

## REG I $\alpha$ PROMOTES PD-L1 EXPRESSION IN ESOPHAGEAL CANCER CELLS

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(received 18 December 2014, accepted 26 December 2014)

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### Abstract

Regenerating gene (REG) I $\alpha$  is known to contribute to carcinogenesis and to be associated with a poor prognosis in various cancers. Programmed death-1 ligand (PD-L1) is a negative regulator of T cell activation thought to play an important role in tumor evasion from host immunity. In the present study, we tested the hypothesis that the pro-survival effects of REG I $\alpha$  in cancer reflect enhanced expression of PD-L1. We found that PD-L1 mRNA expression tended to correspond to REG I $\alpha$  mRNA levels in esophageal squamous cancer cells, and that REG I $\alpha$  expression significantly increased expression of both PD-L1 mRNA and protein in TE-5 and TE-9 squamous esophageal cancer cells transfected with REG I $\alpha$ . In addition, immunohistochemical analysis of squamous cell esophageal cancer specimens revealed that the spatial distribution of PD-L1 expression corresponded to that of REG I $\alpha$ . These findings suggest that REG I $\alpha$  may suppress anti-tumor immunity by inducing PD-L1 expression.

**Key words :** PD-L1, REG I $\alpha$ , esophageal cancer

### Introduction

Regenerating gene (REG) was first identified upon screening a regenerating pancreatic islet-derived cDNA library taken from a 90% depancreatized rat<sup>1)</sup>. Since then it has been learned that the REG family is composed of various acute phase reactants, lectins, anti-apoptotic factors and growth factors affecting pancreatic islet cells, neural cells, and epithelial cells within digestive system<sup>2,3)</sup>. The first REG I genes identified encoded REG I $\alpha$  and REG I $\beta$ . Although the effects of REG I $\alpha$  expression in inflammatory disease<sup>4)</sup> and carcinogenesis in gastroenterological tissues<sup>5-7)</sup> have been investigated, the bi-

ological functions of the protein are still not fully understood. There have been reports that REG I $\alpha$  exerts a trophic effect during carcinogenesis. Consistent with that idea, the survival rate is better among patients with REG I $\alpha$ -negative lung<sup>8)</sup>, stomach<sup>9,10)</sup>, colon<sup>11)</sup>, bile duct<sup>12)</sup> and breast<sup>13)</sup> tumors than among those with REG I $\alpha$ -positive tumors.

Programmed death-1 (PD-1) is a costimulatory molecule expressed on T-cells, B cells and myeloid cells that provides an inhibitory signal during T-cell activation<sup>14,15)</sup>. The PD-1 ligands, PD-L1 and PD-L2, are cell surface glycoproteins belonging to the B7 family<sup>16-19)</sup>. Previous studies have shown that PD-1/PD-L1 ligation inhibits T-cell growth and cytokine secretion<sup>17,19)</sup>. Moreover, recent studies suggest tumor-associated PD-L1 induces apoptosis in tumor-reactive T cells<sup>20)</sup>, thereby enabling tumors to evade host immune defenses and grow. In the present study, we tested the hypothesis that REG I $\alpha$  positively regulates PD-L1 expression resulting in tumor immunotolerance.

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## Materials and Methods

### Cell lines and culture

We obtained the TE-5, TE-9 and TE-12 esophageal cancer cell lines from the RIKEN Bio Resource Center, Tsukuba, Japan and the Cell Resource Center for Biochemical Research Institute of Development, Aging, and Cancer at Tohoku University, Japan. All cells were cultured in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX) and antibiotics (penicillin G/Streptomycin/amphotericin B; Gibco) in a humidified incubator at 37°C under an atmosphere of 5% CO<sub>2</sub>/95% air.

