Reg protein is overexpressed in gastric cancer cells, where it activates a signal transduction pathway that converges on ERK1/2 to stimulate growth

Yasunori Kadowaki[a,*], Shunji Ishihara[a], Youichi Miyaoka[a], Mohammed Azharul Karim Rumi[a], Hiroshi Sato[a], Hideaki Kazumori[a], Kyoichi Adachi[a], Shin Takasawa[b], Hiroshi Okamoto[b], Tsutomu Chiba[c], Yoshikazu Kinoshita[a]

[a] *Second Department of Internal Medicine, Shimane Medical University, Izumo 693-8501, Japan*
[b] *Department of Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan*
[c] *Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan*

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Abstract Reg is a growth factor with mitogenic effects on pancreatic β cells and gastric stem cells. To date, there has been no information available on Reg-mediated intracellular signal transduction pathways. The role of Reg in the gastric carcinogenesis is also unknown. In the current study, the Reg signaling pathway in gastric cancer cell was examined. Reg treatment of MKN45 gastric cancer cells resulted in tyrosylphoshorylation of several cellular proteins and subsequent activation of classical MAPK, ERK1/2. Reg also stimulated thymidine incorporation in MKN45 and AGS gastric cancer cells in a dose-dependent manner. Finally, Reg was shown to be highly expressed in a large number of gastric cancers in vivo. Taken together, these data suggest that gastric cancer cells have gained the ability to overexpress Reg protein, which confer upon themselves added proliferative capacities, resulting in a considerable growth advantage.

Key words: Reg; Gastric cancer; Tyrosine kinase; ERK1/2; Carcinogenesis

1. Introduction

In 1988, *Reg* (regenerating gene) was isolated as a gene upregulated in regenerating pancreatic islet cells, utilizing a differential hybridization technique [1]. The *Reg* gene product, Reg was a lectin-related secretory protein and was shown to be mitogenic to isolated islet cells [2] and cells of a pancreatic β cell line [3]. Thus, Reg is thought to be an endogenous growth factor in pancreatic islets that presumably acts in an autocrine or paracrine manner. On the other hand, *Reg* gene expression has also been found outside the pancreas, mainly in foregut-derived tissues. In particular, Reg was highly expressed in the gastric mucosa, at levels second only to those observed in the pancreas, suggesting a critical role in gastric mucosal proliferation [4]. A series of studies from our laboratory has focused on this issue. We have demonstrated that Reg functions as a growth factor for gastric mucosal stem cells and that it is involved in various important phenomena in the stomach, such as hypergastrinemia and mucosal regeneration [5–7].

Despite these advances, two important issues remain unclear. First, how is the Reg signal transduced in a gastric cell? Since the discovery of the *Reg* gene, no information has been published on the signal transduction pathway evoked by Reg. This is true not only for gastric cells, but also for all other types of cells, including pancreatic β cells. Secondly, it is interesting to hypothesize that Reg may be involved in gastric carcinogenesis. In many cases, malignant transformation results from aberrations in growth factor signal transduction pathways that normally operate to control cell proliferation. A component of the pathway, either extracellular [8] or intracellular [9,10], undergoes a qualitative or quantitative change, leading to constitutive activation of the growth signal and making the cell refractory to external control. Since Reg plays a central role in gastric stem cell proliferation [5–7], an alteration of the Reg signal may contribute to the neoplastic transformation of gastric stem cells.

Thus, the ultimate goals of our research are to delineate the Reg-mediated signal transduction pathway in both normal and cancerous gastric cells and to identify the segment of the pathway altered in the neoplastic process. This could uncover novel mechanisms underlying carcinogenesis in gastric tissue. As a first step towards this goal, we investigated whether gastric cancer cells proliferate in response to extracellular Reg stimuli as normal stem cells do. We also examined the nature of the intracellular signaling pathway evoked during this stimulation. In the course of the study, we found that gastric cancer cells have gained the ability to produce large amounts of the Reg protein, which may act upon themselves causing proliferation, presumably affording them a considerable growth advantage.

