REG Iα protein mediates an anti-apoptotic effect of STAT3 signaling in gastric cancer cells

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Signal transducer and activator of transcription 3 (STAT3) signaling plays roles in inflammation-associated carcinogenesis. Regenerating gene (REG) Iα protein, an interleukin (IL)-6-inducible gene, is suggested to be involved in the gastritis–gastric cancer sequence. We investigated the involvement of IL-6/STAT3 signaling in REG Iα protein expression and examined whether REG Iα protein mediates an anti-apoptotic effect of STAT3 signaling in gastric cancer cells. The effects of IL-6/STAT3 signaling on REG Iα protein expression were examined using a STAT3 small interfering RNA system in gastric cancer cells. The element responsible for IL-6-induced REG Iα promoter activation was determined by a promoter deletion assay. The anti-apoptotic effects of STAT3 signaling and its induced REG Iα protein were examined by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling and caspase assay in vitro. Human gastric cancer specimens were analyzed by immunohistochemistry for phosphorylated signal transducer and activator of transcription 3 (p-STAT3) and REG Iα protein. IL-6 treatment enhanced the expression of REG Iα protein through STAT3 activation in gastric cancer cells. The IL-6-responsive element was determined to lie in the sequence from −142 to −134 of the REG Iα promoter region. REG Iα protein mediated the anti-apoptotic effects of STAT3 signaling in gastric cancer cells by enhancing Akt activation, Bad phosphorylation and Bcl-xL expression. The expression of REG Iα protein was significantly correlated with that of p-STAT3 in gastric cancer tissues. REG Iα protein may play a pivotal role in anti-apoptosis in gastric tumorigenesis under STAT3 activation.

Introduction

Many recent studies have suggested that inflammation plays important roles in the development of various cancers. Signal transducer and activator of transcription 3 (STAT3), a major transcription factor for transmitting inflammatory cytokine signals to the nucleus, has been shown to promote cell proliferation and anti-apoptosis (1–4). Thus, STAT3 activation is suggested to be involved in carcinogenesis under a background of inflammation. In support of this idea, Judd et al. (5,6) have reported that mice showing STAT3 hyperactivation develop gastric tumors in association with chronic gastritis. However, it still remains unclear how activated STAT3 functions in the development of gastric cancer.

The regenerating gene (Reg), encoding a lectin-related protein, was originally isolated from a rat regenerating pancreatic islets, and its human homologue was named REG Iα (7). We previously reported that REG Iα protein is involved in the pathophysiology of gastrointestinal inflammation and its associated cancer (8–11), and furthermore that REG Iα gene expression in gastric cancer cells is strongly induced by interleukin (IL)-6 (9), which is a crucial proinflammatory cytokine for STAT3 signaling (12). Moreover, REG Iα protein is suggested to act as an anti-apoptotic factor in the development of gastric cancer (9). Taken together, it is tempting to speculate that REG Iα protein may be a target of STAT3 signaling and mediate the function of STAT3 signaling. Therefore, in the present study, we examined the mechanism responsible for regulation of REG Iα protein expression by IL-6/STAT3 signaling and tried to elucidate whether REG Iα protein mediates the anti-apoptotic effect of STAT3 signaling in gastric cancer cells. Moreover, to clarify the pathophysiological significance of REG Iα expression linked to STAT3 activation, we examined the expression of STAT3 and REG Iα protein in human gastric cancer tissues.

Materials and methods

Reagents and cell culture

Human IL-6 was purchased from Roche (Mannheim, Germany). Janus kinase inhibitor, AG490, was from Wako Pure Chemical Industries Ltd (Osaka, Japan). Anti-STAT3, tyrosine-phosphorylated signal transducer and activator of transcription 3 (p-STAT3 (Tyr705)), serine-phosphorylated STAT3 (p-STAT3 (Ser727)), extracellular signal-regulated protein kinase (ERK), phospho-specific ERK (p-ERK), Akt, phospho-specific Akt (p-Akt; Ser473) and phospho-specific Bad (p-Bad; Ser112) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-Bcl-2, Bcl-xL and Mcl-1 antibodies were obtained from BD Sciences (San Jose, CA). Anti-β-actin antibody and mitogen-activated protein kinase kinase inhibitor PD 98059 were purchased from Sigma Chemical Co. (St Louis, MO). Gastric cancer cell lines AGS and MKN74 were maintained routinely in Ham’s F12 medium (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Sigma Chemical Co.) and RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum, respectively, in a humidified incubator at 37°C with an atmosphere of 5% CO2.

