Cell Proliferation

Cell Prolif. 2009, **42**, 110–121 doi: 10.1111/j.1365-2184.2008.00577.x

Reg IV enhances peritoneal metastasis in gastric carcinomas

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Received 22 *April* 2008*; revision accepted* 4 *May* 2008

Abstract

Objectives: The role of Regenerating (Reg) IV on peritoneal metastasis was examined in gastric cancer using.

Material and methods: *Reg IV*-transfected human gastric cancer cells (MKN28-R1, MKN28-R2, TMK1-R1), control transfectants (MKN28-R0, TMK1-R0), and *REG4*-knocked down MKN45 cells were examined in *in vitro* and in nude mice peritoneal metastasis models.

Results and Discussion: Increase of expression and secretion of Reg IV, and levels of BCL-2, BCL-XL, survivin, phosphorylated AKT, and phosphorylated EGFR, and decrease of nitric oxide-induced apoptosis were found in *Reg IV*-transfectants, whereas those were abrogated in the knockdown cells. In mice models, increased number and size of peritoneal tumors and decreased apoptosis were found in *Reg IV*-transfectants, whereas those were abrogated by the knockdown cells. Mice survivals were worsened in *Reg IV*-transfectants-inoculated mice, but were improved in *Reg IV*-knockdown cell-inoculated mice. Levels of Reg IV protein in peritoneal lavage fluids increased in *Reg IV*-transfectants inoculated mice, but decreased in *Reg IV*-knockdown cell inoculated mice. In metastasized human gastric cancers, Reg IV positivity in peritoneum-metastasis cases was higher than those in negative cases. Reg IV was detected in peritoneal lavage fluids from human gastric cancer patients, in whose lavages keratin mRNA was detected by reverse transcriptase-polymerase chain reaction. Collectively, Reg IV might accelerate peritoneal metastasis in gastric cancer. Reg IV in lavage fluids might be a good marker for peritoneal metastasis.

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Introduction

Gastric cancer is a leading cause of cancer death in the world, and is the second leading cause of deaths from cancer in Japan (1,2). Approximately 20% of gastric cancer patients show peritoneal and/or liver metastases at surgery (3), and 30% of who have died from gastric cancer suffered from peritoneal metastasis (4). Peritoneal metastasis causes the terminal stage of advanced gastric cancer, and diminishes the quality of patients' life by intestinal obstruction, ascites retention and subsequent malnutrition. Control of peritoneal metastasis is expected to improve patients' quality of life (5,6).

The molecular mechanism of peritoneal metastasis is an ongoing assignment of cancer research. We have identified loss of heterozygosity of chromosome 7q involving 7q35 locus as a peritoneal metastasis-associated event in gastric cancer (7). Overexpression of angiogenic factors, such as vascular endothelial growth factor (VEGF) and interleukin-8, is associated with peritoneal metastasis and ascites production in ovarian cancer (8). The truncated form of fibroblast growth factor/keratinocyte growth factor receptor 2 IIIb (K-*sam*) and c-*met* show gene amplification and/or overexpression in scirrhous-type gastric cancers, which frequently produces peritoneal metastasis (9–11). Gene expression profiling shows alteration in expression of several genes, such as up-regulation of trefoil factor 1, α-1-antitrypsin and galectin 4, and down-regulation of cytidine deaminase (12). Recently, we reported the importance of activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) in suppressing peritoneal metastasis, which provides tumour growth inhibition and apoptosis induction in gastric and colon cancer cells (13,14). The importance of survival factors in peritoneal metastasis formation is emphasized in many reports; RUNX3, survivin, nuclear factor κB and Bcl-2/Bag are also associated with peritoneal metastasis (15–18).

The *Reg* (*regenerating*) gene family belongs to the calcium-dependent lectin superfamily (19,20). *Reg IV* is a new member family, and it is identified as a gene expressed in the gastrointestinal tract and pancreas (21,22). Human

Reg IV gene is located on chromosome 1, unlike other *Reg* family genes, which are located on 2p12 (23). *Reg IV* is expressed in Crohn's disease and ulcerative colitis (21,24) and is revealed to be associated with malignant potential of colorectal adenocarcinomas and malignant transformation of colorectal adenomas (25,26). Recently, Reg IV has been reported to activate epidermal growth factor receptor (EGFR), protein kinase B/Akt and activator protein-1 to accelerate colorectal cancer cell survival by increasing Bcl-2, Bcl-XL and survivin (27). The anti-apoptotic property of Reg IV is associated with colorectal cancer development and drug resistance in gastric cancer (28,29) and its expression is expected to be a marker for highly malignant potential (30–32).

We have identified *Reg IV* as a cancer-affiliated expressed gene, by a serial analysis of gene expression (SAGE) technique, in which results were deposited in the NCBI SAGE Library in the Web (http://www.ncbi.nlm.nih.gov/SAGE/). Reg IV protein is immunohistochemically detected in 36% of colorectal adenocarcinomas and this is associated with tumour stage, whereas Reg IV production is detected in 29% of gastric adenocarcinomas, and is associated with both the intestinal mucin phenotype and neuroendocrine differentiation but not with tumour stage or patient prognosis (33). Thus, the role of Reg IV in gastric cancer is still unclear.

In this study, we attempted to determine the relevance of Reg IV expression in peritoneal metastasis of gastric cancer using *Reg IV*-transfected human gastric cancer cells.

Materials and Methods

Cell culture

Human gastric cancer cell lines, MKN28 and TMK1, were transfected with the *Reg IV* expression vector (29,33). MKN28- and TMK1-*Reg IV* stable transfectants were selected by G418 (Sigma Chemical Co., St. Louis, MO, USA). Three transfectants with marked *Reg IV* expression were used in the present study, which were designated as MKN28-R1, MKN28-R2 and TMK1-R1. For the control, MKN28 and TMK1 cells were transfected with empty vector, which was designated as MKN28-R0 and TMK1-R0. MKN45 cells, which express Reg IV at a high level, was treated with *Reg IV* siRNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or mixed siRNA for control. siRNA (50 nM for 2×10^5 cells) diluted with transfection solution (Santa Cruz Biotechnology) was used for treatment according to the manufacturer's instructions. The cells were routinely maintained in RPMI-1640 (Sigma Chemical) containing 10% foetal bovine serum (Sigma Chemical) and G418 (500 μg/ml) at 37 °C in a 5% CO_2/95% air atmosphere.

Tissue samples

Gastric cancer cases with metastasis were chosen from the patients operated on in Hiroshima University Hospital, Nara Medical University Hospital, and Miyoshi Central Hospital. Among the 85 cases, all showed lymph node metastasis, 8 cases showed liver metastasis, and 43 cases showed peritoneal metastasis at surgery. Of 42 cases without peritoneal metastasis (found via gross and cytological examinations) at the operation, 21 cases showed peritoneal recurrence. For immunohistochemistry, representative formalin-fixed, paraffin-embedded tissue samples were used, which contained the deepest invasive portion of the tumour.