2. Materials and methods

2.1. COS cells transfection and preparation of conditioned media

The vector used to express the rat Reg protein was constructed by modifying the plasmid pEGFP (Promega, Madison, WI, USA), which contains the green fluorescent protein (GFP) gene under the control of the CMV promoter. The plasmid was digested by *Sac*II and *Not*I (to remove the GFP sequence), and ligated to the rat Reg cDNA fragment obtained by RT-PCR from rat stomach RNA, or, self-ligated to

*Corresponding author. Fax: (81)-853-20 2187.
E-mail address: y-kado@momo.so-net.ne.jp (Y. Kadowaki).

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yield the control plasmid. RT-PCR was performed as described below using the following primers that contain the sequence flanking the rat *Reg* coding region: 5′-GCAGAGATTGTTGACTTGCA-3′ and 5′-GATAGATGGTCTAGTTTCAC-3′.

To prepare conditioned media, we cultured COS7 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and transfected them at 30–50% confluence in 9-cm dishes. Transfection was performed using the standard calcium phosphate precipitation method with 15 μg of Reg expression plasmid or control plasmid. The precipitates were removed after 6 h, and the cells were further incubated for 48 h in 10 ml of fresh, serum-free media. The conditioned media were then collected and stored frozen as a source of recombinant Reg protein for use at the time of the assays for phosphorylation or thymidine incorporation.

2.2. Culture of gastric cells

Cells of the gastric cancer cell lines MKN45 and MKN7 were cultured in RPMI medium. AGS cells were cultured in Ham's F12 medium. Both media were supplemented with 10% FCS. The cells were split 36 h before the assay at the indicated cell density. The cells were washed twice with serum-free RPMI media 12 h before the assay and further incubated for 10 h in serum-free media. Then, the cells were again washed, further incubated for 2 h, and assayed for phosphorylation or thymidine incorporation.

2.3. Phosphorylation assay

Media conditioned by Reg-transfected COS cells or mock-transfected COS cells were diluted 100-fold with serum-free RPMI medium. Media of the MKN45 cells or MKN7 cells cultured as described above were aspirated, and the medium conditioned by transfected COS cells was added immediately. At the end of the incubation, the conditioned medium was aspirated, and ice-cold SDS–PAGE sample buffer was added to each plate on ice to obtain cell lysates. The samples were normalized for protein content, and proteins were separated by electrophoresis on a 7.5% SDS–polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked using 5% non-fat dried milk in Tris-buffered saline (pH7.2) and incubated for 3 h with anti-tyr(p) (py20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunoreactive bands were visualized by chemiluminescence.

In the assay for ERK activation, four plates of MKN45 cells or MKN7 cells were prepared as described in Section 2.2. The media of all four plates were aspirated, the medium conditioned by mock-transfected COS cells was added, and cells were further incubated for 2 h. Subsequently, the cell lysates were collected from one plate (0 min). The media from the rest of the plates were aspirated and replaced by the medium conditioned by Reg-transfected COS cells. After the indicated periods (5 min, 10 min, and 15 min), cell lysates were collected. Lysates of all four time points were normalized for protein content and subjected to Western blot analysis as described above, except that a 12% SDS–polyacrylamide gel was employed, and the membrane was probed with a monoclonal anti-phosphorylated ERK antibody (Santa Cruz Biotechnology, Inc.). Immunoreactive bands were visualized by chemiluminescence. As a loading control, the normalized lysates were subjected to Western blot analysis using a rabbit polyclonal anti ERK1/2 antibody (Sigma, St. Louis, MO, USA). In the neutralizing experiment, the Reg-conditioned medium was diluted 100-fold with serum-free RPMI medium. Then the medium was mixed with 100 μg of mouse monoclonal anti-Reg antibody or 3 mg of non-specific mouse IgG and 1 ml of protein A agarose beads and incubated overnight with gentle rocking. The beads were pelleted by centrifugation and discarded. The treated media were added to MKN45 cells as above, and after 10 min of incubation, the lysates were prepared and subjected to Western blot analysis.

2.4. Thymidine incorporation

The media conditioned by Reg-transfected COS cells or mock-transfected COS cells were diluted with serum-free RPMI medium as indicated. The media of the gastric cells cultured as above were aspirated and replaced by the conditioned medium. The cells were further incubated for 24 h. 1 μCi/ml of [methyl-^{3}H]thymidine (Amersham Pharmacia Biotech Co., Buckinghamshire, UK) was added, and the cells were incubated for an additional 6 h. Cells were harvested onto a glass fiber filter mat using a Cell Harvester (Inotech, Switzerland). After the filter was dried, [methyl-^{3}H]thymidine incorporation was measured using a 1450 Microbeta scintillation counter (Wallac, Oy, Turku, Finland). The three independent assays were performed in quadruplicate.

