640 medium (Hyclone) at 37˚C in a humidified atmosphere of 5% CO_2. The media were supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin and was changed every 3-5 days.

Grouping and irradiation. The cells were divided into 4 groups: i) control group (no treatment was performed); ii) gene radiotherapy group (treated with transfection of pcDNA 3.1-pEgr-1-TRAIL and irradiation); iii) gene therapy group (treated with transfection of pcDNA3.1-pEgr-1-TRAIL only); and iv) radiotherapy group (treated with irradiation only). Monolayer of cells in gene radiotherapy group and radiotherapy group were irradiated for 6 Gy ([60]Co γ-ray machine, field = 20 cm x 20 cm, SSD = 80 cm, dose rate = 96 cGy min^{-1}). The cells were cultured for 24 h after irradiation.

Transfection. A total of 5x10^{5} SW480 cells were plated into each well of 6-well plates. When the cells achieved 80-90% confluency, the culture medium was removed. Cells in gene radiotherapy group and gene therapy group were transfected in an appropriate ratio with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Plasmid DNA (4 µg) was mixed with 250 µl of serum-free medium (SFM), then 10 µl of Lipofectamine 2000 mixed with 250 µl of SFM was added, and incubated for 20 min at room temperature. Finally, the resultant mixture was added to cells in each well. The medium was replaced with fresh serum containing medium 5 h after transfection. Positive transfected cells were selected with fresh medium containing 500 µg/ml of G418.

Reverse transcription PCR. Total cellular RNA was isolated with TRIzol as described in the manufacturer's instruction (Molecular Research Center, Inc.). For cDNA synthesis, 1 µg of total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Fermentas) and oligo-dT primer under standard conditions. cDNA (2 µl) was PCR-amplified under the following conditions: incubation at 94˚C for 5 min, followed by 35 cycles denaturing at 94˚C for 40 sec, annealing for 40 sec, elongation at 72˚C for 1 min and a final extension at 72˚C for 10 min. Primers for TRAIL are 5'-CCCAAGCTTATGGCTATGATGGAGG TCCA-3' and 5'-CCGCTCGAGTTAGCCAACTAAAA AGGCC-3' (Tm = 60˚C), for GAPDH: 5'-TTGGTATCG TGGAAGGACTCATG-3' and 5'-GTTGCTGTAGCCAA ATTCGTTGT-3' (Tm = 56˚C).

Amplification products were electrophoresed on 1% agarose gels containing ethidium bromide for visualization under UV light. All PCR reactions were performed at least three times. Densitometric analysis of TRAIL mRNA levels was performed with Gene tools software. GAPDH was used to normalize mRNA levels. Statistical significances were calculated by χ^2 test.

Western blotting. Cells were washed once with ice-cold PBS, exposed to 200 µl of lysis buffer (Biyuntian, Biotech). After centrifugation at 12,000 rpm for 5 min at 4˚C, the supernatants were removed and stored at -80˚C. Samples (50 µg of whole-cell lysates per lane) were separated by 10% SDS-PAGE electrophoresis under denaturing conditions and transferred to PVDF membranes (Biyuntian, Biotech). Membranes were blocked with 5% non-fat milk and probed with rabbit polyclonal antibody against TRAIL (1:333, Santa Cruz Biotechnology, Santa Cruz) or ß-actin (1:333, Beijing Biosynthesis Technology). Goat peroxidase-coupled anti-rabbit IgG (Promega) was used as secondary antibody. Immunoreactive protein was detected with DAB kit (Amresco Biosciences). Densitometric analysis of TRAIL protein levels was performed with Gene tools software. ß-actin was used to normalize protein levels. Statistical significances were calculated by χ^2 test.