Cell population growth

The cells were harvested from 80% confluent monolayer cultures by a brief trypsinization with 0.1% trypsin and 0.1% ethylenediaminetetraacetic acid (Sigma Chemical). Cells were seeded at a density of 10 000 cells per well in 24-well tissue culture plates and treated under the conditions mentioned in the Results section. Cell number was counted using an autocytometer (Sysmecs, Kobe, Japan) at 24, 48 and 72 h. The experiment was repeated three times. For sodium nitropusside (SNP) treatment, cell number was counted 48 h after the treatment.

In vitro *invasion assay*

A modified Boyden chamber assay was performed to examine *in vitro* invasion of MKN28 cells. Polycarbonate filters (pore size 3 μm; diameter 5 mm) were glued to collagen type IV inserts (Becton-Dickinson Labware, Bedford, MA, USA), which were placed in the wells of 24-well tissue culture plates. The cells were suspended in 500 μl of regular medium and placed in the upper part of the chamber. The lower part of the chamber was filled with regular medium. After 24-h incubation at 37 °C, filters were carefully removed from the inserts, stained with haematoxylin for 10 min and mounted on microscope slides. The number of stained cells was counted in whole inserts wide at ×100 magnification. Invasion activity was quantified by average number of cells per insert well. Mean values of invading cells were calculated from the results of three independent experiments.

Animal model

BALB/c nu-nu athymic mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were maintained according to the institutional guidelines approved by the Committee for Animal Experimentation of the Nara Medical University, in accordance with the current regulations and standards

of the Ministry of Health, Labour and Welfare. The mice were used according to the institutional guidelines when they were 5 weeks old. Cells (as above) were briefly trypsinized and washed with Hanks' balanced saline solution (HBSS) three times. They were suspended in HBSS and were injected into the peritoneal cavity (1×10^7) of each mouse; eight or nine mice were injected per group. The mice were sacrificed to count numbers of metastatic foci in the peritoneal cavity. In another experimental set, survival of 10 mice was observed in each cell line until 16 weeks after the inoculation. Mice inoculated with MKN45 cells treated with siRNA were administrated siRNA encapsulated with liposome (34). siRNA (100 pmol) was encapsulated with 2 ml of kationic liposome (EL-C-01, Nippon-Oil_Fats Co., Tokyo, Japan), and 200 µl of the solution was administrated intraperitoneally to each mouse twice a week.

Immunohistochemistry

Consecutive 4-µm sections were cut from each block, and immunostaining was performed by immunoperoxidase technique following antigen retrieval with citrate buffer (pH 6.0) treatment for 10 min (three times). After endogenous peroxidase block by 3% hydrogen peroxide–methanol for 15 min, specimens were rinsed with 5% washing solution (BioGenex, San Ramon, CA, USA). Anti-Reg IV antibody established in our laboratory was used at 0.5 µg/ml primary antibody, and incubated at room temperature for 2 h (33). Specimens were rinsed with 5% washing solution and incubated at room temperature for 1 h with secondary antibody conjugated to peroxidase diluted at 0.5 µg/ml (anti-rabbit IgG, Medical & Biotechnological Laboratories Co., Ltd, Nagoya, Japan). All specimens were then rinsed with 5% washing solution and colour was developed by diaminobenzidine solution (Dako, Glostrup, Denmark). After washing with water, specimens were counterstained with Meyer's haematoxylin (Sigma Chemical). Immunostaining of all specimens was performed to ensure the same condition of antibody reaction and diaminobenzidine exposure.

Preparation of conditioned medium, peritoneal lavage and serum

Cells were cultured in RPMI-1640 containing 1% foetal bovine serum for 12 h. Then, the conditioned medium was filtered with 0.2-µm filter (Becton-Dickinson Labware). The peritoneal cavity of a sacrificed mouse was washed with 2 ml of phosphate-buffered saline and saved phosphate-buffered saline was filtered with 0.2-µm filter (Becton-Dickinson Labware). Mouse blood was obtained by cardiac puncture, mixed with heparin (5% v/v), and centrifuged at 500 ***g*** for 15 min at 4 °C. The supernatant serum was used for immunoblot analysis. For human peritoneal lavage, the entire peritoneal cavity was washed with 100 ml saline after opening of the peritoneal cavity. Of the peritoneal lavage fluid, 10 ml was centrifuged at 500 ***g*** for 15 min at 4 °C. The supernatant was used for immunoblotting and the pellet was used for reverse transcriptase–polymerase chain reaction (RT-PCR). Remnants of lavage fluid were used for cytological examination. Slot blotted lavage fluids and cultured media stained with Coomassie blue dye were served as the control for the sample loading.

Immunoblot analysis

Whole-cell lysates were prepared as described previously (35). The cultured medium and peritoneal lavage fluids were concentrated with the Protein Concentrate kit (TaKaRa Bio Inc., Shiga, Japan). Forty-microgram lysates were subjected to immunoblot analysis in 12.5% sodium dodecyl sulphate–polyacrylamide gels followed by electrotransfer on to nitrocellulose filters. Filters were incubated with primary antibodies and then with peroxidase-conjugated IgG antibodies (Medical and Biological Laboratories, Nagoya, Japan). α-tubulin or β-actin antibodies assessed levels of protein loaded per lane (Oncogene Research Products, Cambridge, MA, USA). The immune complex was visualized by CSA system (Dako) or ECL system (Amersham Biosciences Corp., Piscataway, NJ, USA). Antibodies for Reg IV (33), Bcl-2 (Dako), Bcl-XL, survivin (Santa Cruz Biotechnology), phosphorylated AKT (phospho-Ser473, Upstate Biotechnology Inc., Lake Placid, NY, USA), phosphorylated EGFR (phospho-Tyr992, Cell Signaling Technology, Beverly, MA, USA), and EGFR (Cell Signaling Technology) were used as primary antibodies.

Detection of cytokeratin in peritoneal lavage

Pellet from the peritoneal lavage fluids were used for RT-PCR, which was performed with iScript One-Step RT-PCR Kit (Bio-Rad Laboratories, Hercules, CA, USA). Primers for cytokeratin 20 mRNA were 5′-GAG GTT CAA CTA ACG GAG CT-3′ (forward) and 5′-TCT CTC TTC CAG GGT GCT TA-3′ (reverse) were referred to GenBank NM019010, which were synthesized by Sigma Genosys (Ishikari, Japan).