Western blot analysis

Following treatment with or without reagents, cells were lysed in a protein extraction buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40, 50 mM NaF and 1× proteinase inhibitor (Complete Mini; Roche). Protein extract (20 μg) was fractionated by sodiumdodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with a primary antibody and then with a peroxidase-conjugated secondary antibody. Proteins were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Promoter assay

The fragment of the human REG Iα promoter from −1195 to +78 (−1195/+78) was cloned and then inserted between the Mfu I and Bgl II restriction sites, upstream of the luciferase gene in the pGL3-Basic vector (Promega, Madison, WI) as reported previously (9). To obtain the deletion constructs, REG Iα promoter fragments of −811/+78, −566/+78, −339/+78, −211/+78, −153/+78, −131/+78 and −55/+78 were also cloned into the same sites of the pGL3-Basic vector. Mutant plasmids were constructed to monitor the function of the sequences in the REG Iα promoter corresponding to the potential IL-6 response element. Plasmids −153M1 and −153M2 were generated, in which the IL-6 response element was modified. The following oligonucleotides were used: for −153M1, 5′-CCTCCAGTGGTGCCATCAGAAGG-3′; for −153M2, 5′-CCTCCAGTGCCATGAGAAGG-3′, the underlined sequences corresponding to modified regions. The oligonucleotide used for the opposite orientation was 5′-CCCGAAGATTTTAGATCTAGAGTGC-3′ in all

Abbreviations: ERK, extracellular signal-regulated protein kinase; IL, interleukin; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; PCR, polymerase chain reaction; p-Akt, phospho-specific Akt; p-Bad, phospho-specific Bad; p-ERK, phospho-specific extracellular signal-regulated protein kinase; p-STAT3, phosphorylated signal transducer and activator of transcription 3; REG, regulating gene; REG-R, regulating gene receptor; siRNA, small interfering RNA; ssDNA, single-stranded DNA; STAT3, signal transducer and activator of transcription 3; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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cases. After cloning and confirmation of the nucleotides of the human REG Iα promoter by sequencing, the construct was named hREG Iα-Luc.

MKN74 cells (4 × 10^5) were co-transfected with 700 ng of hREG Iα-Luc and 7 ng of Renilla luciferase plasmid pRL-TK in Lipofectamine 2000 transfection reagent (Invitrogen). To inhibit the STAT3 signaling, 350 ng of human STAT3 small interfering RNA (siRNA) (Qiagen, Hilden, Germany) was also transfected. Forty-eight hours later, the cells were stimulated with IL-6 for 12 h.

Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Both firefly luciferase and Renilla luciferase activities were assayed by a luminometer (Lumat LB 9507; Berthold, Germany). The results obtained were normalized for Renilla luciferase activity and expressed relative to the activity of the untreated cell group transfected with hREG Iα (∼1195/ +78)-Luc.

Electrophoretic mobility shift assay

DNA probes for electrophoretic mobility shift assay were synthesized as oligonucleotides. The sequences of the individual oligonucleotides in the sense orientation were as follows: probe wild type, 5'-AGTGTGTGCCCGG-GAAAAGG-3' corresponding to nucleotides 148 to 130 of the REG Iα promoter gene; probe M1, 5'-AGTGTGTGCCCATCGAAAGG-3' and probe M2, 5'-AGTGTGTGAGTGGAAAGG-3'. AGS cells were pre-treated with or without STAT3 siRNA, followed by stimulation with IL-6 (1000 U/ml) for 30 min or 24 h. Nuclear extract was extracted as described previously (13). Electrophoretic mobility shift assay was carried out with a Gel Shift Assay System (Promega) in accordance with the manufacturer’s recommendation. Briefly, the probes were incubated with 5'-end labeling with [γ-32P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (Promega). The nuclear protein (10 μg) was pre-incubated in a final volume of 9 μl of buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM MgCl2, 50 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid, 4% glycerol, 0.5 mM dithiothreitol and 0.5 mg of poly (dl-dc) for 10 min at room temperature. Then, the 32P-labeled probe was added to the mixture, followed by incubation for 20 min at room temperature. The protein–DNA complexes were electrophoresed on a 6% acrylamide gel, and the gels were dried before autoradiography.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay

The full-length human REG Iα cDNA was inserted in the pIRE2-EGFP vector (Clontech, Palo Alto, CA). The construct was named pIRE2-hREG Iα, and the pIRE2-EGFP vector without an insert was used as control. AGS cells, stably transfected with pIRE2-hREG Iα (AGS-REG Iα) or pIRE2-EGFP (AGS-EGFP) vectors, were prepared as described previously (9). After AGS-REG Iα or AGS-EGFP, cells were transfected with STAT3 siRNA (or non-silencing siRNA as a control), and the cells were cultured in four-well culture slides (Falcon, Bedford, MA) in serum-free medium with or without an anti-REG Iα antibody (50 μg/ml) for 18 h. The slides were washed with phosphate-buffered saline, fixed with 10% buffered formalin for 15 min and then treated with deoxyribonuclease- and neuraminidase- treated REG Iα by a STAT3-dependent mechanism

The effect of IL-6 treatment on p-STAT3 (Tyro705) and pSTAT3 (Ser727), p-ERK and REG Iα protein expression was examined in two gastric cancer cell lines. In both AGS and MKN74 cells, the expression of p-ERK, p-STAT3 (Tyro705) and p-STAT3 (Ser727) was enhanced from 5 min after IL-6 stimulation (10–1000 U/ml) and then remained constant up to 24 h after IL-6 stimulation (Figure 1A and D). The expression of REG Iα was also increased in AGS and MKN74 cells at 12 and 24 h after IL-6 stimulation (Figure 1C and D).

We next examined the link between ERK and STAT3 signaling in IL-6-treated gastric cancer cells. As shown in Figure 2A and B, IL-6 stimulation enhanced p-STAT3 (Ser727) and p-ERK expression in gastric cancer cells simultaneously. In addition, the inhibition of ERK phosphorylation by PD 98059 strongly decreased not only the increased expression of p-ERK but also p-STAT3 (Ser727) in IL-6-stimulated gastric cancer cells, suggesting that ERK signaling is associated with the enhancement of STAT3 (Ser727) phosphorylation by IL-6 stimulation. On the other hand, it was noteworthy that inhibition of ERK signaling did not affect STAT3 (Tyro705) phosphorylation (Figure 2A and B) and REG Iα protein expression (Figure 2C and D) in IL-6-stimulated gastric cancer cells. Together, these findings suggest that enhancement of REG Iα protein expression
IL-6-stimulated gastric cancer cells is due mainly to activation of STAT3 (Tyr705) phosphorylation.

We then examined whether inhibition of STAT3 signaling affected REG Iα expression in gastric cancer cells. As shown in Figure 3A and B, the basal expression of p-STAT3 (Tyr705) in untreated gastric cancer cells was abolished by treatment with the Janus kinase inhibitor tyrphostin AG490. Moreover, the increased level of p-STAT3 (Tyr705) in IL-6-treated gastric cancer cells was inhibited by concomitant administration of AG490 (25–100 μM). The increased expression of REG Iα protein in IL-6-treated gastric cancer cells was decreased by concomitant administration of AG490 at a dose of 25 μM. Furthermore, 100 μM AG490 abolished REG Iα expression in not only untreated but also IL-6-treated gastric cancer cells (Figure 3C and D).

Using an siRNA system for STAT3, we also confirmed the contribution of STAT3 signaling to IL-6-induced REG Iα protein expression in gastric cancer cells. Indeed, treatment with STAT3 siRNA abolished the increase of p-STAT3 (Tyr705) and REG Iα expression (Figure 3E and F).