Clonogenic assays. Clonogenicity was examined by a colony-forming assay. Exponentially growing cells of group 2 and 4 were irradiated at the dose point of 0, 1, 2, 3, 4, 6, 8, 10 Gy respectively. An appropriate number of cells were seeded into culture flasks in order to obtain similar number of colonies for different doses of irradiation. After 2-3 weeks of incubation, the colonies were fixed and stained by crystal violet. Those colonies containing >50 cells were scored as clonogenic survivors. The data were fit into the single-hit multi-target model, and survival curve of both groups were drawn by SPSS 13.0. Radiobiological parameters, such as SF2 (survival fraction at 2 Gy), D_0, D_q and SER (sensitization enhancement ratio) were calculated according to the survival curves. All experiments were performed independently at least three times.

Cell cycle analysis. Control and 6 Gy irradiated cells with or without transfection were harvested by trypsinisation, washed with ice-cold PBS, fixed in 1 ml 75% ethanol at 4˚C for 1-2 h in the dark. Ethanol was removed by centrifugation (2000 rpm, 4˚C), and DNA content was labeled with 1 ml of dye buffer which contains 0.05 mg/ml propidium iodide (PI) (centrebio) and 0.02 mg/ml RNAse (sigma) at 4˚C in darkness for 20 min. Cells were centrifuged (2000 rpm, 4˚C) and resuspended in 500 µl PBS. Cell cycle analysis was performed by flow cytometry analysis (Beckman Coulter).

Apoptosis quantification. Cells were harvested by trypsinisation, washed with ice-cold PBS and resuspended in 200 µl Binding buffer (centrebio), stained with 10 µl of 20 µg/ml Annexin-V-FTIC (centrebio) and 10 µl of 20 µg/ml PI (centrebio), and incubated in the dark for 15 min at room temperature, then added 300 µl binding buffer. Cell apoptotic profile was analysed immediately in flow cytometry analysis (Beckman Coulter).The percentage of apoptotic cells was obtained from a bivariate histogram of Annexin-V labeled-cells versus propidium iodide labeled-DNA.
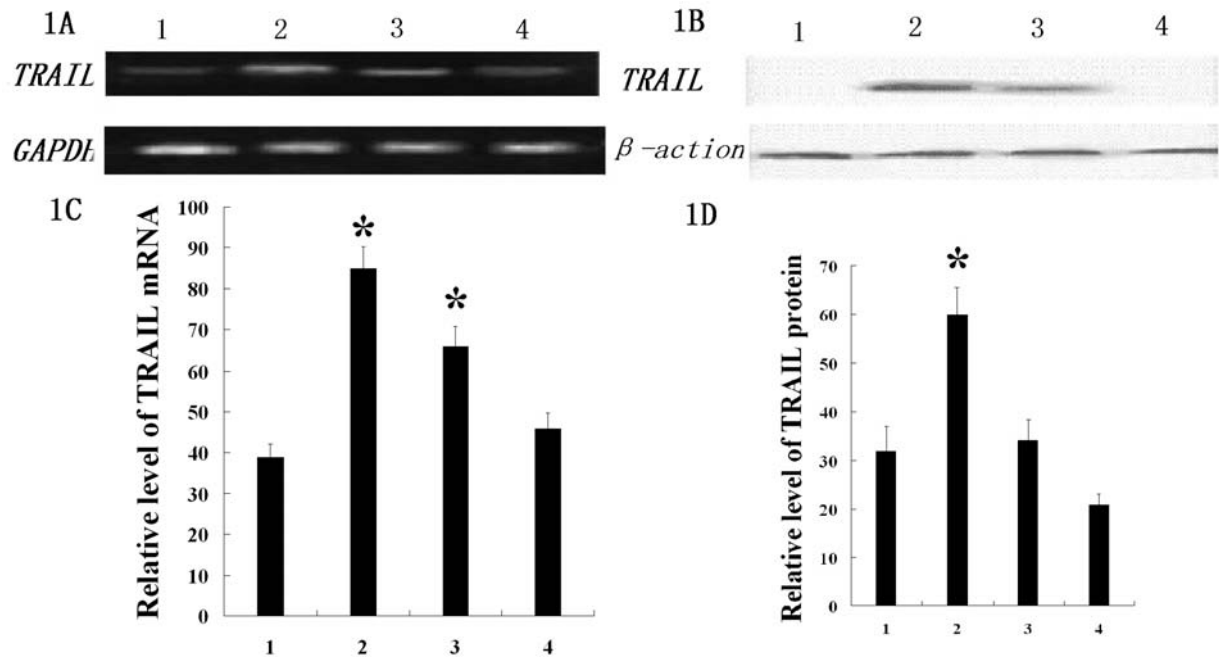
Figure 1. Expression level of TRAIL. (A) Expression level of TRAIL detected by RT-PCR. (B) Expression level of TRAIL detected by Western blotting. 1, Control group; 2, gene radiotherapy group; 3, gene therapy group; 4, radiotherapy group. (C) A graphical representation of the mean data presented in (A). (D) A graphical representation of the mean data presented in (B). The SW480 cell line showed nearly the same expression level of GAPDH mRNA and ß-action protein, while displayed evidently increased TRAIL levels in gene radiotherapy group (P<0.05), as compared with the other three groups. *P<0.05 compared with control (Dunnett test, n=3). Columns represent mean data ± SD.
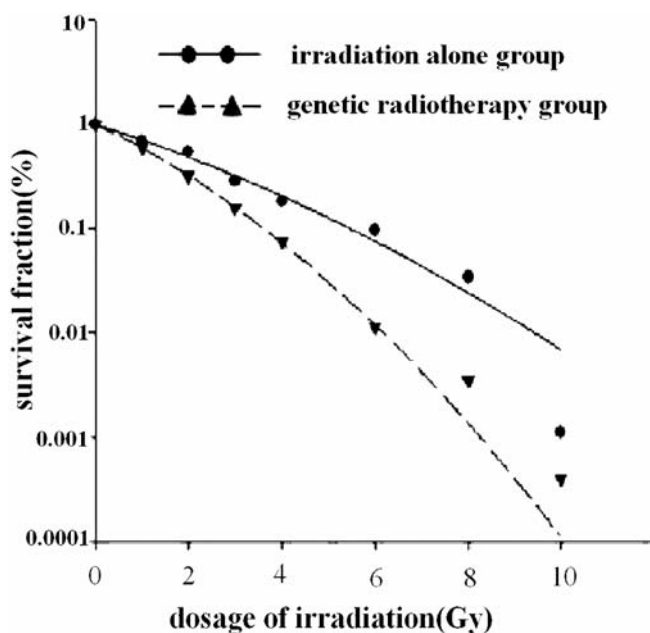