Statistical analysis

Statistical significance was examined by two-tailed Fisher's exact test, two-tailed *chi*-squared test, and two-tailed, unpaired Mann–Whitney test by using InStat software (GraphPad Software, Los Angels, CA, USA). Survival

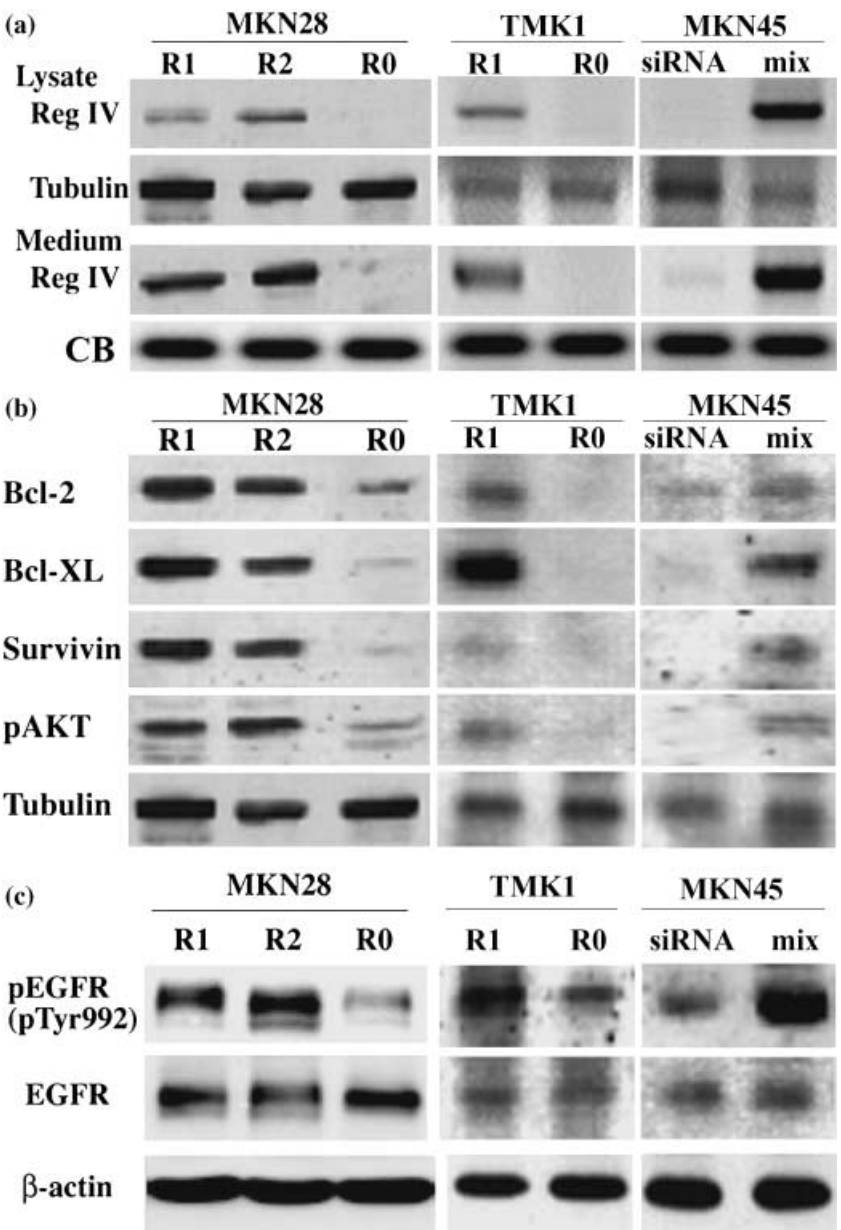


Figure 1. Production and secretion of Reg IV and survival-related proteins in gastric cancer cells. (a) Production and secretion of Reg IV protein were examined in lysates and culture media by immunoblotting. Tubulin and total loading protein detected by Coomassie blue staining (CB) served as loading controls. (b) Production of cell survival-related factors (Bcl-2, Bcl-XL, survivin and phophorylated AKT) was examined by immunoblotting. Tubulin served as loading control. (c) Phosphorylation levels of Tyr992 of EGFR were examined by immunoblotting. (a–c) MKN28-R1, MKN28-R2 and TMK1-R1: stable *Reg IV* transfectants. MKN28-R0 and TMK1-R0: empty vector-transfectant. MKN45-siRNA: *Reg IV* siRNA-treated MKN45 cells. MKN45-mix: siRNA mixture-treated MKN45 cells. β-actin or tubulin served as loading controls.

curves were calculated by Kaplan–Meier model (Statview 4.5, Abacus Concepts Inc., Berkeley, CA, USA). Difference of survivals was calculated by Cox proportional hazard model (Statview 4.5). Statistical significance was defined as a two-sided *P*-value of less than 0.05.

Results

Production of Reg IV protein and survival factors in gastric cancer cells

We first confirmed expression of Reg IV protein in *Reg IV*-transfected MKN28 and TMK1 cells and Reg IV siRNA-treated MKN45 cells (Fig. 1a). Three *Reg IV*-transfected cells (MKN28-R1, MKN28-R2 and TMK1-R1) and control vector-transfected cells (MKN28-R0 and TMK1-R0) were examined to detect Reg IV protein in the cell lysates and cultured media. MKN28-R0 and TMK1-R0 cells showed undetectable levels of Reg IV in the lysate and cultured media, whereas MKN28-R1, MKN28-R2 and TMK1-R1 cells produced Reg IV in the lysates and media. siRNA mixture-treated MKN45 cells expressed Reg IV at high level, whereas Reg IV siRNA-treated MKN45 cells did not express Reg IV. Second, levels of survival-related proteins in these cells were examined (Fig. 1b). Protein levels of Bcl-2, Bcl-XL, survivin and phorphorylated