IL-6/STAT3 signaling enhances REG Iα promoter activity in gastric cancer cells

The effect of IL-6/STAT3 signaling on REG Iα promoter activity was analyzed by transient expression assay. In MKN74 cells transfected with the hREG Iα (−1195/+78)-Luc construct, IL-6 stimulation significantly enhanced the level of luciferase activity (Figure 4A). However, the increased level was attenuated by concomitant treatment with STAT3 siRNA. Furthermore, the level of REG Iα promoter activity in STAT3 siRNA-treated MKN74 cells was significantly lower than that in untreated control cells (Figure 4A). These findings suggest that IL-6/STAT3 signaling upregulates the transcriptional activity of the REG Iα gene.

We next determined the element responsible for IL-6-dependent REG Iα promoter activation in a series of promoter deletion assays. While extending deletion of the 5′ REG Iα promoter gene from position −1195 to −153, a significant increase of luciferase activity was sustained. However, the luciferase activity returned to the control level when the promoter region was additionally deleted up to −131 (Figure 4B). These findings suggest that the promoter region between −153 and −131 is critical for REG Iα promoter activation in response to IL-6.
IL-6-induced REG Ixa promoter activation, we used the mutant constructs −153M1 and −153M2 (Figure 4C). As shown in Figure 4D, neither −153M1 nor −153M2 responded to IL-6 stimulation, suggesting that the element from position −142 and −134 (TGCCGGGAA) is responsible for IL-6-induced REG Ixa promoter activation.

The binding activity of the nuclear extract to candidate nucleotides for the IL-6 responsible element was examined by electrophoretic mobility shift assay. IL-6 enhanced the binding activity of nuclear extracts to the probe, and its enhanced activity was decreased by inhibiting the STAT3 signal with the siRNA system (Figure 4E). Moreover, disruption of the responsible element resulted in complete loss of the binding activity (Figure 4F).

REG Ixa protein mediates the anti-apoptotic effects of STAT3 in gastric cancer cells

Inhibition of STAT3 signaling by STAT3 siRNA significantly increased TUNEL positivity in AGS cells, suggesting that STAT3 contributes to anti-apoptosis in gastric cancer cells under unstimulated conditions (Figure 5A). On the other hand, we found that REG Ixa gene induction significantly decreased the TUNEL positivity accelerated by STAT3 inhibition (Figure 5A). We next examined intracellular change of apoptosis-related enzyme in gastric cancer cells. Compatible with data from TUNEL assay, inhibition of STAT3 signaling significantly increased caspase-3 activity, and REG Ixa gene induction significantly decreased the caspase-3 activity (Figure 5B).

We further examined whether STAT3 signaling contributes to anti-apoptosis through REG Ixa protein induction in gastric cancer cells. Treatment with STAT3 siRNA clearly suppressed the expression level of not only p-STAT3 (Tyr705) but also REG Ixa protein in AGS cells (Figure 5C). Moreover, inhibition of STAT3 signaling was accompanied by reduction of p-Akt, Bcl-xL and p-Bad expression in untreated cells (Figure 5C), suggesting that these anti-apoptotic molecules act downstream of STAT3 signaling. On the other hand, induction of REG Ixa protein clearly enhanced the expression of the anti-apoptotic molecules p-Akt, Bcl-xL and p-Bad in the presence or absence of STAT3 siRNA. Thus, the enhancing effects of REG Ixa on p-Akt, Bcl-xL and p-Bad do not involve STAT3 activation. In addition, REG Ixa induction did not affect p-STAT3 (Tyr705) expression in AGS cells, ruling out the presence of a positive functional loop between REG Ixa and STAT3 activation. Taken together, STAT3 inhibition might promote apoptosis by not only inhibiting the Akt/Bad/Bcl-xL pathway directly but also by suppressing REG Ixa protein expression and its resulting activation of the Akt/Bad/Bcl-xL pathway.

