REG I enhances chemo- and radiosensitivity in squamous cell esophageal cancer cells

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Identification of reliable markers of chemo- and radiosensitivity and the key molecules that enhance the susceptibility of squamous esophageal cancer cells to anticancer treatments would be highly desirable. To test whether regenerating gene (*REG*) I expression enhances chemo- and radiosensitivity in esophageal squamous cell carcinoma cells, we used MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays to compare the chemo- and radiosensitivities of untransfected TE-5 and TE-9 cells with those of cells stably transfected with REG Iα and Iβ. We then used flow cytometry to determine whether REG I expression alters cell cycle progression. No REG I mRNA or protein were detected in untransfected TE-5 and TE-9 cells. Transfection with REG Iα and Iβ led to strong expression of both REG I mRNA and protein in TE-5 and TE-9 cells, which in turn led to significant increases in both chemo- and radiosensitivity. Cell cycle progression was unaffected by REG I expression. REG I thus appears to enhance the chemo- and radiosensitivity of squamous esophageal cancer cells, which suggests that it may be a useful target for improved and more individualized treatments for patients with esophageal squamous cell carcinoma. (*Cancer Sci* 2008; 99: 2491–2495)

Thoracic squamous cell esophageal carcinoma is known for its rapid clinical progression and poor prognosis.[1,2] Esophagectomy with extensive lymph node dissection is performed as a standard treatment for thoracic esophageal cancer; however, this procedure induces severe surgical stress and depression of host immunological defenses, and is associated with various surgical complications.[3,4] On the other hand, there recently have been remarkable advances in definitive chemoradiotherapy (CRT) for thoracic esophageal cancer that offer patients a choice between surgery and definitive CRT as their first-line and primary treatments.[5] Esophageal squamous cell carcinomas generally show some degree of radiosensitivity, but individual tumors can exhibit widely differing susceptibility to radiotherapy; although CRT may be effective in some patients, others may show either no response or experience adverse effects. For those individuals, valuable time has been wasted, and the opportunity to obtain a potentially curative surgery may be lost.[6] Thus, identification of reliable markers of chemoradiosensitivity and the key molecules that enhance chemoradiosensitivity in esophageal cancer cells would be highly desirable and has long been sought.

The human regenerating gene (*REG*) family belongs to the lectin superfamily and encodes five small, secreted proteins. REG I was originally isolated as an endogenous growth factor from pancreatic islet β cells.[7–9] Since then, there have been many reports suggesting that REG plays important roles in tissue regeneration, cell proliferation, differentiation, mitogenesis, and carcinogenesis in various gastric and enteric tissues.[10–16] Among the various functions of REG I, we focused on the relationship between REG I and chemo- and radiosensitivity because our earlier clinical study suggested that REG Iα expression in squamous cell esophageal carcinoma cells was a reliable marker of chemoradiosensitivity in advanced esophageal squamous cell cancer patients.[17,18] That study was the first clinical report to show a correlation between survival and/or chemoradiosensitivity and REG I expression in thoracic squamous cell esophageal cancer. To confirm those clinical results and to test whether REG I expression enhances chemo- and radiosensitivity in esophageal squamous cell carcinoma cells, we have now carried out the present *in vitro* study.

Materials and Methods

Cell lines and culture. We obtained the TE-5 and TE-9 esophageal squamous cell carcinoma lines from the Cell Resource Center for Biochemical Research Institute of Development, Aging, and Cancer at Tohoku University, Japan[19] All cells were cultured in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics (penicillin G/streptomycin/amphotericin B; Gibco) in a humidified incubator at 37°C under an atmosphere of 5% CO_2/95% air.