Figure 2. Survival curves. An appropriate number of cells in both groups were seeded into culture flasks for different doses of irradiation. After 2-3 weeks of incubation, the colonies were fixed and stained. Those colonies containing >50 cells were scored as clonogenic survivors and the survival fraction was calculated. The data were fit into the single-hit multi-target model, and survival curves of both groups were drawn by software SPSS 13.0. All experiments were performed at least three times. Data were obtained from triplicate samples and expressed as the mean ± SD.

Statistical analysis. Data were obtained from triplicate samples and expressed as the mean ± standard deviation (SD). Statistical analysis was performed with software SPSS

13.0, and the comparison of radiobiological parameters was analyzed by Student's t-test. A value of P<0.05 was considered to be statistically significant.

Results

Expression level of TRAIL. As shown in Fig. 1A and C, cells in pEgr.1-TRAIL transfected groups displayed increased TRAIL mRNA levels compared to control and radiotherapy group (P<0.05), especially cells in gene radiotherapy group showed the most manifest up-regulation of mRNA level. The result was confirmed by Western blot analysis (Fig. 1B and D). TRAIL up-regulation mediated by Egr-1 promoter could be observed, which proved that Egr-1 promoter is irradiation inducible.

Radiobiological parameters of clonogenic assays. The survival curves describe the radiobiological parameters of both groups (Fig. 2). Compared to the radiotherapy group, the survival fractions of the gene radiotherapy group were much lower at each dose point. The radiobiological parameters calculated are shown in Table I. D_0 and SF2 values in gene radiotherapy group are significantly lower than the control group (P<0.001). The results suggested that pEgr-1-TRAIL plasmid enhanced the radiosensitivity of SW480 cells.