2.5. RNA isolation and cDNA synthesis by reverse transcription

Total RNA was extracted by the acid guanidinium-phenol chloroform method using Isogen (Nippon Gene, Tokyo, Japan). Then, 10 μg of total RNA extracted from each cell line was reverse transcribed to cDNA using a First Strand cDNA Synthesis kit (Toyobo, Tokyo, Japan) and amplified directly by the PCR method employing LA-Taq polymerase (Takara, Kyoto, Japan) in 25 cycles.

2.6. Immunohistochemistry and Western blot analysis of gastric tissues

Tissue slices of gastric cancer specimens were dehydrated in a graded ethanol series and embedded in paraffin. Tissue sections were cut at a 50-μm thickness and mounted on poly-L-lysine-coated glass slides. After being stored at 20°C, the slides were air-dried for 30 min, and the cells in the area corresponding to the tumor mass were dissected and collected into microfuge tubes and extracted once with xylene and twice with ethanol. The pellet was dissolved in RIPA buffer, boiled for 10 min and normalized for protein content. The samples were subjected to SDS–PAGE on a 15% gel and transferred to a PVDF membrane. The membrane was probed with anti-human Reg monoclonal antibodies [4].

For immunohistochemistry, the cryostat samples were prepared on glass slides at a 6-μm thickness and fixed in 100% acetone at 4°C for 10 min. Samples were incubated with normal goat serum for 30 min, followed by incubation for 120 min with a monoclonal antibody [4] that the recognized the human Reg protein. After being washed with phosphate-buffered saline, the sections were incubated with biotinylated goat anti-mouse IgG for 30 min. Bound antibody was detected by the avidin–biotin–peroxidase method (ABC kit; Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was subsequently detected by incubation with 3,3′-diaminobenzidine (Sigma, St. Louis, MO, USA) in 0.05 M Tris–HCl for 10 min at room temperature, followed by hematoxylin staining. After dehydration in a graded ethanol series, the sections were cleared in xylene.

3. Results

3.1. Reg induced tyrosyl phosphorylation of several proteins and activation of ERK1/2 in gastric cancer cells

COS cells were transiently transfected with a rat Reg cDNA expression plasmid. Release of a large amount of the Reg protein into the conditioned medium was confirmed by Western blot analysis with an anti-rat Reg monoclonal antibody [11], and this medium served as the source of the Reg protein (Fig. 1a). As a negative control, the medium conditioned with COS cells transfected with the mock plasmid was also prepared (Fig. 1a). These conditioned media were added to cultures of cells of a poorly differentiated gastric cancer cell line, MKN45, and the phosphorylation state of the cellular proteins was examined by immunoblotting with anti-phosphotyrosine antibodies (PY20). Tyrosine phosphorylation of several proteins was enhanced as early as 2 min after Reg treatment (Fig. 1b). Phosphorylation of three major proteins of molecular masses 170, 125 and 47 kDa was markedly enhanced (Fig. 1b). Of note, with increasing cell density, these proteins became basally phosphorylated (compare lanes 1 and 3 of Fig. 1b).

To explore whether the ERK1/2 pathway [12,13] lies downstream of Reg signaling, the activation of kinases during Reg stimulation was evaluated using the same experimental conditions as outlined above. Lysates obtained 5 min, 10 min and 15 min after Reg treatment were subjected to Western blot analysis using an anti-phospho-ERK antibody to detect phosphorylated (and therefore activated) forms of ERK proteins. ERK1/2 activity was slightly increased 5 min after Reg