Isolation of stable transfectants expressing REG I protein. cDNA fragments encoding human REG Iα or REG Iβ (nucleotides 15–597 of M18963 and nucleotides 58–619 of D16816, respectively) were inserted into the XhoI/XbaI site of the pCI-neo mammalian expression vector (Promega, Madison, WI, USA). The expression vectors or control vector (without insert DNA) were then introduced into TE-5 and TE-9 cells by electroporation, after which the cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum and 500 μg/mL Geneticin (Invitrogen, Grand Island, NY, USA) for 2 weeks. Stable transfectants expressing REG I protein were identified by immunoblot analysis of the culture medium prior to their isolation.[20–22]

Immunoblot analysis. Cells were cultured in 10-cm dishes for 24 h, after which serum-free RPMI-1640 medium was added, and the cells were cultured for an additional 48 h. The supernatant was then collected, and the protein concentration was determined. Samples of extract containing 20 μg of protein were then fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA, USA), after which the membranes were incubated first with anti-human REG I antibody (diluted 1:500 in TBS) for 1 h and then with peroxidase-conjugated secondary anti-mouse IgG (diluted 1:1000 in TBS) for 1 h. Immunodetection was accomplished

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Table 1. Primer sequences used for real-time reverse transcriptase–polymerase chain reaction

	Primer sequence (5′–3′)	Size
REG Iα	5′-AACATGAATTCGGGCAACC-3′ 5′-AGGAGAACTTGTCTTCACAA-3′	480
REG Iβ	5′-GCAAGAGATTCACTGCCGCTAA-3′ 5′-GCAGGACCAGTTCTAGACATCC-3′	397
GAPDH	5′-CGGAGTCAACGGATTTGGTCGTAT-3′ 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′	306

using an ECL Western blotting detection reagents and analysis system (GE Healthcare, Waukesha, WI, USA). The membranes were subsequently exposed to X-ray film.

Reverse transcriptase–polymerase chain reaction. Total RNA was isolated from each cell type using ISOGEN (Nippon Gene Co., Tokyo, Japan). cDNA was then reverse-transcribed from 1-µg samples of total RNA using a SuperScriptIII reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was then carried out using the primers shown in Table 1 and Platinum Taq DNA polymerase (Invitrogen). The amplification protocol entailed incubation at 94°C for 2 min, followed by 35 cycles (for REG Iα and REG Iβ), 55°C for 30 s, and 30 cycles (for glyceraldhyde-3-phosphate dehydrogenase [GAPDH]) at 94°C for 30 s or 72°C for 60 s. The amplified products were subjected to 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Radiotherapy and chemotherapy for cultured cells. Cells were exposed to 0, 5, or 10 Gy radiation using a SOFTEX M-100WE operating at 100 KV and 5 mA, which delivered the dose at 0.01 Gy/min. Chemotherapy involved the application of 5-fluorouracil (5-FU; Kyowa Hakko Kogyo Co., Tokyo, Japan) and cisplatin (CDDP; Nihonkayaku Co., Tokyo, Japan) to the cultures to final concentrations of 0.1, 1.0, 5.0, or 10 µM.

Cell proliferation assay. Cell proliferation was assessed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cells were plated in 96-well plates to a density of 1×10^3 cells/well and incubated for 0, 1, 2, 3, or 4 days. After addition of 10 µL of Cell Counting Kit-8 reagent, the cells were incubated for 2 h, after which the optical densities of the plates were read at 450 nm using a Model 550 Microplate Reader (Bio-Rad Laboratories).

Cell viability was assessed using colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays (Sigma-Aldrich). Cells were plated in 96-well plates to a density of 1×10^3 cells/well and incubated for 24 h at 37°C. They were then irradiated at 0, 5, or 10 Gy, and treated with one of the indicated concentrations of either CDDP or 5-FU. For concurrent therapy, the cells were plated as described above, and CDDP (0–10 µM) was added to the cultures; then 4 h later the cells were irradiated (5 Gy). Following incubation for an additional 72 h, 10 µL of 5.5 mg/mL MTT was added to the cultures, which were then incubated for 4 h at 37°C. Thereafter, 90 µL of extraction solution (40% N,N-dimethylformamide, 2% CH_3COOH, 20% SDS, and 0.03 N HCl) was added, and the mixture was incubated for 2 h at room temperature. Finally, cell viability was determined by measuring the optical density at 570 nm using a microplate reader.