### Establishment of transfectants stably expressing REG Ia

cDNA fragments encoding human REG Ia (nucleotides 15-597 of M18963) were inserted into the *XhoI/XbaI* site in pCI-neo (Promega, Madison, WI, USA). The resultant mammalian expression vector or the control vector (without inserted DNA) was then introduced into TE-5 and TE-9 cells using electroporation, after which the cells were cultured for 2 weeks in RPMI-1640 supplemented with 10% FBS and 500 µg/mL Geneticin (Invitrogen, Grand Island, NY, USA). The selected Geneticin-resistant clones were then harvested (TE-5 REG Ia and TE-9 REG Ia cells) using cloning cylinders.

### Real-time RT-PCR assays

Our past investigation indicates that high expression levels of REG Ia mRNA are admitted in TE-12 cells while small quantity of expressions are detected in TE-5 cells. To seek the relationships between expressions of REG Ia and PD-L1, we first assessed these two cell lines. Subsequently, we applied TE-5 and TE-9 REG Ia transfected cells to analyze the effect of REG Ia on PD-L1 expressions. Endogenous expression of REG Ia and PD-L1 mRNA was assessed by determining the REG Ia/ $\beta$ 2-microglobulin and PD-L1/ $\beta$ 2-microglobulin mRNA ratios. The primer sequences used to amplify human REG Ia, PD-L1 (CD274) and  $\beta$ 2-microglobulin mRNA and their Universal Probe Library (Roche Applied Science) numbers were follows: for REG Ia, CCTCCAT

GACCCCAAAAAG (F) and AGCCAGGATTAACACT GCTTG (R), Universal Probe Library #54; for PD-L1 GGCATCCAAGATACAAACTCAA (F) and CAGAAGTT CCAATGCTGGATTA (R), Universal Probe Library #25; for  $\beta$ 2-microglobulin, TTCTGGCCTGGAGGC TATC (F) and TCAGGAAATTTGACTTTCCATTC (R), Universal Probe Library #42. Total RNA was isolated from each cell type using TRIzol reagent (Invitrogen, Grand Island, NY) and a Purelink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. After quantifying the isolated RNA using a spectrophotometer, 2-µg aliquots were reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using a Light Cycler 480 Real-time PCR System (Roche Diagnostics). After 5 min of initial denaturation at 95°C, the cycling protocol entailed 45 cycles of denaturation at 95°C for 10 s and annealing and elongation at 60°C for 30 s. The ddCT method was employed for comparative mRNA analysis. As an internal control, all samples were normalized to the endogenous housekeeping gene  $\beta$ 2-microglobulin. All experiments were repeated three times for each cell line with consistent results ( $n=3$ ).

### Immunoblot analysis

Cells were cultured in 10-cm dishes for 24 h, after which serum-free RPMI1640 medium was added and the culture was continued for an additional 48 h. The cells were then collected and lysed in lysis buffer supplemented with protease inhibitors. Aliquots containing 10 µg of protein were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (ATTO, Tokyo, Japan), which were then blocked for 1 h with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). The membranes were then incubated with anti-human REG I antibody (1 : 500 dilution), which we recently purified, or with mouse monoclonal anti- $\beta$ -actin antibody (1 : 5,000, A5441; SIGMA, St. Louis, MO) overnight at 4°C. This was followed by incubation for 1 h with peroxidase-conjugated anti-mouse IgG as the secondary antibody (1 : 1,000 dilution, P0447; DAKO, Glostrup, Denmark). Membranes were also

probed using rabbit anti-PD-L1 (1 : 1,000 dilution, 13684 ; Cell Signaling Technology) monoclonal antibody diluted in TBS-T containing 5% BSA. Peroxidase-conjugated anti-rabbit IgG (1 : 3,000 dilution, P0399 ; DAKO) served as the secondary antibody in that case. Immunodetection was accomplished using an ECL Western Blotting Detection System (GE, Healthcare, Waukesha, WI).