Cell cycle distribution. As shown in Fig. 3, higher fractions of cells in S phase and lower fractions of cells in G1 phase (P<0.01) after irradiation were observed in SW480 cells. In addition, expression of TRAIL induced accumulation in the G1 (P<0.05) phase and decreased the percentage of cells in the S phase (P<0.05).

Table I. Radiobiological parameters.

Grouping	D_0[a]	D_q	SF2[a]
Radiotherapy group	2.2589±0.0679	0.1724±0.0272	0.4701±0.0114
Gene radiotherapy group	1.4575±0.0235	0.1654±0.0101	0.3159±0.0014
SER	1.55	1.09	1.49

Data from triplicate clonogenic assays were fit into the single-hit multi-target model, and survival curves of both groups were drawn by software SPSS 13.0, the SF2, D_0, D_q and SER values were calculated (P_{D0}, P_{SF2}<0.001). Data were expressed as the mean ± SD. [a]Indicates statistic significances (P<0.001). SF, surviving fraction; D_0, mean lethal dose, is also known as final slope; D_q, quasi-threshold dose.
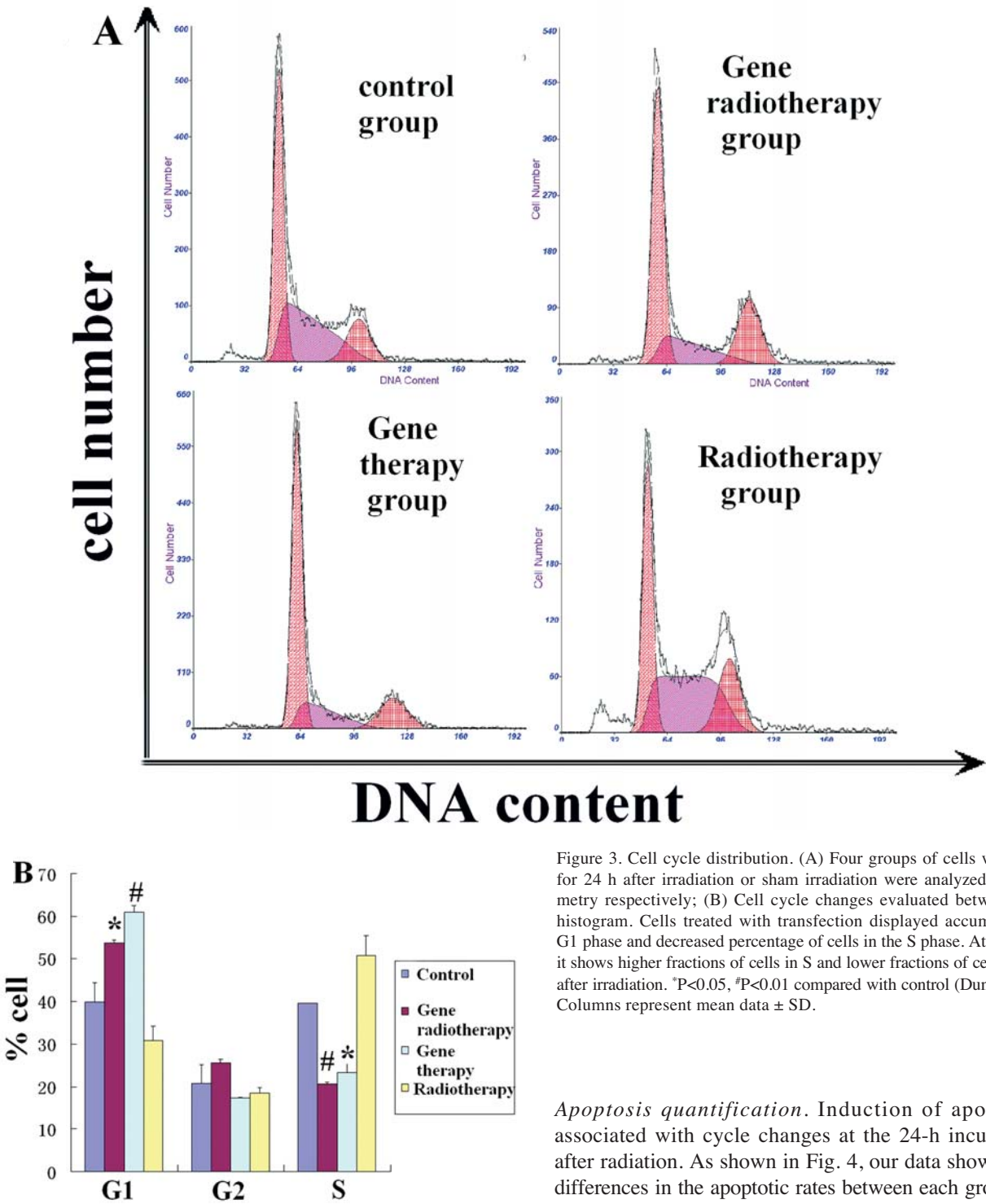

