COLORECTAL CANCER

Enhanced expression of hepatocyte growth factor activator inhibitor type 2-related small peptide at the invasive front of colon cancers

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Background: Hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP) is a small nuclear protein abundantly expressed in the gastrointestinal epithelium. However, its functions remain unknown.
Aims: To investigate the expression and localisation of H2RSP in normal, injured and neoplastic human intestinal tissue.
Methods: Immunohistochemical examination and in situ hybridisation for H2RSP were performed using normal and diseased intestinal specimens. Its subcellular localisation and effects on the cellular proliferation and invasiveness were examined using cultured cells.
Results: In the normal intestine, H2RSP was observed in the nuclei of surface epithelial cells and this nuclear localisation was impaired in regenerating epithelium. In vitro, the nuclear translocation of H2RSP was observed along with increasing cellular density, and an overexpression of H2RSP resulted in a reduced growth rate and enhanced invasiveness. H2RSP expression was down regulated in well-differentiated colorectal adenocarcinomas. However, a marked up regulation of the cytoplasmic H2RSP immunoreactivity was observed in cancer cells at the invasive front. These cells showed low MIB-1 labelling, an enhanced p16 expression and nuclear β-catenin. The number of H2RSP-positive cells in the invasive front of well-differentiated adenocarcinomas was considerably higher in the cases with lymph node metastases than in node-negative ones.
Conclusion: In the normal intestine, the nuclear accumulation of H2RSP is a marker of differentiated epithelial cells. Although H2RSP was down regulated in colorectal adenocarcinomas, a paradoxical up regulation was observed in actively invading carcinoma cells. H2RSP immunoreactivity at the invasive front may serve as a marker of invasive phenotype of well-differentiated colon cancers.

The proliferation and differentiation of gastrointestinal epithelial cells are complex events and this mechanism has been widely investigated.[1 2] The normal gastrointestinal epithelium shows a rapid cell turnover whereby pluripotential stem cells provide a constant supply of daughter cells that pursue maturation pathways along the crypt.[2–4] This extremely short-term renewal of the cells is fundamental for the maintenance of normal gastrointestinal epithelial architecture.[5] The intestinal epithelium is an attractive biological model for stem cell experiments as the topographical position along the crypt–villous axis is directly related to the cells in a lineage.[3–9] Although the precise molecular mechanisms in the initiation and the regulation of gastrointestinal proliferation and differentiation remain to be clarified, several molecules have been reported to be involved in the regulation of stem cell function and differentiation. For example, β-catenin is located in the nuclei of the stem cells at the crypt base and mediates cell positioning in the intestinal epithelium by controlling the expression of EphB/EphrinB.[10 11] In well-differentiated colorectal adenocarcinomas, genetic changes in the Wnt pathway are often observed, thus leading to the abnormal accumulation of β-catenin in the nucleus, particularly at the invasive front.[12 13]

Hepatocyte growth factor activator inhibitor type 2 (HAI-2)-related small peptide (H2RSP) is a small nuclear peptide that was originally cloned and characterised in the process of the search for splicing variant forms of HAI-2, a proteinase inhibitor possibly involved in the regulation of hepatocyte growth factor activity.[14 15] H2RSP is identical to immortalisation up regulated protein-1 (IMUP-1), which has been identified as one of the mRNAs up regulated in SV40-immortalised compared with senescent fibroblasts.[16] The human *H2RSP/IMUP-1* gene consists of four exons spanning approximately 1 kbp, and is located 11 kbp downstream of HAI-2 gene (19q.13.11; brief gene organisation is shown in fig 1C).[15 17] An engineered expression of the deleted series of H2RSP cDNAs fused to enhanced green fluorescent protein in HeLa cells disclosed the nuclear localisation signal in the lysine-rich region of exon 4.[15 18] A chimeric mRNA transcribed from both *HAI-2* and *H2RSP* genes was present in some human tissues with a northern blot analysis.[15] However, this transcript was hardly detectable in gastrointestinal tissue and was absent in mice.[15 18] To date, little is known about the biological role of H2RSP in vivo. As IMUP-1, an identical protein to H2RSP, was up regulated in SV40-immortalised compared with senescent fibroblasts,[16] H2RSP/IMUP-1 may thus have a role in cellular proliferation or differentiation.

Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovary; FCS, fetal calf serum; HAI-2, hepatocyte growth factor activator inhibitor type 2; H2RSP, HAI-2-related small peptide; IMUP-1, immortalisation up regulated protein-1; ISH, in situ hybridisation; LCM, laser-captured microdissection; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; TBS-T, Tris-buffered saline-Tween 20

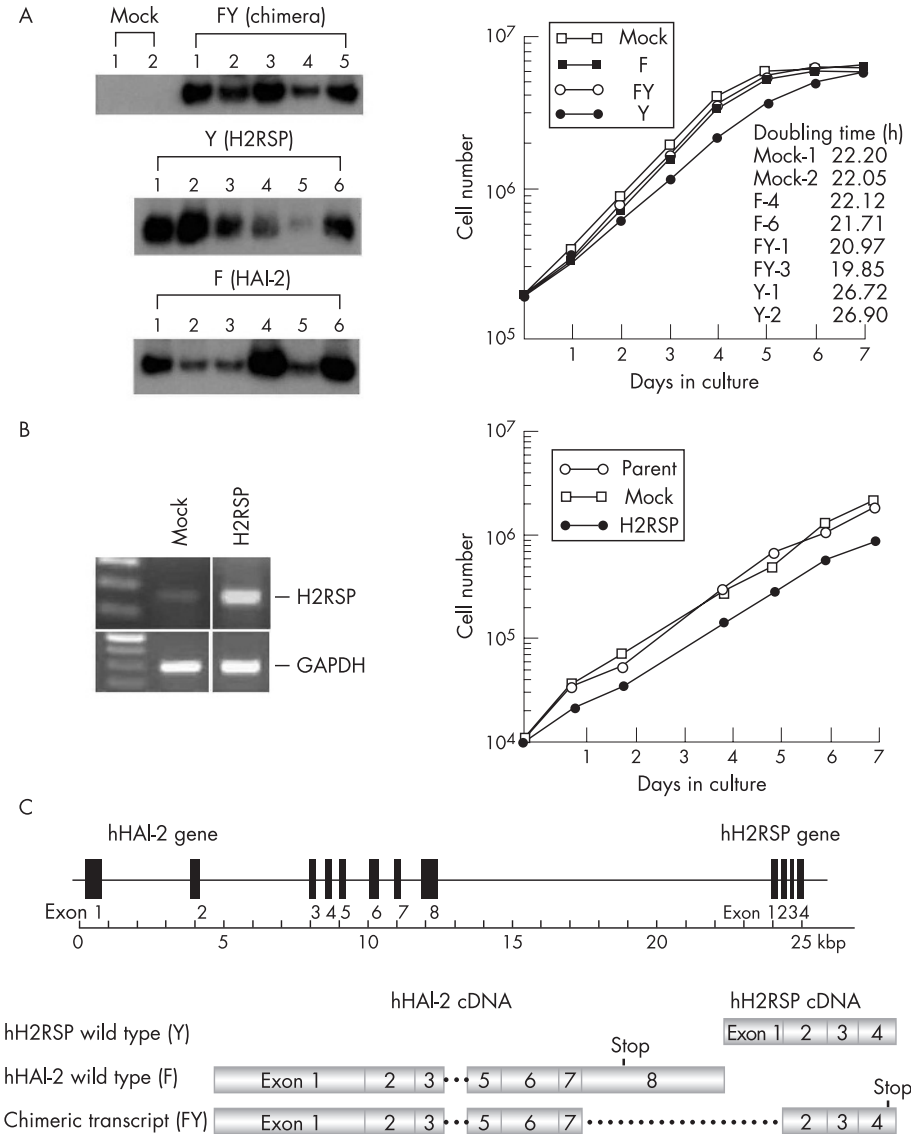

Figure 1 (A) A Northern blot analysis and growth characteristics of stable transfectants using Chinese hamster ovary (CHO) cells. F (6 clones), FY (5 clones), Y (6 clones) and mock (2 clones) were isolated and their mRNA expression levels were examined by Northern blotting using a full-length chimeric cDNA as a probe. F4 and 6, FY1 and 3, and Y1 and 2 clones abundantly expressed transfected mRNAs and were selected for following cell proliferation assay. Growth curve (semilogarithmic plot) of a representative clone from each transfectant was shown. The doubling time of each clone was also indicated in the figure. A decreased growth rate compared with mock clone was noted only in Y clones ($p<0.01$). (B) Effect of hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP) overexpression on growth of DLD-1 cells. The level of H2RSP expression was confirmed by reverse transcriptase-polymerase chain reaction. (C) Schematic representation of human hepatocyte growth factor activator inhibitor 2 (HAI-2) and H2RSP gene structure and their transcripts.