Flow cytometry. Cells (1×10^3) were cultured in 6-cm dishes for 2 days at 37°C, trypsinized, rinsed in phosphate-buffered saline (PBS), and fixed in 70% ethanol. Prior to analysis, the cells were centrifuged to remove the ethanol and resuspended in 500 µL of 50 µg/mL propidium iodide, 20 µg/mL RNase A, 0.1% (w/v) sodium citrate, and 0.3% (w/v) Nonident P-40. After incubation for 1 h at 37°C, the cells were examined using a flow cytometer (Cytomics FC500; Beckman Coulter, Miami, FL, USA). The data obtained were analyzed using CXP software version 2 (Beckman).

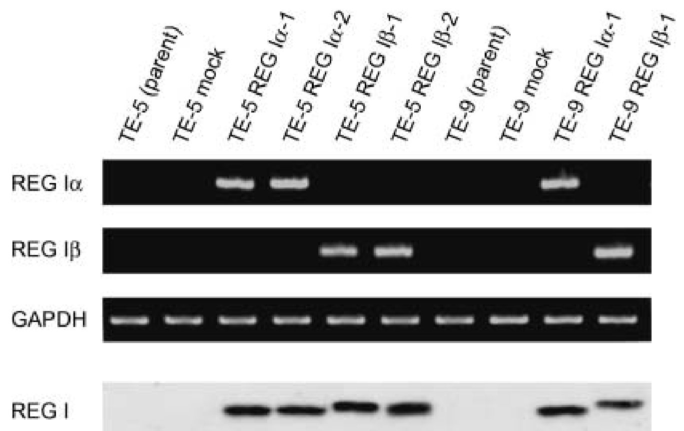

Fig. 1. TE-5 and TE-9 cells stably transfected with regenerating gene (REG) Iα and REG Iβ DNA expressed REG I mRNA and protein.

Statistical analysis. Data were expressed as mean values. Significant differences between groups were assessed using one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test and χ^2-test (Stat View J-5.0; Abacus Concepts, Berkeley, CA, USA). Values of $P < 0.01$ were considered significant.

Results

Chemo- and radiosensitivity of cells stably transfected with REG I. We stably transfected TE-5 and TE-9 cells with REG Iα and REG Iβ DNA, after which the expression of REG I mRNA and protein was assessed. TE-5 and TE-9 transfectants (TE-5 REG Iα/TE-5 REG Iβ and TE-9 REG Iα/TE-9 REG Iβ cells) showed stronger expression of REG I than control cells, or cells transfected with the neomycin-resistance gene alone (mock-transfected) (Fig. 1). When we measured the chemo- and radiosensitivities of the transfectants in MTT assays, we found that there were no differences between the growth rates of cells stably transfected with REG I and the control cells (TE-5 mock and TE-9 mock) (Fig. 2a). On the other hand, both TE-5 REG Iα/TE-5 REG Iβ and TE-9 REG Iα/TE-9 REG I β cells showed a significant increase in radiosensitivity (5 and 10 Gy) and chemosensitivity (10 µM CDDP), as compared with TE-5 mock and TE-9 mock cells (Fig. 2b,c). And in concurrent experiments, TE-5 REG Iα/TE-5 REG Iβ and TE-9 REG Iα/TE-9 REG I β cells showed significantly higher chemoradiosensitivity than control cells (Fig. 2d). Thus exogenous expression of REG I by esophageal cancer cells enhanced their chemo- and radiosensitivity.

Cell cycle progression in TE cells stably transfected with REG I. Next, we used flow cytometry to test whether cell cycle progression was altered by REG I transfection. We found that all of the cell lines had very similar cell cycle distribution profiles, indicating that REG I expression does not affect cell cycle progression (Table 2).

Discussion

In the present study, we showed that REG I transfection enhanced both chemo- and radiosensitivity in squamous cell esophageal cancer.