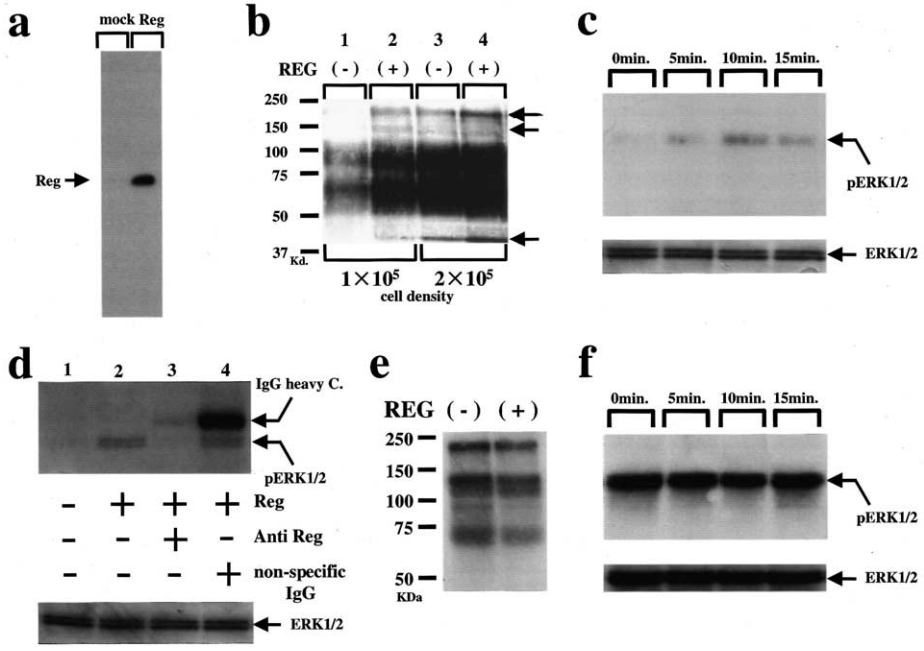

Fig. 1. Reg-mediated signal transduction in cells of the gastric cancer line MKN45. a: The source of the Reg protein. COS cells were transiently transfected with a rat Reg cDNA expression plasmid or a control plasmid (mock), and the medium conditioned by these cells was collected. The release of large amounts of Reg protein into the conditioned medium of the rat Reg cDNA-transfected cells was confirmed by Western blot analysis with an anti-rat Reg monoclonal antibody. b: Tyrosine phosphorylation of cellular proteins in response to Reg in MKN45 cells. The medium conditioned by Reg-expressing COS cells (Reg medium) (+) or by mock-transfected COS cells (mock medium) (−) were diluted 100-fold with serum-free culture media and added to MKN45 gastric cancer cells seeded at the indicated cell density (per 9-cm dish). After incubation for 2 min, cell lysates were prepared. Proteins were separated by SDS–PAGE and immunoblotted with an anti-phosphotyrosine monoclonal antibody. c: ERK1/2 activation in response to Reg stimulation in MKN45 cells. The mock medium was diluted 100-fold with serum-free culture media, added to MKN45 gastric cancer cells seeded at a cell density of 10^5 per 9-cm dish, and cells were further incubated for 2 h. Then, the media were replaced by the Reg medium, also diluted 100-fold. After incubation for the indicated periods (0 min, 5 min, 10 min, and 15 min), cell lysates were prepared. Proteins were separated by SDS–PAGE and immunoblotted with an anti-phospho-ERK monoclonal antibody. The lower panel shows the immunoblot with the anti-ERK1/2 polyclonal antibody as the loading control. d: A neutralizing experiment using the anti-rat Reg monoclonal antibody. Mock medium (lane 1), Reg medium (lane 2), Reg medium precleared with the anti-Reg antibody (lane 3), or Reg medium treated with an excess amount of non-specific mouse IgG (lane 4) were diluted 100-fold and added to MKN45 gastric cancer cells seeded at a cell density of 10^5 per 9-cm dish. After incubation for 10 min, cell lysates were prepared and subjected to Western blot analysis using an anti-phospho-ERK monoclonal antibody. The contaminated anti-Reg monoclonal antibody and non-specific mouse IgG were recognized and visualized by anti-mouse IgG-HRP, which was employed as the secondary antibody in the Western blot analysis (lanes 3 and 4). The lower panel shows the immunoblot with the anti-ERK1/2 polyclonal antibody as a loading control. e: Tyrosine phosphorylation of cellular proteins in response to Reg stimulation in MKN7 cells. Reg medium (+) or mock medium (−) was diluted 100-fold with serum-free culture media and added to cultures of MKN7 gastric cancer cells seeded at a cell density of 10^5 per 9-cm dish. After incubation for 2 min, the cell lysates were prepared and analyzed by Western blot analysis with an anti-phosphotyrosine monoclonal antibody. f: ERK1/2 activation in response to Reg stimulation in MKN7 cells. The mock medium was diluted 100-fold with serum-free culture media and added to cultures of MKN7 gastric cancer cells seeded at a cell density of 10^5 per 9-cm dish, and further incubated for 2 h. Then, the media were replaced by the Reg medium, also diluted 100-fold. After incubation for the indicated periods (0 min, 5 min, 10 min, and 15 min), cell lysates were prepared. Proteins were separated by SDS–PAGE and immunoblotted with an anti-phospho-ERK monoclonal antibody. The lower panel shows the immunoblot with the anti-ERK1/2 polyclonal antibody as a loading control.