### Immunohistochemistry

To assess the association between PD-L1 and REG Ia expressions, we enrolled squamous cell esophageal cancer patient who received primary esophagectomy. Resected tumors were fixed in formalin and embedded in paraffin. After cutting the tumors into 4- $\mu$ m-thick sections, the sections were deparaffinized in xylene and ethanol, placed in 10 mmol/L Tris buffer (pH 9.0) containing 1 mmol/L EDTA and irradiated with microwaves (750 W) for 5 min. Endogenous peroxidase activity was blocked by incubating the sections for 15 min in 3% H<sub>2</sub>O<sub>2</sub>, and nonspecific binding was blocked by incubation for 30 min in 10% goat serum (Nichirei, Tokyo, Japan). The specimens were then incubated for 60 min with rabbit monoclonal anti-PD-L1 antibody (1 : 200 dilution, 13684 ; Cell Signaling Technology) as the primary antibody. This was followed by incubation first in blocking buffer and then with a peroxidase-conjugated, anti-rabbit antibody (Histofine Mouse stain Kit®, Nichirei) for 30 min each.

The sections were then developed by incubation for 5 min with 3,3'-diaminobenzidine tetrahydrochloride (Nichirei). For REG Ia staining, all immunohistochemical processes were performed automatically using a VENTANA BenchMark XT IHC/ISH Staining Module (Roche Applied Science, Penzberg, Germany). Briefly, tissue sections were incubated with anti-human REG Ia antibody (2.5  $\mu$ g/mL, rabbit polyclonal, BioVendor, Candler, NC) for 32 min at 37°C. The antigen was then visualized using biotin, HRP-conjugated streptavidin and DAB peroxidase substrate according to manufacturer's instructions. Finally, the sections were counterstained with Gill hematoxylin, dehydrated and mounted.

### Statistical analysis

Data are expressed as the mean  $\pm$  the standard deviation. The significance of differences between two groups was assessed using Student's *t* test. All analyses were performed using JMP 10 (SAS Institute, Cary, NC), which yielded two-sided *p* values. Values of *p* < 0.05 were considered significant.

### Results

#### Expression of REG Ia and PD-L1 mRNA in esophageal cancer cell lines

TE-5 and TE-12 cells expressed detectable levels of

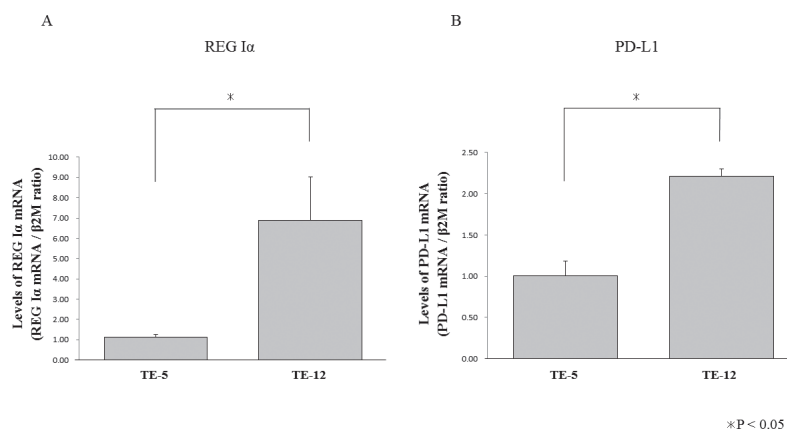


Fig. 1. Expression of REG Ia (Fig. 1A) and PD-L1 (Fig. 1B) mRNA. Three esophageal cancer cell lines were applied for analysis. Correspondence between expression levels of REG Ia and PD-L1 mRNA was detected. Significantly elevated levels of REG Ia and PD-L1 mRNAs expression were seen in TE-12. *p* < 0.05 for TE-12 vs. TE-5 cells.

REG  $\alpha$  and PD-L1 mRNA. Moreover, there tended to be correspondence between expression levels of PD-L1 and REG  $\alpha$  mRNA, and significantly higher levels of both REG  $\alpha$  and PD-L1 mRNA were seen in TE-12 cells than TE-5 cells (Fig. 1).