Cellular responses to DNA damage constitute an important field in cancer biology. Most therapeutic modalities currently used to treat malignancies, including radiation therapy and many chemotherapeutic agents, target DNA. When normal cells are damaged by radiation or chemotherapeutic agents, cell-cycle checkpoints sense DNA damage and activate pathways that lead to DNA repair.[23,24] Cancer cells that are highly proliferative are

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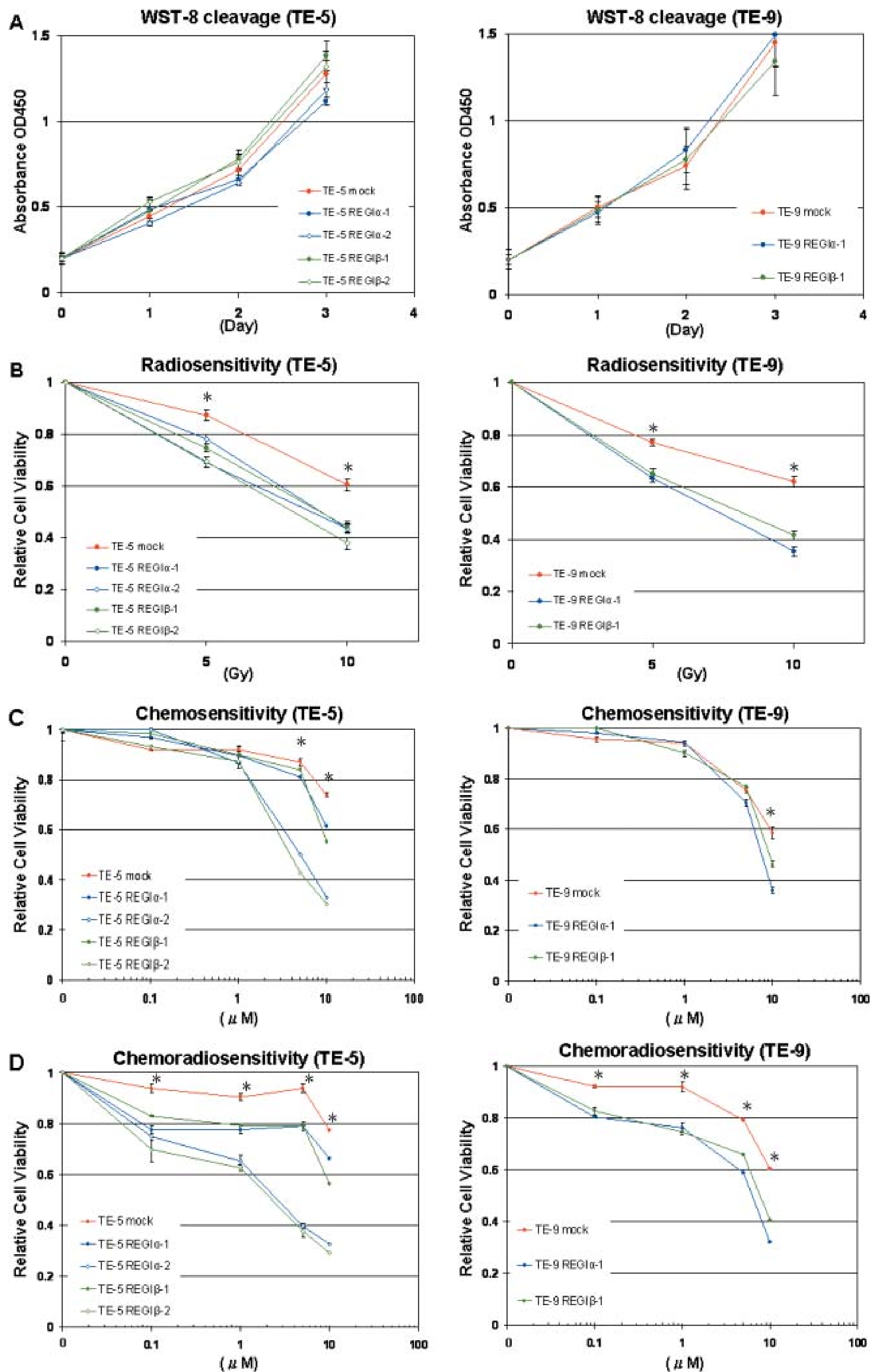