treatment, peaked at 10 min, and declined after 15 min (Fig. 1c).

In order to rule out the possibility that the observed effects were mediated by some other undetermined factors secreted in the Reg-conditioned medium, we performed a neutralizing experiment employing the anti-Reg antibody. The Reg-conditioned medium was mixed with the anti-Reg monoclonal antibody and protein A beads and incubated overnight. Then, the medium was cleared of the resulting immune complexes by centrifugation and added to MKN45 cells as described above. This Reg-depleted medium did not stimulate activation of ERK1/2 (Fig. 1d, lane 3). As a control, Reg-conditioned medium was also treated with an excess amount of non-specific mouse IgG, and was shown to retain its stimulating activity (Fig. 1d, lane 4).

We also examined Reg-mediated signaling events in the other cell line MKN7, which is derived from a well-differentiated gastric carcinoma. We could not observe obvious changes in the tyrosyl-phosphorylation state of the cellular proteins (Fig. 1e) nor ERK activation (Fig. 1f) in response to Reg.

3.2. Reg-stimulated growth of gastric cancer cells

The MKN45 cancer cells stimulated by the Reg-conditioned medium as in Fig. 1b,c were cultured for an additional 30 h and assayed for cell proliferation using the thymidine incorporation method. Addition of Reg stimulated the growth of MKN45 cells in a dose-dependent manner, reaching a maximum nearly two-fold that of basal levels (Fig. 2a). To examine whether ERK activation is required for Reg-mediated cell growth, the MAPK/MEK inhibitor PD98059 was employed. In the presence of PD98059, the rate of Reg-mediated growth induction was significantly inhibited (Fig. 2b). Next, we investigated the effects of Reg on AGS cells, which also originate