### Transfection of esophageal cancer cells with REG $\alpha$

The established TE-5 REG  $\alpha$  transfectants showed significantly stronger expression of REG  $\alpha$  protein than cells transfected with empty vector (mock-transfected). REG  $\alpha$  expression was also much stronger in TE-5 REG  $\alpha$  cells than TE-9 REG  $\alpha$  cells.

### Expression of PD-L1 mRNA in REG $\alpha$ transfectants

We found that TE-5 REG  $\alpha$  cells showed significantly stronger expression of PD-L1 mRNA than mock-trans-

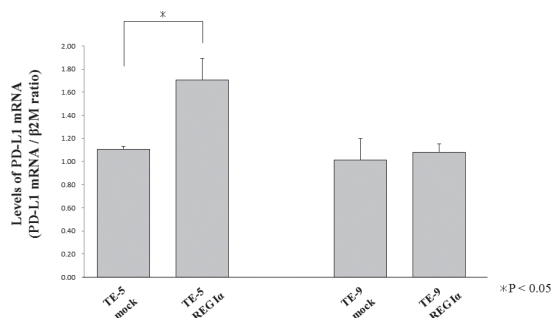


Fig. 2. TE-5 and TE-9 cells transfected with REG  $\alpha$  DNA showed stronger expression of PD-L1 mRNA than mock-transfected control cells. Expression levels were lower in TE-9 REG  $\alpha$  cells, which corresponds to the lower levels of REG  $\alpha$ .  $p < 0.05$  for REG  $\alpha$  transfected vs. mock-transfected cells.

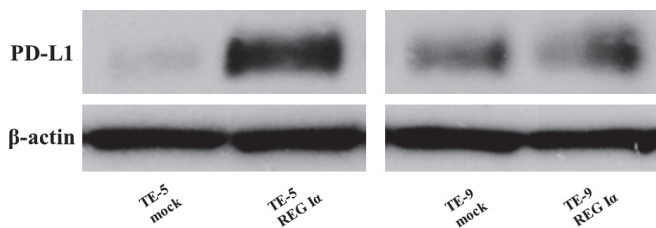


Fig. 3. Cells expressing REG  $\alpha$  DNA showed stronger expression of PD-L1 protein than mock-transfected control cells. Expression levels were lower in TE-9 REG  $\alpha$  cells, which corresponds to the lower levels of REG  $\alpha$ .

fected TE-5 cells (Fig. 2). TE-9 REG  $\alpha$  cells also expressed PD-L1 mRNA, but at much lower level than TE-5 cells. Western blot analysis of TE-5 REG  $\alpha$  and TE-9 REG  $\alpha$  cell lysates revealed PD-L1 levels to be elevated in both transfectant cell lines (Fig. 3). Moreover, there was good correspondence between the expression levels of REG  $\alpha$  and PD-L1.

### Expression of REG $\alpha$ and PD-L1 in clinicopathological specimens

Finally, we used immunohistochemistry to examine the distribution of REG  $\alpha$  and PD-L1 expression in three primary esophageal cancer specimens (Fig. 4). Note that the spatial distribution of PD-L1 within the tissue tended to correspond to the distribution of REG  $\alpha$ .

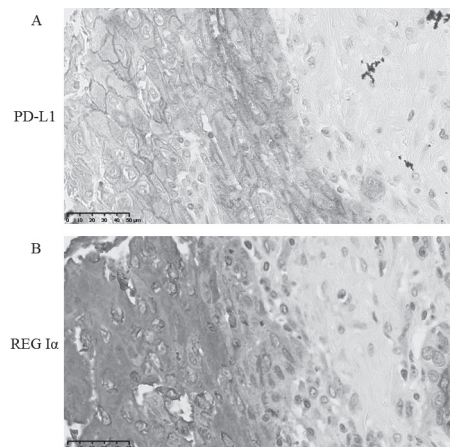


Fig. 4. Immunohistochemical analysis showing PD-L1 expression in squamous cell esophageal cancer tissue specimens. Areas of PD-L1 expression tended to correspond to the REG  $\alpha$ -positive areas.