H2RSP is abundantly expressed in gastrointestinal tissue.[15 18] Our recent study using murine intestinal tissue showed the nuclear localisation of H2RSP in differentiated surface epithelial cells of the mucosa.[19] Interestingly, although H2RSP was also expressed in the epithelial cells at the crypt base, its localisation was confined in the cytoplasm of these cells.[19] Moreover, nuclear localisation was impaired in the epithelial cells showing epithelial restitution on the injured mucosal surface in a mouse experimental colitis model.[19] Hence, the expression and nuclear translocation of H2RSP might in some way be involved in the differentiation of the intestinal epithelial cells. We tried to extend the above study to human materials. To obtain insight into the possible role of H2RSP in vivo, we examined the expression and subcellular localisation of H2RSP using surgically resected normal, injured and neoplastic human intestinal tissues. We also analysed the effect of H2RSP on cellular proliferation and invasiveness in vitro.

MATERIALS AND METHODS

Antibodies

Two kinds of anti-human H2RSP rabbit polyclonal antibody were used in this study. One was generated against a synthetic peptide corresponding to the N-terminal portion of H2RSP, ^{25}DPKLSPHKVQGRSEAG^{40}, whereas the other was generated against recombinant full-length H2RSP.[15 19] Anti-human β-catenin (Sigma, Steinheim, Germany), lamin A/C (Cell Signaling Technology, Danvers, Massachusetts, USA), heat shock protein 70 (HSP70; Cell Signaling Technology) rabbit polyclonal antibodies, anti-human p16 (BD Bioscience, San