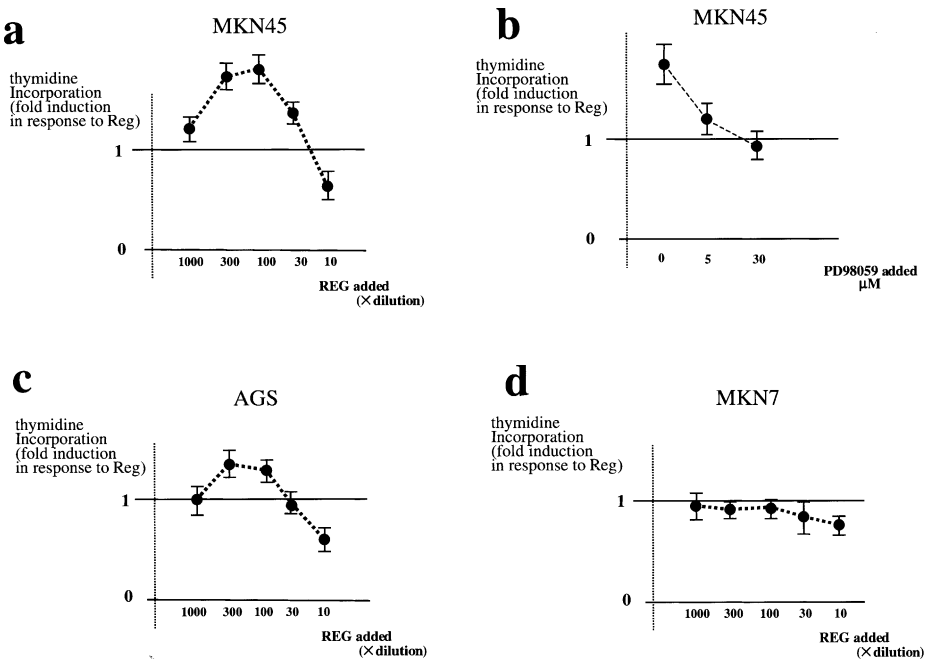

Fig. 2. Growth stimulation of cultured gastric cells in response to Reg. a,c,d: Reg medium or mock medium were diluted as indicated with the serum-free culture media and added to cultured gastric cells MKN45 (a), AGS (c) or MKN7 (d). After incubation for 24 h, [^{3}H]thymidine was added to the medium and the incubation was continued for a further 6 h. The cells were collected and assayed for thymidine incorporation. The amount of incorporated thymidine in the cells treated with the Reg medium relative to that of the cells treated with the mock medium is shown. b: Reg medium or mock medium was diluted 100-fold with serum-free culture media and added to MKN45 cells in the presence of the indicated concentrations of PD98059. After incubation for 24 h, [^{3}H]thymidine was added to the medium and the incubation was continued for a further 6 h. The cells were collected and assayed for thymidine incorporation. The amount of incorporated thymidine in the cells treated with the Reg medium relative to that of the cells treated with the mock medium is shown.

from a poorly differentiated gastric carcinoma. Reg enhanced thymidine incorporation in AGS cells, though levels were $<150\%$ of that observed in the control (Fig. 2c). Finally, MKN7 cells were tested, and no increase in Reg-stimulated thymidine incorporation was observed (Fig. 2d).

3.3. *Reg protein is overexpressed in gastric cancer cells*

To assess the expression of Reg in gastric cancer cells, cells of the MKN45, AGS, and MKN7 lines were analyzed by RT-PCR (Fig. 3a). MKN45 and AGS cells showed some level of expression. No expression of *Reg* was detected under these experimental conditions in the MKN7 cell line (Fig. 3a).

In order to provide in vivo evidence, Western blot analyses with anti-human Reg monoclonal antibody [4] were performed on nine gastric cancer samples (Fig. 3b). The cells in the tumor masses were dissected from paraffinized blocks and analyzed for expression of the Reg protein. Five of nine tumors showed significant levels of expression of the Reg protein. The variation of molecular masses of the Reg proteins can be explained by the heterogeneity of their sugar chains [4,14]. Immunohistochemical analyses were also performed. Fig. 4 shows a representative section of a poorly differentiated adenocarcinoma. In the portion of normal tissue (Fig. 4b,c), Reg immunostaining was detected only in the small cells located at the bottom of the gastric pit, which may be ECL cells, as previously shown in rat [5] and human tissue [15] (Fig. 4b). A basally located population of chief cells is also stained, though faintly, for the Reg protein in human tissue, as reported in the previous report [15] (Fig. 4b). No stain was detected in the other normal parts, including the neck zone, which harbors the gastric stem cells from which poorly differ-