## Discussion

Our findings demonstrate that transfecting esophageal cancer cells with REG  $I\alpha$  gene enhances their expression of PD-L1. This suggests that REG  $I\alpha$  activity may assist esophageal cancer cells to evade host immune defenses through PD-L1 expression.

Evidence from numerous preclinical models indicates that PD-L1 blockade using neutralizing antibodies has antitumor effects. For example, using a multistage model of squamous cell carcinoma, Belai, et al. found that immune neutralization of PD-1 delayed development and reduced the incidence of papillomas and suggested that PD-1/PD-L1 interaction contributes to the progression of squamous cell carcinoma through downregulation of antitumor responses<sup>21</sup>. Moreover, ligation of PD-L1 expressed on cancer cells to PD-1 expressed on T-cells suppresses T-cell activation and proliferation, and induces T-cell apoptosis<sup>20</sup>. In a clinical setting, stronger tumoral expression of PD-L1 correlates with a poor prognosis in several cancers, including esophageal cancer<sup>22-28</sup>. Based on these and other findings, it has been suggested that PD-L1 is key to a tumor's ability to evade the host immune system and that by precluding an optimal immune response, PD-1/PD-L1 signaling promotes tumor growth and metastasis.

In an earlier report, we demonstrated that REG  $I\alpha$ -transfected squamous cancer cells secrete excessive amounts of IL-6, a multifunctional cytokine originally characterized as a regulator of immune and inflammatory responses<sup>29</sup>. Notably, elevated expression of IL-6 has also been detected in various epithelial tumors, including esophageal cancer<sup>30-32</sup>. IL-6 increases tumor growth by upregulating pro-survival and pro-angiogenic genes via activation of STAT3<sup>33</sup> and induces cancer cell invasion through activation of the c-Src/RhoA/ROCK signaling pathway<sup>34</sup>. IL-6 levels also correlate with clinicopathological features of gastric and esophageal cancer, including tumor stage, depth of tumor, invasion and the presence of lymph node metastasis<sup>35,36</sup>. Expression of PD-L1 on LPS-treated tolerogenic antigen-presenting cells is reportedly regulated in an IL-6, IL-10 and STAT3 dependent manner<sup>37</sup>. In addition, Jin et al. observed that an IL-6-dependent signal is involved in PD-L1 expres-

sion in the central nervous system after Theiler's murine encephalomyelitis virus infection<sup>38</sup>. In that context, our present findings suggest REG  $I\alpha$  may regulate expression of PD-L1 by inducing increases in IL-6 secretion.

Patrella et al. previously reported that IL-1 $\beta$  activates C/EBP, which is a downstream mediator of p38 MAPK signaling<sup>39</sup>. In preliminary experiments, we observed that IL-1 $\beta$  mRNA expression and p38 MAPK phosphorylation are both elevated in REG  $I\alpha$  transfectants (data not shown). Although the pro-apoptotic and anti-proliferative actions of p38 MAPK have been well characterized<sup>40-42</sup>, the enzyme also mediates growth promoting and anti-apoptotic signaling<sup>43-45</sup>. Given the complexity of p38 MAPK's activities, it is not surprising that although REG  $I\alpha$  appears to increase levels of activated p38 MAPK, its contribution to malignancy in cancer remains controversial. We suggest REG  $I\alpha$  stimulates IL-6 secretion by inducing IL-1 $\beta$  and activating p38 MAPK. Consistent with that idea, recent studies have shown that p38 MAPK activation is associated with expression and secretion of IL-6 in myocardial cells, osteoblasts, bone marrow cells and gastric cancer cells<sup>46-48</sup>. This suggests that REG  $I\alpha$  plays a key role in the upregulation of PD-L1 and that its activities may also include induction of IL-1 $\beta$  and regulation of the p38 MAPK signaling pathway.

In conclusion, we suggest REG  $I\alpha$  suppresses antitumor immunity and promotes tumor progression by inactivating T cells through induction of PD-L1.

## Acknowledgements

This work was supported, in part, by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan.

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