Fig. 2. Proliferation of esophageal cancer cells stably transfected with regenerating gene (REG) I was measured as a function of WST-8 cleavage (a). Cell viability was assessed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays. Cells were treated with radiation at dose of 5 Gy or 10 Gy (b), with 0.1, 1.0, 5.0, or 10 μM cisplatin (c), or with a combination of 5 Gy radiation and 0.1, 1.0, 5.0, or 10 μM cisplatin (d). REG I transfectants were significantly more susceptible to chemo-, radio- and chemoradiotherapy than control cells. All results are expressed as the means ± SD of 10 samples; $^*P < 0.01$.

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Table 2. **Cell-cycle distribution in control cells and regenerating gene (REG) I stable transfectants**

Cell lines	G1/G0 (%)	S (%)	G2/M (%)
TE-5 (parent)	34	23	43
TE-5 mock	38	25	37
TE-5 REG Iα-1	40	36	34
TE-5 REG Iα-2	40	25	35
TE-5 REG Iβ-1	42	24	34
TE-5 REG Iβ-2	38	27	35
TE-9 (parent)	39	28	33
TE-9 mock	44	25	31
TE-9 REG Iα-1	42	27	31
TE-9 REG Iβ-1	40	29	31

There were no significant differences.

generally considered to be more susceptible to DNA damage because checkpoint dysfunction leads to enhanced cell cycle progression, which makes it more likely that there will be insufficient time to repair DNA damage and circumvent apoptosis.[25] Thus the loss of cell-cycle checkpoint responses likely results in increased cellular susceptibility to chemoradiotherapy, particularly if the affected checkpoint controls the G2 transition.

The molecular basis of both radiosensitivity and chemosensitivity is a complex product of both cellular and tissue responses. Among the affected molecules, p53 is reported to have a major impact on the cellular responses to ionizing radiation and cytotoxic drugs, but there are inconsistencies in the reported mechanisms by which p53 affects cell survival.[26] When we compared the expression of p53 mRNA and protein in REG-I transfected and mock-transfected TE-5 cells, we found no difference in the p53 status (data not shown). Moreover, Barnas *et al*. reported that TE-9 cells express truncated forms of p53 as a result of frameshift mutations and that these truncated forms did not exhibit p53-DNA binding activity.[27] We therefore suggest that REG I enhances chemo- and radiosensitivity in a manner that is independent of p53. Numerous other molecules are known to be involved in the cellular responses to anticancer therapy, including those encoded by cyclin D, p21, p16, ras, raf-1, bcl-2, Ki-67, EGFR, RUNX3, TRAIL, and PARP, among others, but the molecular mechanisms involved remain unclear.[28–38] Overall, there is much that is still not understood about the complex roles played by many proteins during the various responses to treatment.

The results of this *in vitro* study demonstrate that REG I could enhance the sensitivity of squamous cell esophageal carcinoma cells to anticancer treatments. By contrast, Mitani *et al*. reported that overexpression of REG IV inhibited 5-FU-induced apoptosis.[39] While the detailed mechanisms are unclear, it appears that the REG family can have opposing effects on chemo- and/or radiosensitivity. For instance, Takasawa *et al*. demonstrated that REG activates cyclin D1 signaling, which correlates with cell cycle progression and radiosensitivity,[40] whereas Sekikawa *et al*. reported that the REG I mediates the anti-apoptotic effects of STAT3 signaling by enhancing Akt activation, Bad phosphorylation, and Bcl-xL expression.[41] Considering that both the cyclin D1 and Akt/Bad/Bcl-xL pathways can be activated by REG, the sensitivity of cancer cells to particular chemotherapeutic agents may reflect the relative impacts of these and other signaling pathways.

In conclusion, the present results demonstrate that REG I enhances the susceptibility of cancer cells to anticancer treatments. REG I may thus be a useful target for anticancer therapy, enabling us to design better, more individualized treatments for patients with advanced esophageal squamous cell carcinoma.

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