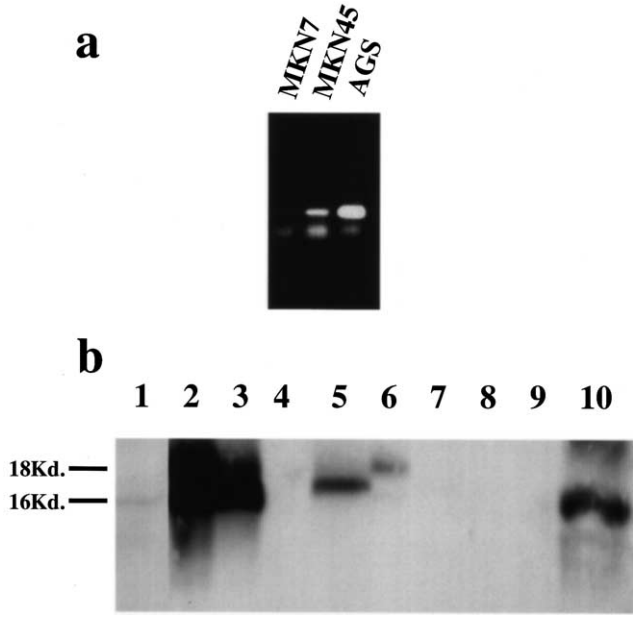

Fig. 3. Expression of Reg in gastric cancers. a: RT-PCR analysis of expression of *Reg* in cultured gastric cancer cells. b: Western blot analysis for expression of the Reg protein in gastric cancers in vivo. The cells corresponding to tumor masses were dissected from nine samples of paraffin-embedded gastric cancers. The samples were extracted with xylene and ethanol, and analyzed for the expression of the Reg protein by Western blot analysis with an anti-human Reg monoclonal antibody. Lane 1, normal gastric epithelium; lanes 2–5, well-differentiated adenocarcinoma; lanes 6–9, poorly differentiated adenocarcinoma; lane 10, signet ring cell adenocarcinoma.

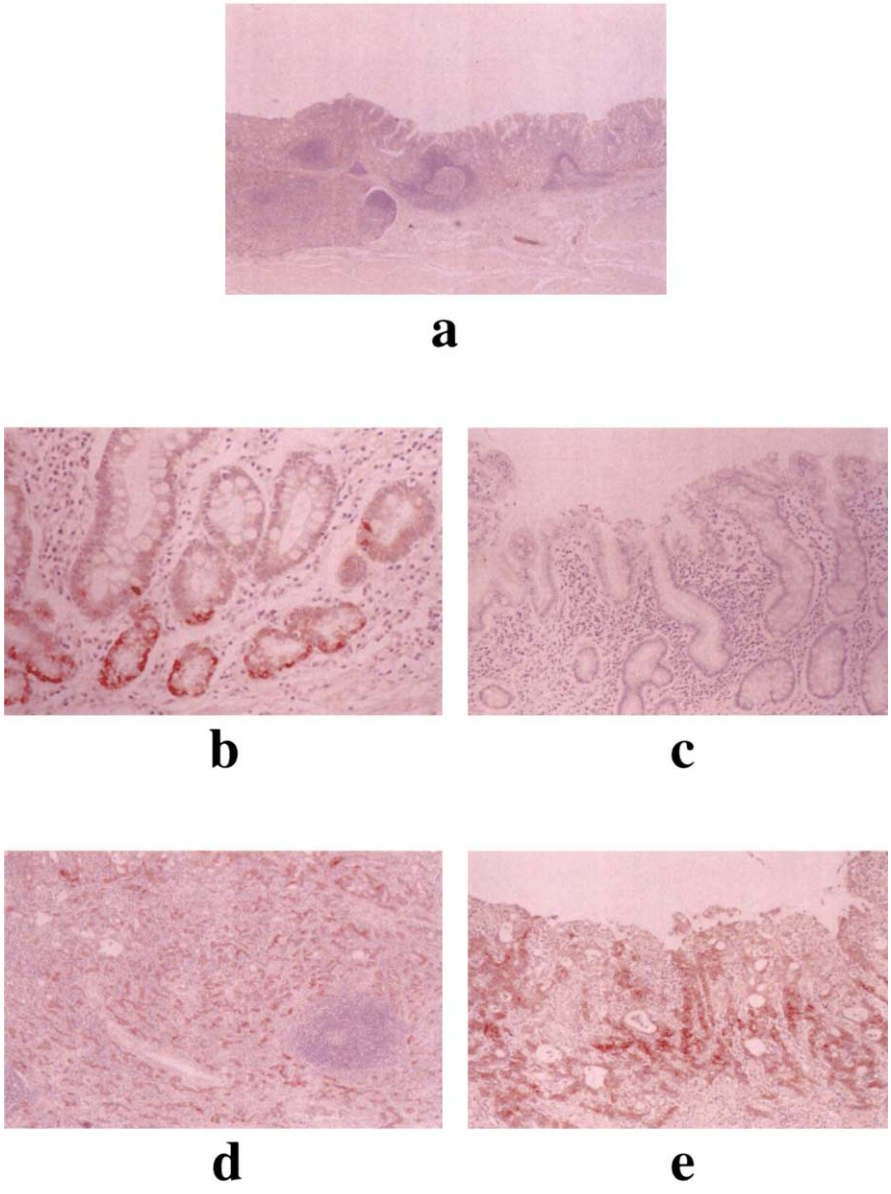

Fig. 4. Immunohistochemical staining for the Reg protein in a poorly differentiated adenocarcinoma. Sections of a poorly differentiated adenocarcinoma (a–e) were stained with an anti-human Reg monoclonal antibody. a: Whole section of the lesion, including normal and cancerous parts. b: Basal half of the normal part. c: Upper half of the normal part. d,e: Cancerous parts.

entiated adenocarcinomas presumably originate (Fig. 4c). Strong expression of the Reg protein was found in the cancer cells, as shown in Fig. 4d,e.