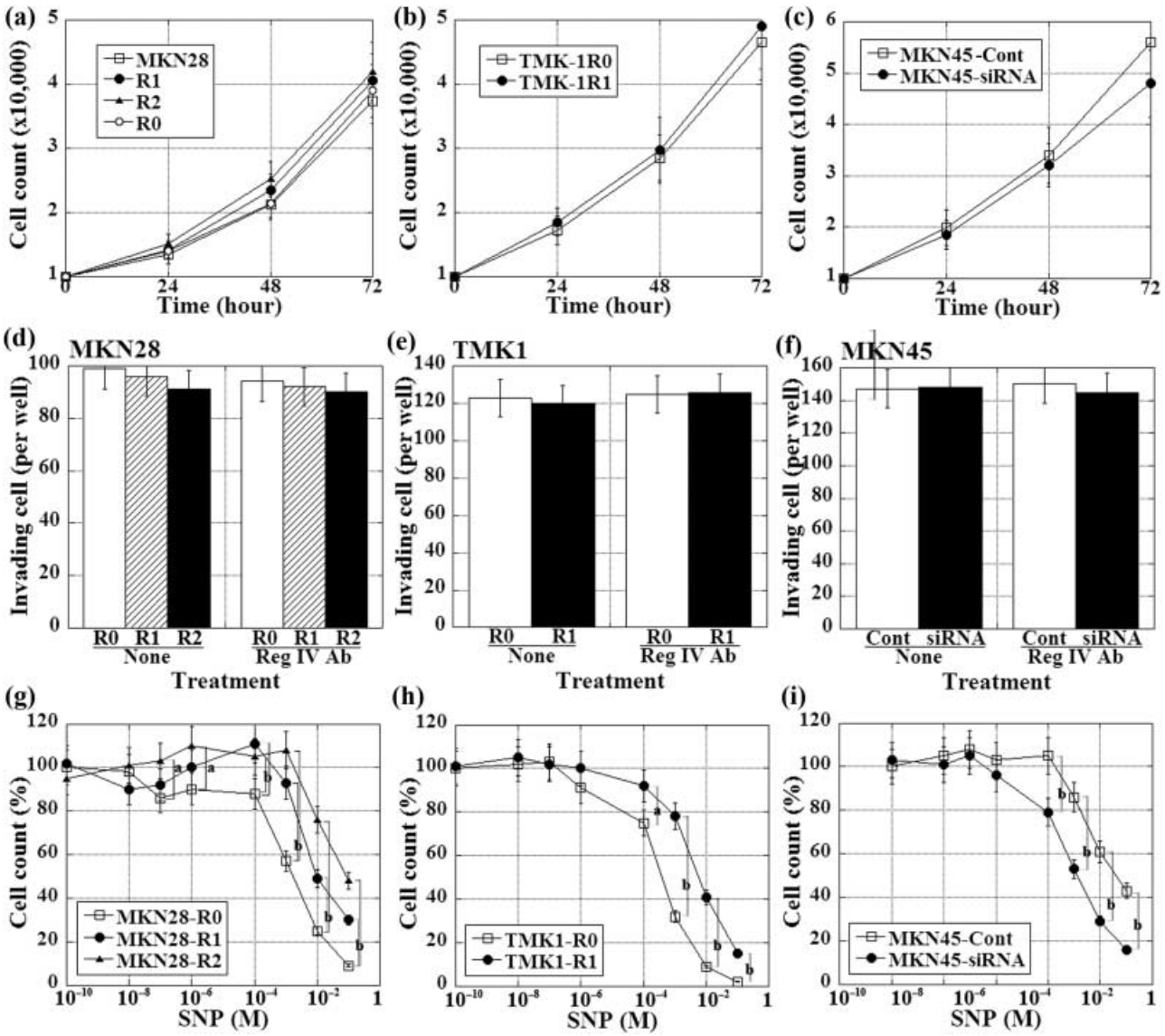


Figure 2. Effects of Reg IV transfection on cell population growth, *in vitro* invasion and nitric oxide-induced apoptosis in gastric cancer cells. (a–c) Cell growth of Reg IV transfectants and Reg IV siRNA-treated cells. (d–f) Invasion activity was examined by *in vitro* invasion assay with type IV collagen-coated insert. Anti-Reg IV polyclonal antibody was added to culture media for neutralizing secreted Reg IV at 0.5% v/v ('Reg IV Ab'). (g–i) Sensitivity to nitric oxide-induced cytotoxicity was examined. Sodium nitroprusside (SNP) was used as nitric oxide donor. (a) $P < 0.001$; (b) $P < 0.0001$. (a–c) MKN-R1, MKN-R2 and TMK1-R1: stable *Reg IV*-transfectants. MKN28-R0 and TMK1-R0: empty vector-transfectant. MKN45-siRNA: *Reg IV* siRNA-treated MKN45 cells. MKN45-Cont: siRNA mixture-treated MKN45 cells. Error bar: standard deviation.

AKT in MKN28-R1, MKN28-R2, TMK1-R1 and siRNA mixture-treated MKN45 cells were higher than those in MKN28-R0, TMK1-R0 and Reg IV siRNA-treated MKN45 cells. Third, phosphorylated form of EGFR (phosphotyrosin 992) in these cells was examined (Fig. 1c). pEGFR levels in MKN28-R1, MKN28-R2, TMK1-R1 and siRNA mixture-treated MKN45 cells were higher than those in MKN28-R0, TMK1-R0 and Reg IV siRNA-treated MKN45 cells.

Effect of Reg IV transfection on cell population growth, invasion and survival of gastric cancer cells

Next, biological effects of *Reg IV* transfection on MKN28 cells were examined (Fig. 2). As shown in Fig. 2(a), growth of MKN28-R1 and MKN28-R2 cells was not different from MKN28-R0 and MKN28 parental cells. Numbers of MKN28-R1 and MKN28-R2 cells invading into type IV collagen-coated membranes was not different

Table 1. Peritoneal metastasis of *Reg IV* transfectants

Cells	Treatment	Metastasis[a]	Number	Size (mm)
MKN28				
R0	Vehicle	2/9[b]	1.5 ± 0.7[b]	1.0 ± 0.1[d]
R1	*Reg IV*	9/9[b]	5.3 ± 1.0[b]	4.2 ± 1.2[d]
R2	*Reg IV*	9/9[b]	5.6 ± 0.9[b]	5.2 ± 1.4[d]
TMK1				
R0	Vehicle	2/8[b]	0.7 ± 0.6[c]	0.6 ± 0.1[d]
R1	*Reg IV*	8/8[b]	9.8 ± 1.5[c]	4.6 ± 0.8[d]
MKN45				
Control	siRNA mix	8/8	14.4 ± 2.1[d]	3.5 ± 0.7[d]
siRNA	*Reg IV* siRNA	5/8	0.55 ± 0.7[d]	0.9 ± 0.5[d]

[a]Metastasis determined at 2 weeks post inoculation.
[b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.0001$.

from those of MKN28-R0 cells. Anti-Reg IV antibody added to the culture media to neutralize secreted Reg IV did not affect invasion of three types of MKN28 cells (Fig. 2d). In TMK1 and MKN45 cells, transfection or knockdown of *Reg IV* did not affect the cell growth or invasion (Fig. 2b,c,e,f). We next treated these cells with nitric oxide (NO) using SNP as an NO donor. NO cytotoxicity depends on NO concentration and sensitivity of the cells (36). MKN28-R0 cells were decreased by SNP treatment in a dose-dependent manner (Fig. 2g). In contrast, MKN28-R2 and MKN28-R1 cells attenuated cell decrease in SNP concentration higher than 10^{-7} M. At 10^{-1} M, the relative cell numbers compared to untreated MKN28 parental cells were $28 \pm 3\%$ and $57 \pm 5\%$ in MKN28-R1 and MKN28-R2 cells, respectively, which were higher than that in MKN28-R0 cells ($13 \pm 2\%$) (both $P < 0.0001$). TMK1-R1 cells also showed lower sensitivities to SNP-induced cytotoxicity than that in TMK1-R0 cells. In contrast, MKN45-siRNA cells showed higher SNP sensitivities than that in MKN45-Cont cells (Fig. 2h,i).