To confirm how REG Ixa protein acts on gastric cancer cells, we additionally examined the level of REG Ixa protein in the culture medium. As shown in Figure 5D, a small amount of REG Ixa protein was secreted from AGS cells under unstimulated conditions, but its secretion was completely abolished by the inhibition of STAT3 activation. On the other hand, we found an abundant amount of REG Ixa protein in a medium of REG Ixa-transfected AGS cells, suggesting that the anti-apoptotic effect of REG Ixa overexpression is mainly mediated by the REG Ixa protein secreted from the cells. We then inhibited the

Fig. 3. Effects of Janus kinase inhibitor (A–D) and STAT3 siRNA treatment (E and F) on IL-6-induced STAT3 phosphorylation (Tyr705) and REG Ixa protein expression in gastric cancer cells. AGS cells (3 × 10⁵) and MKN74 cells (1 × 10⁶) were pre-treated with 25 or 100 μM AG490 for 45 min and then stimulated with IL-6 (1000 U/ml) for 15 min to detect STAT3 phosphorylation (Tyr705) (A and B) and for 12 or 24 h to detect REG Ixa expression (C and D). AGS (E; 3 × 10⁵) and MKN74 cells (F; 1 × 10⁶) were transfected with STAT3 siRNA (or non-silencing siRNA as a control) for 48 h (E and F). After transfection, the cells were stimulated with IL-6 (1000 U/ml) for 15 min to evaluate STAT3 phosphorylation (Tyr705) and for 12 h to evaluate REG Ixa expression. Extracted protein was analyzed by western blotting as described in Materials and Methods. Representative data from four experiments are shown.
function of the secreted REG Iα protein using anti-REG Iα antibody. Although REG Iα gene induction significantly suppressed the TUNEL positivity and the caspase-3 activity accelerated by STAT3 inhibition, additional treatment with anti-REG Iα antibody abolished the inhibitory effect of REG Iα gene induction on TUNEL positivity and caspase-3 activity (Figure 5A and B). With regard to intracellular signaling, induction of the REG Iα gene enhanced the expression of p-Akt, p-Bad and Bcl-xL in AGS cells. However, when the cells were

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**Fig. 4.** Determination of the element responsible for IL-6-induced REG Iα promoter activation. (A) Effects of STAT3 siRNA transfection on IL-6-induced REG Iα promoter activity in MKN74 cells. MKN74 cells (4 × 10^5) were seeded in 12-well plates 24 h before transfection. The cells were then co-transfected with 700 ng of hREG Iα (−1195/+78)-Luc construct, 7 ng of Renilla luciferase plasmid pRL-TK and 350 ng of STAT3 siRNA. Forty-eight hours later, the cells were stimulated with IL-6 (1000 U/ml) for 12 h. Luciferase activity was measured in extracts from transfected MKN74 cells, normalized for Renilla luciferase activity and expressed relative to the activity of the untreated group. (B) Deletion analysis of the REG Iα promoter. Constructs listed on the ordinate are numbered according to their 5′ term in the REG Iα promoter. MKN74 cells were transfected with the corresponding plasmids for 48 h and then stimulated with IL-6 (1000 U/ml). Relative luciferase activity in extracts from each cell group was measured. In all the constructs except for −131 and −55, significant increases in luciferase activity by IL-6 stimulation were retained (P < 0.01). (C) Alignment of the REG Iα gene promoter. Nucleotide substitutions in the cis-element are indicated by underlining. Dots indicate residues that are identical to the REG Iα gene promoter. (D) Effects of site-directed mutagenesis of the cis-element within the REG Iα promoter. Relative luciferase activity was measured by using mutant plasmids. Significant increase in luciferase was retained in the −153 plasmid but not in −153M1 or −153M2 (P < 0.01). The binding activity of nuclear extracts to the responsible element for IL-6-induced REG Iα promoter activation was analyzed by electrophoretic mobility shift assay. (E) Nuclear extracts were obtained from MKN74 cells stimulated with IL-6 (1000 U/ml) for 30 min in the presence or absence of pre-treatment with 25 μM PD 98059. The nuclear proteins were incubated with a 32P-labeled wild type probe including the responsible element for IL-6-induced REG Iα promoter activation for 20 min. (F) The nuclear extracts from MKN74 cells stimulated with IL-6 (1000 U/ml) for 30 min were incubated with 32P-labeled wild type, M1 or M2 probe for 20 min. All results in A, B and D are represented as the mean ± SEM of four samples. *Significantly greater than the control group (P < 0.01). §Significantly lower than the IL-6-treated group (P < 0.01).
treated with anti-REG Iα antibody, this enhancement was clearly suppressed (Figure 5C). Taken together, it is suggested that REG Iα protein, whose production is regulated by STAT3 signaling, acts on gastric cancer cells in a paracrine and/or autocrine manner and functions as an anti-apoptotic factor by activating the Akt/Bad/Bcl-xL pathway.