4. Discussion

In many cases, the intracellular signaling pathway for a growth factor involves tyrosine kinases. Therefore, we hypothesized that this is also the case for the Reg signaling pathway. We tested this possibility and observed tyrosine phosphorylation of several cellular proteins in response to Reg treatment (Fig. 1b). The phosphorylation began within 2 min of stimulation. Recently, a 105-kDa protein was isolated as a cellular Reg receptor from an expression cDNA library [16]. As judged by sequence homology [17,18] this protein belongs to the EXT family, the members of which have glycosyl transferase activity to assemble peptide glycans [19]. It is unlikely that this protein is located upstream of the tyrosine kinases, which are activated very rapidly in response to Reg. Therefore two distinct Reg signaling pathways may exist. One is the growth signaling pathway in gastric cells discovered and presented in this study, and the alternative pathway is the one evoked by the 105-kDa receptor, which may mediate other important effects of Reg. About 10 min after Reg treatment, ERK1/2, a member of the MAP kinase family, was activated (Fig. 1c) [12,13]. Activation of the ERK1/2 pathway is thought to result in transcriptional activation of several growth regulatory genes, ultimately leading to cell proliferation [12,13]. The representative members of the MAP kinase family are ERK1/2, JNK, and p38. Many intracellular signaling pathways converge on one of these MAP kinases [12,13]. The character of the signaling pathway can

be predicted by knowing which MAPK is selected. ERK1/2, known as the classical MAPK, lies downstream of many growth factor signaling pathways. In contrast, JNK and p38 mediate stress-induced apoptotic signals. We have also tested the activity of JNK and p38; however, no activation of these kinases was seen in response to Reg stimulation (data not shown). Taken together, involving some tyrosine kinases and ERK1/2, the Reg signaling pathway seems to have a representative and classical nature as a signal transduction pathway for a growth factor. This is the first report to delineate an intracellular Reg signaling pathway in any type of cell.

We have demonstrated that some gastric cancer cells proliferate in response to Reg stimulation. Reg stimulated thymidine incorporation in cells of the gastric cancer cell lines MKN45 and AGS (Fig. 2). As described above, Reg evoked the growth signaling pathway that converges on the ERK1/2 pathway in the MKN45 gastric cancer cells. Thus, it is suggested that these cancer cells have the ability to respond to the Reg signal, which leads to cell proliferation. MKN7 cells, which are derived from a well-differentiated gastric carcinoma, did not respond to the Reg growth signal. It is speculated that the gastric stem cells may have retained their responsiveness to Reg in the course of malignant transformation to mainly undifferentiated carcinomas.

We have demonstrated that the Reg protein is highly expressed in some parts of gastric cancer cells in vivo (Figs. 3b and 4). Taken together with the results presented in Fig. 2, the data raise the possibility that gastric cancer cells produce Reg proteins that act upon themselves causing proliferation. During the multistep neoplastic progression, the cancer cells might acquire the ability to produce the Reg protein, resulting in a considerable growth advantage and development of the malignant phenotype. The precise mechanisms by which the Reg proteins are distributed among cancer cells remain to be elucidated. We speculate that the distribution occurs in a paracrine manner or through cell-to-cell contact. Consistent with this speculation, basal tyrosine kinase activity was observed when the cells were grown at a higher density (Fig. 1b, lane 3). Also, MKN45 cells grow exponentially at a normal cell density in serum-free medium without adding any growth factors, but cease to grow when cultured at a low cell density.

In conclusion, we began research to delineate the intracellular Reg-mediated signal transduction pathway, and eventually found that, in gastric cancer cells, the extracellular part of the pathway has undergone a quantitative change, which presumably leads to a growth advantage. In a future study, delineation of the intracellular Reg signal will be completed, and additional mechanisms that may have profound effects on gastric carcinogenesis will be uncovered.

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