Peritoneal tumours of Reg IV-transfectants

Reg IV-transfected MKN28 cells were inoculated into the peritoneal cavities of nude mice. Peritoneal tumours of *Reg IV*-transfected MKN28 cells were compared to MKN28-R0 tumours (Table 1, Fig. 3). Tumorigenicity of MKN28-R1 and MKN28-R2 cells was significantly higher (both 9/9) than that of MKN-R0 cells (2/9) ($P = 0.0023$). Numbers of peritoneal tumours in MKN28-R1 and MKN28-R2 cells were higher (5.3 ± 1.0 and 5.6 ± 0.9 foci, respectively) than of MKN28-R0 cells (1.5 ± 0.7 foci) ($P = 0.0364$). Sizes of tumours of MKN28-R1 and MKN28-R2 cells were significantly larger (4.2 ± 1.2 mm and 5.2 ± 1.4 mm, respectively) than of MKN28-R0 cells (1.0 ± 0.1 mm) ($P = 0.0005$). MKN28-R2 cells formed larger tumours in the peritoneum than MKN28-R0 cells (Fig. 1a). *Reg IV*-transfected TMK1-R1 cells also showed higher tumorigenicity (8/8) than TMK1-R0 cells (2/8). Numbers and sizes of the peritoneal tumours were larger (9.8 ± 1.5 foci and 4.6 ± 0.8 mm, respectively) in TMK1-R1 cells than those in TMK1-R0 cells (0.7 ± 0.6 foci and 0.6 ± 0.1 mm, respectively) ($P < 0.001$ and $P < 0.0001$). In contrast, knockdown of *Reg IV* decreased tumourigenicity, tumour number and tumour growth in MKN45 cells. *Reg IV* siRNA-treated MKN45 cells showed lower tumorigenicity (5/8) than siRNA mixture-treated MKN45 cells (8/8). The number and sizes of the peritoneal tumours in *Reg IV* siRNA-treated MKN45 cells were smaller (0.55 ± 0.7 foci and 0.9 ± 0.5 mm, respectively) than those in siRNA mixture-treated MKN45 cells (14.4 ± 2.1 foci and 3.5 ± 0.7 mm, respectively) ($P < 0.0001$ and $P < 0.0001$).

Histologically, MKN28-R0 tumours showed large areas of necrosis, whereas no necrosis was found in the MKN28-R2 tumours (Fig. 3b). Production of Reg IV in the tumours was confirmed by immunohistochemistry (Fig. 3b, Table 2). MKN28-R0 tumours contained few Reg IV-positive cells, whereas MKN28-R2 tumours showed marked Reg IV immunoreactivity in the cytoplasm in all tumour cells (Allred's grade 8). As shown in Table 2, MKN28-R1, TMK1-R1 and siRNA mixture-treated MKN45 cells showed marked Reg IV expression (grades 7, 8 and 8, respectively), whereas MKN28-R0, TMK1-R0 and *Reg IV* siRNA-treated MKN45 cells showed no Reg IV expression (grade 0).

Cell proliferation and apoptotic properties of MKN28-R1 and -R2 tumours were compared to those of MKN28-R0 tumours (Fig. 3c, Table 2). Proliferating cell nuclear antigen (PCNA) indices in MKN28-R1 and MKN28-R2 tumours were $87 \pm 5\%$ and $83 \pm 4\%$, respectively, which were similar to those in MKN28-R0 tumours ($84 \pm 5\%$). In contrast, TUNEL indices in MKN28-R1 and MKN28-R2 tumours were $0.8 \pm 0.8\%$ and $0.5 \pm 0.7\%$, respectively, which were significantly lower than in MKN28-R0 tumours ($6.2 \pm 1.6\%$, $P < 0.0001$). We examined labelling indices of PCNA and TUNEL in TMK1-R1 and *Reg IV* siRNA-treated MKN45 tumours, which were compared to those in TMK1-R0 and siRNA mixture-treated MKN45 tumours. PCNA indices in TMK1-R0 and TMK1-R1 tumours were 58 ± 7 and 65 ± 7, respectively, which were similar to each other. In contrast, the TUNEL index in *Reg IV*-transfected TMK1-R1 tumours was significantly lower (2.3 ± 1.8) than that in TMK1-R0 tumours (8.6 ± 2.4) ($P < 0.01$). TUNEL-positive apoptotic cells were significantly increased by *Reg IV* knockdown in MKN45 cells. In contrast, PCNA indices were not affected by *Reg IV* knockdown.