Figure 3. Cell cycle distribution. (A) Four groups of cells were incubated for 24 h after irradiation or sham irradiation were analyzed by flow cytometry respectively; (B) Cell cycle changes evaluated between groups in histogram. Cells treated with transfection displayed accumulation in the G1 phase and decreased percentage of cells in the S phase. At the same time, it shows higher fractions of cells in S and lower fractions of cells in G1 phase after irradiation. *P<0.05, #P<0.01 compared with control (Dunnett test, n=3). Columns represent mean data ± SD.

Apoptosis quantification. Induction of apoptosis was associated with cycle changes at the 24-h incubation time after radiation. As shown in Fig. 4, our data show significant differences in the apoptotic rates between each group (Fig. 4).
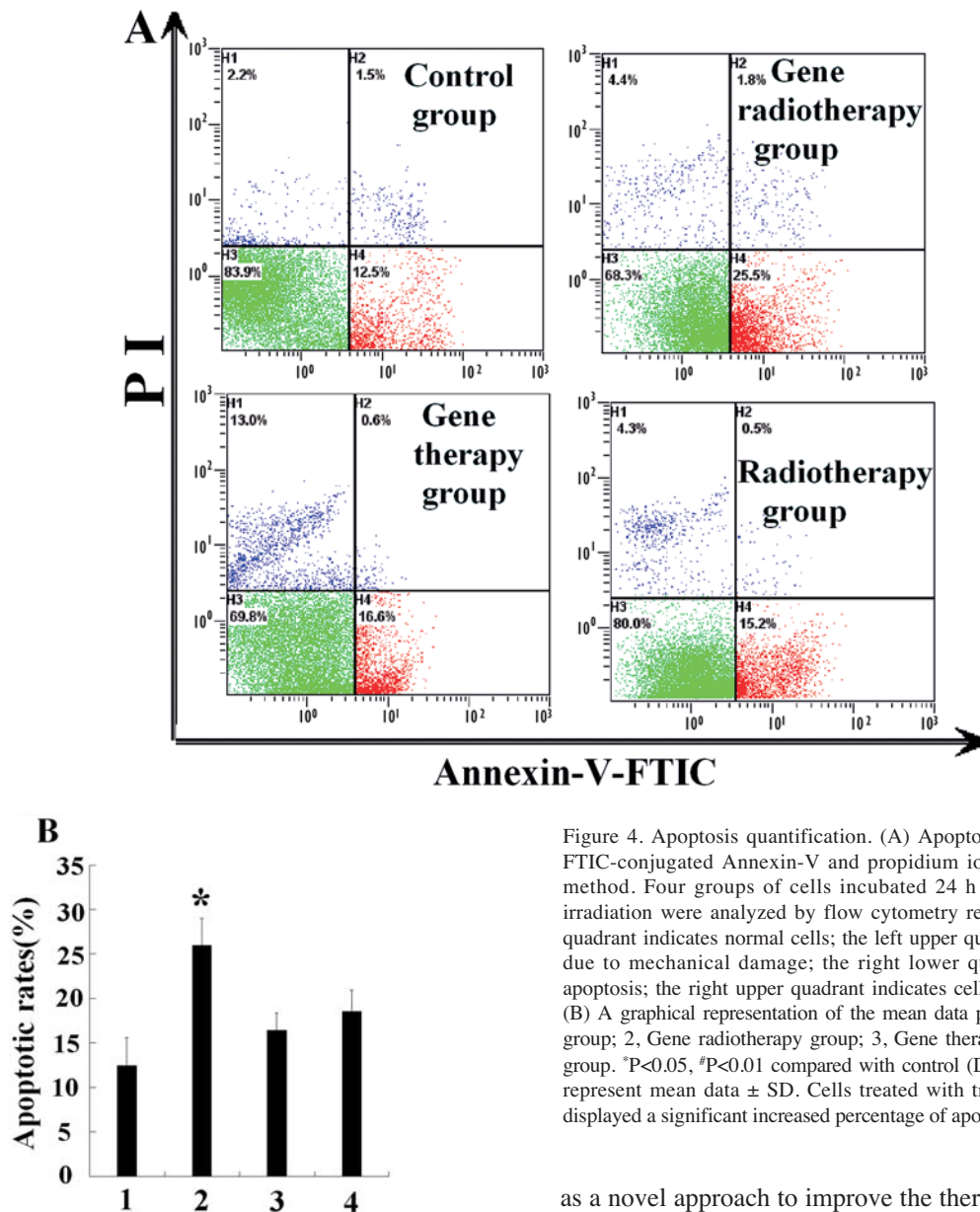
Figure 4. Apoptosis quantification. (A) Apoptotic cells were detected by FTIC-conjugated Annexin-V and propidium iodide (PI) double labeling method. Four groups of cells incubated 24 h after irradiation or sham irradiation were analyzed by flow cytometry respectively. The left lower quadrant indicates normal cells; the left upper quadrant indicates dead cells due to mechanical damage; the right lower quadrant indicates cells of apoptosis; the right upper quadrant indicates cells of necrosis or apoptosis. (B) A graphical representation of the mean data presented in (A). 1, Control group; 2, Gene radiotherapy group; 3, Gene therapy group; 4, Radiotherapy group. $^*P<0.05$, $^\#P<0.01$ compared with control (Dunnett test, n=3). Columns represent mean data ± SD. Cells treated with transfection and irradiation displayed a significant increased percentage of apoptotic cells.