On the other hand, in this series of in vitro studies we also examined the expression of REG-R mRNA in gastric cancer cells by real-time reverse transcription–PCR. However, we found that neither STAT3 inhibition nor REG Iα gene induction affected the level of REG-R mRNA in gastric cancer cells (Figure 5E).

REG Iα expression is associated with p-STAT3 expression in human gastric cancer tissues

Among 38 samples of gastric cancer tissue, 16 (42.1%) were positive for REG Iα protein and 20 (52.6%) were positive for p-STAT3 (Tyr705) (Figure 6). Positivity for both REG Iα and p-STAT3 (Tyr705) was significantly higher in gastric cancers with lymphatic invasion than in those without (51.7 versus 11.1% and 62.1 versus 22.2%, respectively). In contrast, expression of neither REG Iα nor p-STAT3 (Tyr705) was significantly related to parameters including age, gender, tumor location, tumor size, histological type, venous invasion, lymph node metastasis or tumor-node-metastasis stage.

Fig. 5. Effects of STAT3-induced secretory REG Iα protein on anti-apoptosis in AGS–REG Iα cells. Effects of STAT3 siRNA transfection and/or REG Iα gene induction on (A) TUNEL positivity and (B) caspase-3 activity in AGS cells. (A) AGS–REG Iα cells (2 × 10⁴) and AGS–EGFP cells (2 × 10⁴) transfected with STAT3 siRNA or non-silencing siRNA were cultured in four-well culture slides in serum-free medium with or without an anti-REG Iα antibody (50 μg/ml) for 18 h. After the slides were fixed with 10% buffered formalin, TUNEL assay was performed. All results are expressed as the mean ± SEM of four samples. (B) AGS–REG Iα (1 × 10⁶) and AGS–EGFP cells (1 × 10⁶) transfected with STAT3 siRNA or non-silencing siRNA were cultured in 6 cm culture dishes in serum-free medium with or without an anti-REG Iα antibody (50 μg/ml) for 18 h. After these cells were lysed in the buffer for caspase assay, the lysate was reacted with Ac-DEVD-pNA in the 96-well microplate according to the manufacturer’s protocol. (C) Effects of STAT3 siRNA transfection and/or REG Iα gene induction on the expression of Akt and Bcl family proteins in AGS cells. Anti-REG Iα antibody (50 μg/ml) was added to evaluate the effects of secretory REG Iα protein. Extracted protein was examined for the phosphorylation of Akt and the expression of Bcl family proteins by western blotting. (D) Detection of REG Iα protein in the medium of AGS–REG Iα cells. The medium was collected and the release of REG Iα protein was evaluated by western blotting. (E) Changes in REG-R mRNA expression by STAT3 inhibition or REG Iα gene induction. The data are expressed as fold change in REG-R mRNA/18S rRNA mRNA ratio relative to the control group. All results in Figure A, B and E are represented as the mean ± SEM of four samples.
Twelve (75%) of the 16 REG Iα-positive gastric cancers were positive for p-STAT3 (Tyr705). Conversely, 12 (60%) of the 20 p-STAT3 (Tyr705)-positive gastric cancers were positive for REG Iα protein. Positivity for p-STAT3 (Tyr705) was significantly correlated with positivity for REG Iα protein in gastric cancer tissues (P = 0.019; Table I).

REG Iα protein and p-STAT3 expression is associated with anti-apoptosis in gastric cancer tissues

P-STAT3 (Tyr705)-positive gastric cancers showed a significantly lower ssDNA index (1.77 ± 0.27) than p-STAT3 (Tyr705)-negative ones (3.35 ± 0.73) (Figure 7A). REG Iα-positive cancers also showed a significantly lower ssDNA index (1.59 ± 0.27) than REG Iα-negative ones (3.19 ± 0.61) (Figure 7B). These data suggest that both p-STAT3 (Tyr705) and REG Iα protein expression are associated with anti-apoptotic behavior of tumor cells in gastric cancer tissue.