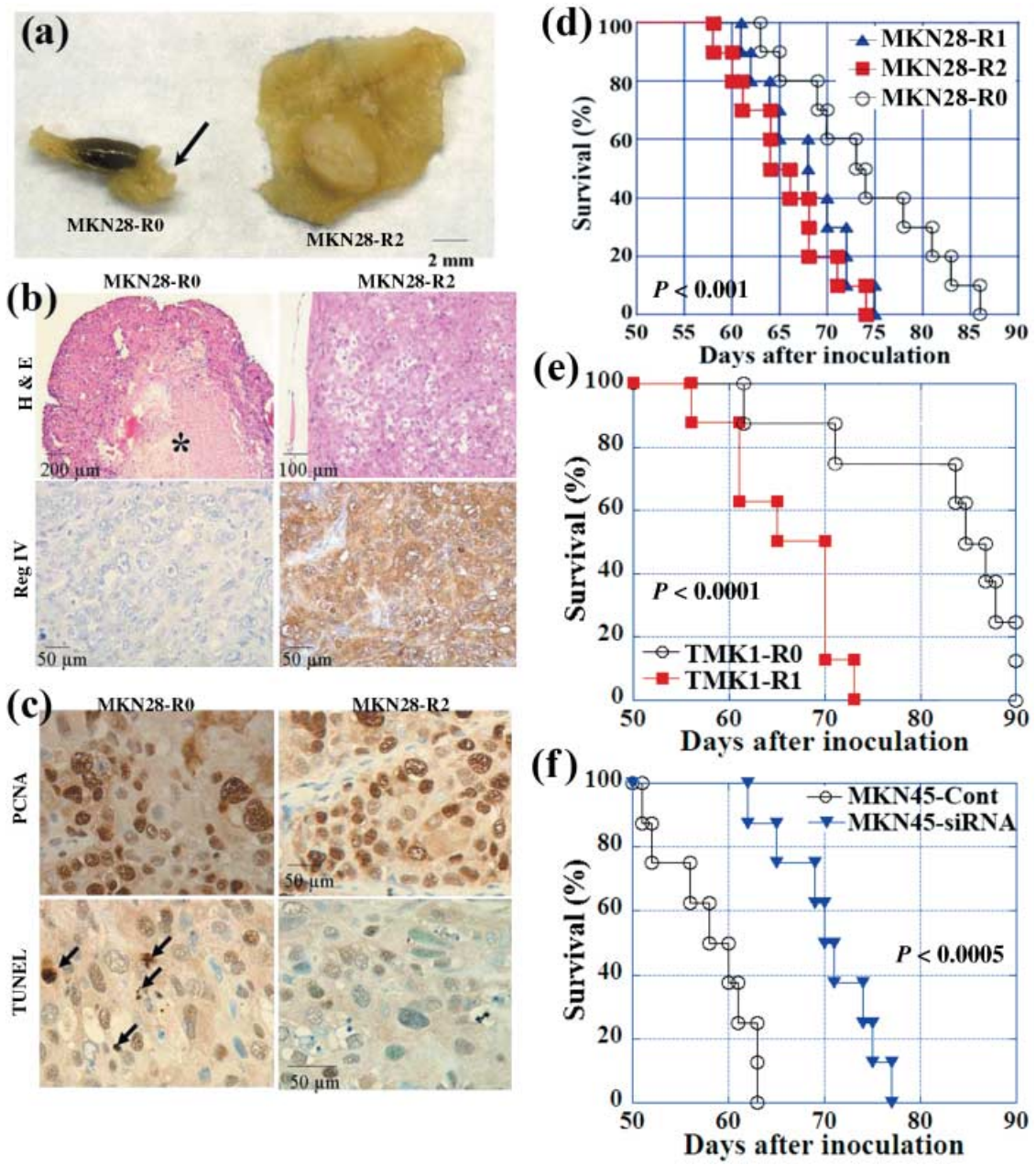


Figure 3. Peritoneal tumours of Reg IV transfectants. (a) Macroscopic appearance of MKN28-R0 cell tumour (at the mesocolon, arrow) and MKN28-R2 tumour (at the abdominal wall). (b) Histological findings using haematoxylin and eosin staining (upper panels). Necrotic area (asterisk). Immunostaining of Reg IV (lower panels). Immunoreactivity was observed in cytoplasm in MKN28-R2 cells. (c) Immunostaining of PCNA and TUNEL assay in MKN28-R2 and MKN28-R0 tumours. Arrow, TUNEL-positive apoptotic cells. (d–f) Survival of mice inoculated with MKN28-R1, MKN28-R2 and MKN28-R0 cells (d), TMK1-R0 and TMK1-R1 (e), and MKN45 treated with *Reg IV*-siRNA (MKN45-siRNA) or siRNA mixture (MIN45-Cont) (f) were calculated by Kaplan–Meier model and compared by Cox proportional hazard model. Survival of mice inoculated with MKN28-R1/MKN28-R2, TMK1-R1 and MKN45-Cont were significantly worse than those of mice inoculated with MKN28-R0, TMK-R0, MKN45-siRNA ($P < 0.001$, $P < 0.0001$, $P < 0.0005$, respectively).

Survival of mice burdened with peritoneal tumours of Reg IV-transfected gastric cancer cells

The survival of a further set of mice inoculated with *Reg IV*-transfected MKN28 and TMK1 cells or *Reg IV* siRNA-treated MKN45 cells into the peritoneal cavity was analysed (Fig. 3d–f). Mice inoculated with MKN45 cells were continuously administrated liposome-encapsulated siRNA into the peritoneal cavity. Survival of mice inoculated with MKN28-R1 and MKN28-R2 cells, or

Table 2. PCNA and TUNEL indices in peritoneal metastasis of *Reg IV* transfectants in mice

Cell line	Immunohistochemistry		
	Reg IV grade[a]	PCNA (%)	TUNEL (%)
MKN28			
R0	0	84 ± 5	6.2 ± 1.6[b,c]
R1	7	87 ± 5	0.8 ± 0.8[b]
R2	8	83 ± 4	0.5 ± 0.7[c]
TMK1			
R0	0	58 ± 7	8.6 ± 2.4[d]
R1	8	65 ± 7	2.3 ± 1.8[d]
MKN45			
Control	8	76 ± 8	3.7 ± 0.5[d]
siRNA	0	72 ± 9	10.6 ± 2.3[d]

[a]According to Allred grading. Grade 0, no staining;
Grade 7, intermediates immunoreactivity was found in all cells;
Grade 8, strong immunoreactivity was found in all cells.
[b,c]$P < 0.0001$, [d]$P < 0.01$.

TMK1-R1 cells was significantly worse than of those of mice inoculated with MKN28-R0 and TMK-R0 cells ($P < 0.001$ and $P < 0.0001$, respectively). In contrast, mice inoculated with *Reg IV* siRNA-treated MKN45 cells showed significantly better survival than those inoculated with siRNA mixture-treated MKN45 cells ($P < 0.0005$). All mice died from extended peritoneal tumours which lead to malnutrition.

Reg IV levels in peritoneal lavage fluid and serum of Reg IV transfectants-inoculated nude mice and human gastric cancer patients

Next, we detected Reg IV protein in peritoneal lavage fluid and serum from *Reg IV*-transfected MKN28 and TMK1 cells or siRNA-treated MKN45 cells (Fig. 4a,b). Reg IV protein levels in peritoneal lavage fluid from mice inoculated with MKN28-R1, MKN28-R2 and TMK1-R1 cells increased 12.3, 19.6 and 1.5 times, respectively, higher than that from mice inoculated with MKN28-R0 or TMK1-R0 cells. In contrast, Reg IV protein levels in peritoneal lavage fluid from mice inoculated with *Reg IV* siRNA-treated MKN45 cells were 9% of that in mice inoculated with siRNA mixture-treated MKN45 cells. Reg IV protein levels in serum from mice inoculated with MKN28-R1, MKN28-R2 and TMK1-R1 increased 2, 3.4 and 12 times, respectively, higher than that in mice inoculated with MKN28-R0 or TMK1-R0 cells. In contrast, Reg IV protein levels in serum from mice inoculated with *Reg IV* siRNA-treated MKN45 cells were 0.6% of that in mice inoculated with siRNA mixture-treated MKN45 cells.