In particularly, gene radiotherapy group demonstrated the highest apoptotic rate (P<0.05), suggesting that increasing apoptosis is involved in the mechanism of radiosensitisation induced by TRAIL.

Discussion

In the present study, we showed that TRAIL expression mediated by Egr-1 promoter sensitized SW480 to radiotherapy via apoptosis pathway. For the radiotherapy (6 Gy) only group, the killing effect on tumor cells was minimal, mean apoptosis ratio is 18.0% (Fig. 4). There is no statistical difference compared with control group. These data suggest that SW480 cell line is resistant to radiotherapy at this dose. Similarly, transfection of pEgr.1-TRAIL alone moderately diminished the tumor resistance phenotype. However, combination of radiotherapy and pEgr.1-TRAIL displayed a synergetic effect on the apoptotic rate (Fig. 4) and clone formation assay (Fig. 2). The combined treatment may serve

as a novel approach to improve the therapeutic ratio of radiotherapy in colon cancer.

Radiotherapy commonly causes acute and chronic adverse effects, which are the major dose-limiting toxicities of radiotherapy (14), especially in colon cancer with critical surrounding organs. While gene therapy causes different toxicity profiles, such as fevers (15). Therefore, combined gene therapy and radiotherapy may increase therapeutic efficacy on cancer cells, with tolerable toxicities. Among the family of death receptor ligands, tumor necrosis factor and Fas have been extensively investigated, a phase I clinical trial has been completed with TNF-α (16). TRAIL is an attractive candidate for cancer treatment due to its ability to induce apoptosis selectively in cancer cells (11), which is related to differential distribution of TRAIL-receptors. TRAIL interacts with four homologous TRAIL receptors. Both DR4 and DR5 expressed mainly on tumor cells transducing apoptotic signals when binding with TRAIL, while DcR1 and DcR2 expressed mainly on normal cells functioning as decoys or transduce anti-apoptotic signals (17). DR4 and DR5 expressed at high levels in the majority of colon carcinomas (18). Therefore, TRAIL is a more potent approach with more specificity and safety in treatment of colon carcinoma. More-