Discussion

It has been suggested that IL-6 and its responsive signaling molecule, STAT3, are involved in the process of inflammation and play pivotal roles in carcinogenesis under inflammatory condition by modulating its downstream gene products (15–17). We recently reported that the REG Iα gene is overexpressed in gastritis tissues (8) and that its expression is induced by IL-6 stimulation (9). In the present in vitro studies, we confirmed that IL-6 stimulation enhances REG Iα protein expression through STAT3 activation and also demonstrated that the IL-6-responsive element is present in the REG Iα promoter at the position from −142 to −134, which corresponds to the consensus STAT3-binding site (17). IL-6 is known to activate not only STAT3 but also the mitogen-activated protein kinase (MAPK) pathway (18–20). However, by using the MAPK inhibitors PD98059, we found that the MAPK pathway is not involved in IL-6-induced REG Iα gene and protein expression in AGS and MKN74 cells. Interestingly, Judd et al. (5,6) reported that mice with STAT3 hyperactivation but without SHP2-MAPK activation showed reg I gene overexpression, being compatible with the present in vitro studies. Taken together, STAT3 signaling appears to have crucial roles in REG Iα expression in gastric cancer cells.

In the present study, we clearly demonstrated that the STAT3/REG Iα pathway has a significant role in anti-apoptosis of gastric cancer cells. Moreover, we suggested that REG Iα protein mediates the anti-apoptotic effect of STAT3 signaling via activation of the Akt/Bad/Bcl-xL pathway. However, it has been reported previously that STAT3 activation can directly enhance the expression of Bcl family proteins in myeloma, leukemia and intestinal epithelial cells (21–23). Moreover, in addition to REG Iα protein, STAT3 activation appears to enhance other anti-apoptotic molecules such as survivin or IAP2 (inhibitor of apoptosis protein-2) (24–27). These findings appear to be reflected in our present results, showing that REG induction partly but not completely rescued gastric cancer cells from apoptosis by STAT3 inactivation. However, since REG Iα protein induced strong activation of Akt and enhancement of Bcl-xL expression in AGS cells, it is probably that REG Iα protein is a downstream molecule of STAT3 signaling and plays an important role in anti-apoptosis of gastric cancer cells.

We showed previously that REG Iα protein plays important roles in the process of wound healing in non-neoplastic gastric mucosa (28,29). Therefore, one might question how REG Iα protein is involved in the development of gastric cancer. In the non-neoplastic gastric mucosa, REG Iα expression is maintained at a low level but markedly augmented in gastric epithelial cells under inflammatory

Table I. Relationship between p-STAT3 (Tyr705) and REG Iα expression in 38 gastric cancer tissues

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<td>Total</td>
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Values are the number of cases (P = 0.019).
conditions (8). Also importantly, the level of REG Iα expression normalizes soon after inflammation has resolved (8). Thus, since REG Iα expression is strictly regulated, its anti-apoptotic effect appears essential for gastric mucosal regeneration. In contrast, dysregulated REG Iα expression may function as a secreted protein and accordingly REG Iα protein appears to act in a paracrine/autocrine fashion in gastric cancer tissues. On the other hand, we considered the possibility of a positive functional loop between STAT3 signaling and REG Iα protein. However, REG Iα gene induction did not affect p-STAT3 expression in AGS cells, possibly ruling out such a functional loop.

In summary, we have clarified in this study that REG Iα protein is an important downstream molecule of STAT3 signaling and mediates the anti-apoptotic effect of STAT3 by activating the Akt/Bad/Bcl-XL pathway. Moreover, we have shown that REG Iα overexpression is linked to STAT3 activation in human gastric cancer tissues. These data suggest that REG Iα protein plays a critical anti-apoptotic role in gastric tumorigenesis under STAT3 hyperactivation. Therefore, whether REG Iα protein is a candidate target of anticancer therapy is an interesting question to be elucidated in future studies.

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References

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