Table 3. Reg IV expression in metastatic gastric cancer

	n	Reg IV expression	
		Positive	Negative
At operation			
Peritoneal metastasis (+)	43	29[a]	14
Peritoneal metastasis (–)	21	1[a,b]	20
Peritoneal recurrence	21	18[b]	3
Total	85	48	37

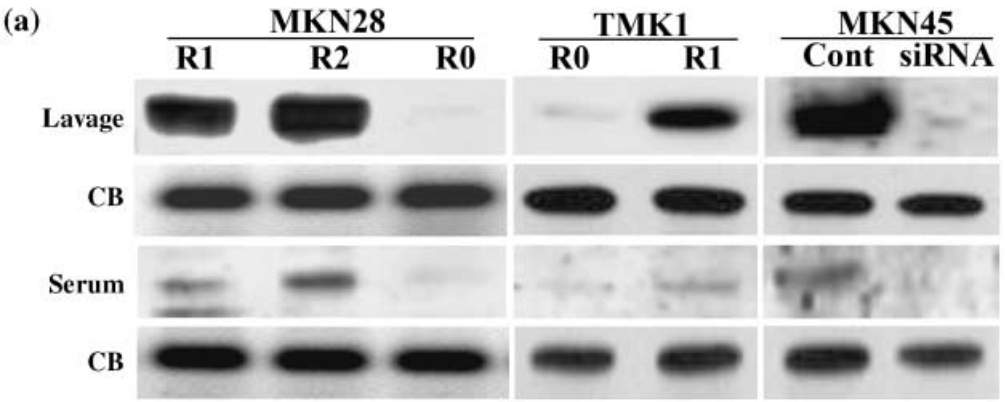
[a,b]$P < 0.0001$ (Fisher's exact test).

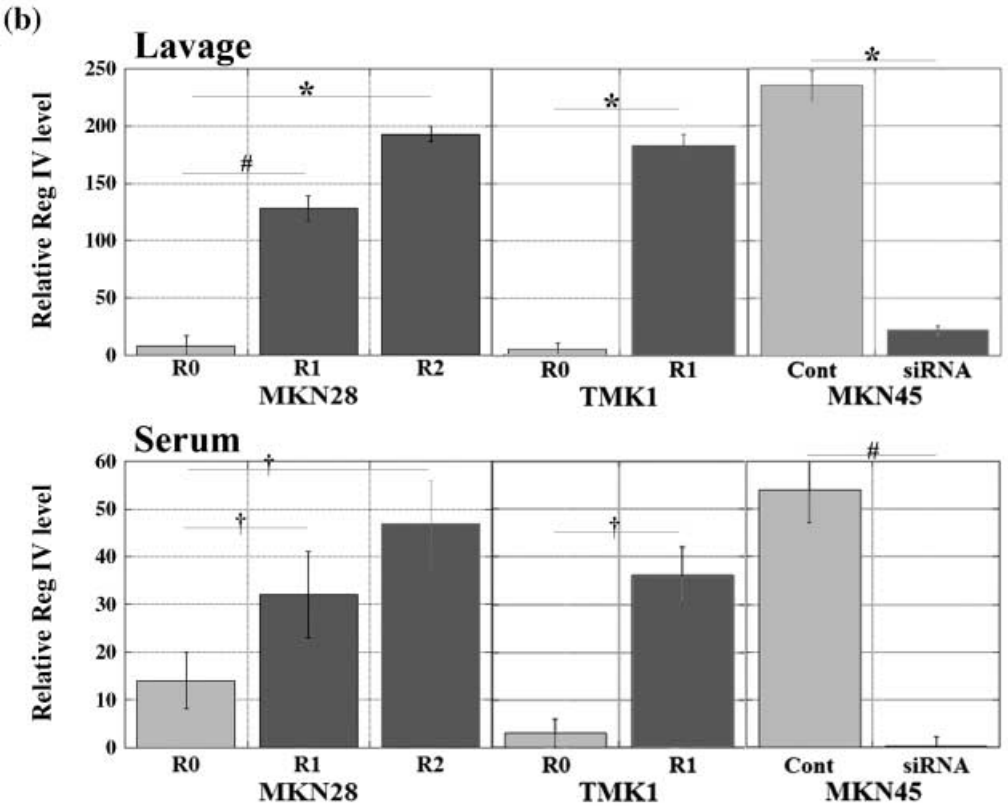
We next examined expression of Reg IV in 85 human gastric cancer patients with metastasis to the lymph nodes, liver or peritoneum (Table 3, Fig. 4c). Reg IV expression was detected in 30 of 64 (47%) gastric cancers at the time of surgery. In these cases, Reg IV was detected in 29 of 43 (67%) peritoneal metastasis-positive cases at the operation, whereas 1 of 21 (5%) peritoneal metastasis-negative cases showed Reg IV expression ($P < 0.0001$). In 21 peritoneal recurrent cases, 18 (86%) were positive for Reg IV. In 48 Reg IV-positive cases, 29 showed peritoneal metastasis at the time of operation. In contrast, the 18 out of the 48 cases showed peritoneal recurrence after the operation despite no peritoneal metastasis at the operation. It suggested that Reg IV-positive peritoneal lavage might be a marker for peritoneal recurrence.

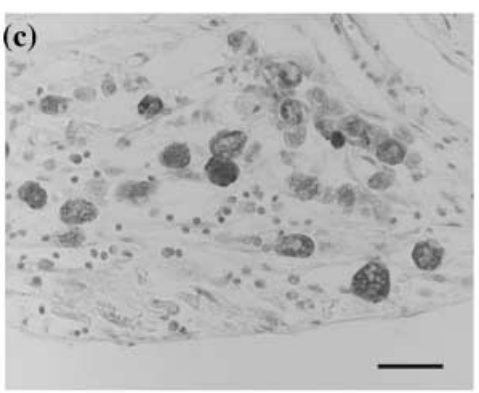
We further detected Reg IV protein in peritoneal lavage fluid from 14 gastric cancer cases, which were found to be invading into the serosa (Fig. 4c). In 8 out of the 14 cases, Reg IV protein, keratin mRNA, and cancer cells were detected in the peritoneal lavage fluids (Fig. 4d). PCR examination of epithelial cell-specific keratin was also positive in the above 8 cases, which supported the evidence that cancer cells existed in the lavage fluid. In 6 cytology-negative cases, Reg IV protein was detected in 2 cases in which keratin was detected. The other 4 cases were negative for Reg IV protein, cytology and keratin.