over, TRAIL gene therapy may overcome problems observed with the use of soluble recombinant TRAIL regarding protein instability and resistance (19,20), and untransfected neighboring cancer cells can be killed by a TRAIL-mediated bystander effect (21,22). The only reported toxicity to normal cells is the induction of apoptosis in human primary hepatocytes (23) and brain cells (24) *in vitro*. To minimize such toxicities, we developed a radioresponsive gene expression system by controlling TRAIL expression mediated by Egr.1 promoter following irradiation. The six CArG (CC(A/T) 6GG) elements in the upstream sequence of Egr.1 promoter can mediate transcription of downstream gene (25). In our previous study (26), the induction response to irradiation peaked at 6 Gy, then decreased in a dose-dependent manner. Consistently, the activation of Egr-1 promoter was the highest after 24 h with 6 Gy irradiation, then decreased in a time-dependent manner. As in this study, the expression of TRAIL 24 h after 6 Gy irradiation was significantly higher (Fig. 1, P<0.05), and TRAIL under the control of radioresponsive promoter induced apoptosis and sensitized radiotherapy in SW480 cell line.

An enhanced anticancer effect of the combination of TRAIL gene therapy with radiotherapy has been reported in various cancer cells, including breast cancer (27), neurogliocytoma (28), leukemia, and lymphoma (29) cells. Our results also showed a similar effect on colon cancer cells. We thought such effects could be explained by the following mechanisms. First, TRAIL and radiation activate distinct apoptotic pathways but result in an amplified response (30). TRAIL binds with DR4/DR5 and activates caspase-8, subsequently activates caspase-3, resulting in activation of a cascade of caspases (31). In addition, caspase-8 can also activate Bid that triggers cytochrome c release, with subsequent activation of caspase-9 and caspase-3, thereby strongly amplifying the initial apoptotic signal (32). However, radiation activates the p53-dependent apoptosis pathway by direct DNA damage (33), loss of mitochondrial membrane potential and cyto-chrome c releasing (34,35), which led to caspase-9 activation (36). Alternatively, radiation may directly damage the cellular membrane resulting in the release of ceramide (37), which can directly damage mitochondrial membrane and then stimulate apoptosis by releasing cytochrome c. Therefore, distinct apoptotic pathways induced by combination of TRAIL with radiotherapy will exert synergistic effects on trans-formed cells. Second, It is reported that DR5 overexpression in TRAIL resistant cancer cells could restore TRAIL sensitivity (38,39). Other studies and our previous study confirmed ionizing radiation could up-regulate expression of DR5 (12,13), which indicates radiotherapy may overcome the TRAIL resistance in malignant cells. Moreover, ionizing radiation can improve transfection efficiency (40). Finally, TRAIL gene therapy and radiotherapy target different phases of the cell cycle. The G1 or S phases being most responsive to TRAIL (41,42) and the G1 and G2 phases being most radiosensitive (43,44). Our data showed a higher proportion in S phase after irradiation and a higher proportion in G1 phase with transfection of pEgr.1-TRAIL (Fig. 3), which demonstrated a synergistic and additive effect on inducing apoptosis.

In summary, our data showed that tumor-specific TRAIL gene mediated by radioresponsive Egr.1 promoter sensitized SW480 cells to radiotherapy. pEgr.1-TRAIL may increase the therapeutic efficacy of ionizing radiation or reduce radiation-mediated side effects, and radiotherapy may also overcome the TRAIL resistance in highly malignant cancer cells. It indicates that radioresponsive gene therapy may have great potential as a novel therapy because this therapeutic approach can be spatially or temporally controlled by radiotherapy and appears to have better specificity and safety.

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