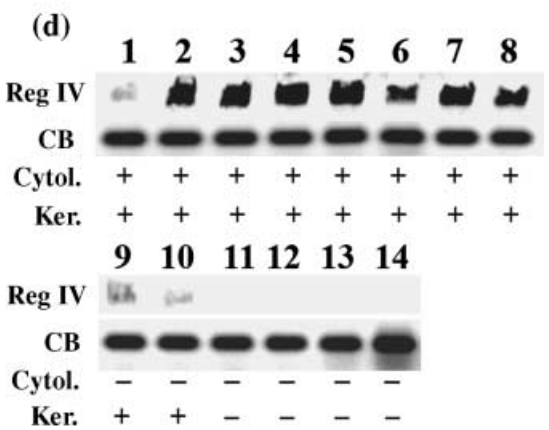
Discussion

Our data have shown that Reg IV increased expression levels of anti-apoptotic BCL-2, BCL-XL and survivin, and phosphorylation levels of AKT in *Reg IV*-transfected MKN28 and TMK1 gastric cancer cells. Moreover, *Reg IV* knockdown decreased these apoptotic factors in *Reg IV*-expressing MKN45 cells; Reg IV protein levels paralleled apoptotic factors in these cells. Although NO is a strong inducer of apoptosis, increments of anti-apoptotic factors reduced NO-induced cytotoxicity in these cells (36). Anti-apoptotic property of Reg IV has been reported in several studies, and this gives cancer cells a









survival advantages for progression and metastasis (24,27,29,37).

We have previously confirmed that EGF and the receptor formed autocrine and paracrine loops in MKN28 and TMK1 cells (38). MKN28 and TMK1 cells showed high phosphorylation levels of EGFR and Reg IV is reported to be associated with phosphorylation of EGFR (27). Our data confirmed increased phophorylation levels of EGFR in *Reg IV*-transfected MKN28 and TMK1 cells, whereas cell population growth and invasive capacity were not enhanced in the transfectants. Reg IV enhanced phosphorylation of EGFR, however, downstream signals might be preferentially associated with cell survival but not with growth and invasion in the *Reg IV*-transfected MKN28 and TMK1 cells. *Reg IV* knockdown inversely suppressed EGFR phosphorylation in MKN45 cells but the intracellular signalling pathway of Reg IV needs to be elucidated.

Reg IV-transfected and TMK1 cells produced peritoneal metastasis with larger diameter tumours and higher multiplicity than those of control cells. Proliferative activity of transfectant tumours was not different from that of the control cell tumours, whereas transfectant tumours had reduced necrosis and apoptosis in comparison to control tumours. These findings suggest that anti-apoptotic property of Reg IV renders more pronounced potential for peritoneal metastasis of MKN28 and TMK1 cells. Moreover, remarkable progression of metastatic tumours worsened survival of mice inoculated with the *Reg IV*-transfected MKN28 cells than that of mice inoculated with control cells. In contrast, *Reg IV* knockdown significantly suppressed peritoneal metastasis of MKN45 cells. Tumourigenicity was still sustained in *Reg IV* siRNA-treated MKN45 cells. *In vivo* knockdown using liposome encapsulation of siRNA might be less efficient than that of *in vitro* treatment (34). MKN45 cells express c-*met* at high levels with gene amplification, which is associated with the scirrhous phenotype and peritoneal metastasis (7,9,10).

Our previous report shows that Reg IV expression is not associated with peritoneal metastasis in the overall gastric cancer cases (33). However, examination of metastatic gastric cancer shows that Reg IV expression is significantly associated with peritoneal metastasis and peritoneal recurrence. In establishment of peritoneal metastasis, several mechanisms are proposed. Cell-to-cell adhesion between cancer cells and peritoneal mesothelial cells is an initial step of peritoneal metastasis, expression of CD44 and β1 integrin and intercellular adhesion molecule-1 playing a role in cancer cell adhesion to mesothelial cells (39,40). However, CD44 expression is silenced in MKN28 cells and also in the *Reg IV*-transfected MKN28 cells (data not shown) (41). The anti-apoptotic property of cancer cells is emphasized in formation of peritoneal metastasis (15–18). In our data, *Reg IV* transfectants showed up-regulation of several anti-apoptotic proteins: Bcl-2, Bcl-XL, survivin, and phosphorylated AKT. *Reg IV* transfectants acquired resistance to NO-induced apoptosis. TMK1 cells, which are sensitive to various apoptotic inducers, show the anti-apoptotic phenotype after *Reg IV* transfection (35). Reg IV is associated with anti-apoptotic phenotype in MKN45 cells, which carry wild-type p53 differently from MKN28 and TMK1 cells (41) and the anti-apoptotic property is not specific to peritoneal metastasis; however, enhanced survival potential might be a relevant advantage for peritoneally disseminated cancer cells to form metastatic foci.

Because Reg IV is a small secretory protein, its detection in ascites might be expected as a marker for peritoneal metastasis (22). We examined Reg IV protein in peritoneal lavage of gastric cancer cell-inoculated mice. Reg IV in peritoneal lavage fluid was at higher levels in *Reg IV*-transfected MKN28 and TMK1 cells than in control cells. In contrast, lavage Reg IV was significantly lower in mice inoculated with *Reg IV*-knocked down MKN45 cells. We then examined Reg IV protein in peritoneal lavage fluid of gastric cancer patients at the operation. Reg IV protein was detected in all cases with macroscopical and cytological peritoneal metastasis. Moreover, all cases with keratin mRNA-positive ascites showed Reg IV protein in the ascites. Ascites keratin detected by RT-PCR is a sensitive marker for scanty cancer cells in ascites in cytologically metastasis-negative cases (42). These findings suggest that ascites Reg IV might be a sensitive marker for peritoneal metastasis of gastric cancer.

In the present study, we have reported the pivotal role of Reg IV in peritoneal metastasis of gastric cancer. Reg IV is expected to be a marker for early detection of peritoneal metastasis and a prognostic marker for gastric cancer.

Figure 4. Reg IV protein levels in peritoneal lavage fluids and serum of Reg IV-transfected gastric cancer cells and human gastric cancer cases. (a) Reg IV protein levels were examined in peritoneal lavage fluids and serum of *Reg IV*-transfected MKN28 and TMK1 cells and *Reg IV* siRNA-treated MKN45 cells by immunoblotting (CB: loaded protein detected by Coomassie blue). (b) Reg IV signals of mice lavage fluids and serum were semiquantified. Reg IV signal of peritoneal lavage fluid of MKB28-R0 inoculated mice was set to 10. Error bar: standard deviation. (c) Immunohistochemistry of Reg IV in serosa-invading human gastric cancer. Signet ring cells showed strong Reg IV immunoreactivity. Bar: 50 μm. (d) Reg IV protein levels were examined in peritoneal lavage fluids from human gastric cancers by immunoblotting. Cytol., cytological examination of the lavage fluid; +, cancer cell positive; –, cancer cell negative; Ker., PCR examination of keratin in the lavage fluid.

Acknowledgement

This study was supported by Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare, Japan.

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