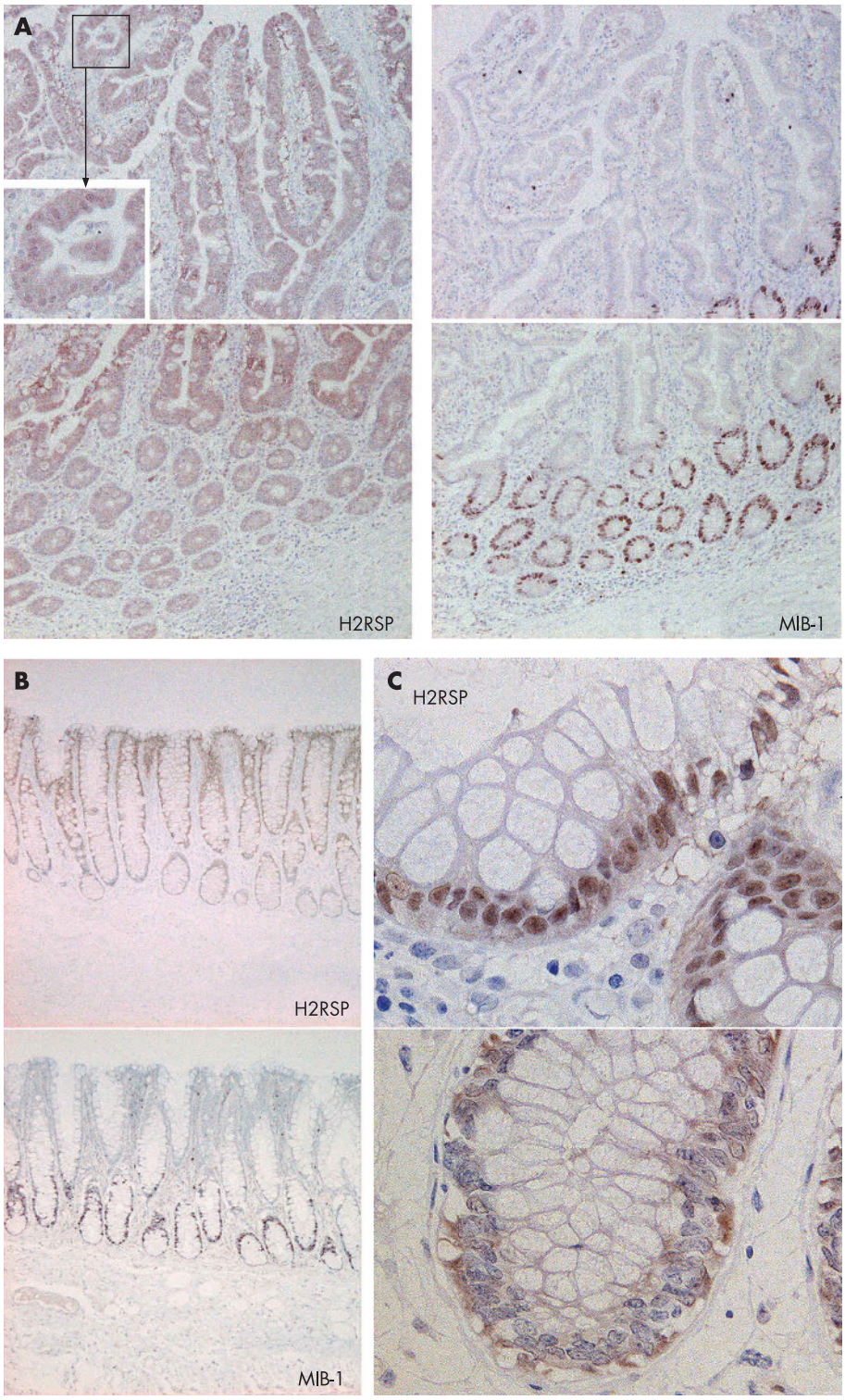

Figure 2 Immunohistochemical localisation of hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP) in the jejunum (A) and colon (B,C). H2RSP was detected mainly in the nucleus of the surface epithelial cells (C, upper panel), which were negative for MIB-1. On the other hand, at the crypt base, the cytoplasm of the epithelial cell was weakly positive for H2RSP and most of the nuclei of these cells were negative (C, lower panel).

Jose, California, USA) and Ki-67 (clone MIB-1; DAKO, Glostrup, Denmark) monoclonal antibodies were also used.

Immunohistochemical analyses and immunofluorescence

A total of 46 samples of colorectal carcinomas were used, including 34 cases of differentiated (well to moderately differentiated) adenocarcinoma (12 cases of mainly well-differentiated and 22 cases of mainly moderately differentiated adenocarcinomas), 5 cases of poorly differentiated adenocarcinoma, and 5 cases of mucinous carcinoma. Formalin-fixed and paraffin-wax-embedded sections were subjected to antigen retrieval by autoclaving for 5 min in 10 mM citrate buffer, pH 6.0. After peroxidase blocking, the sections were blocked in 3% bovine serum albumin (BSA) and 10% goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature.

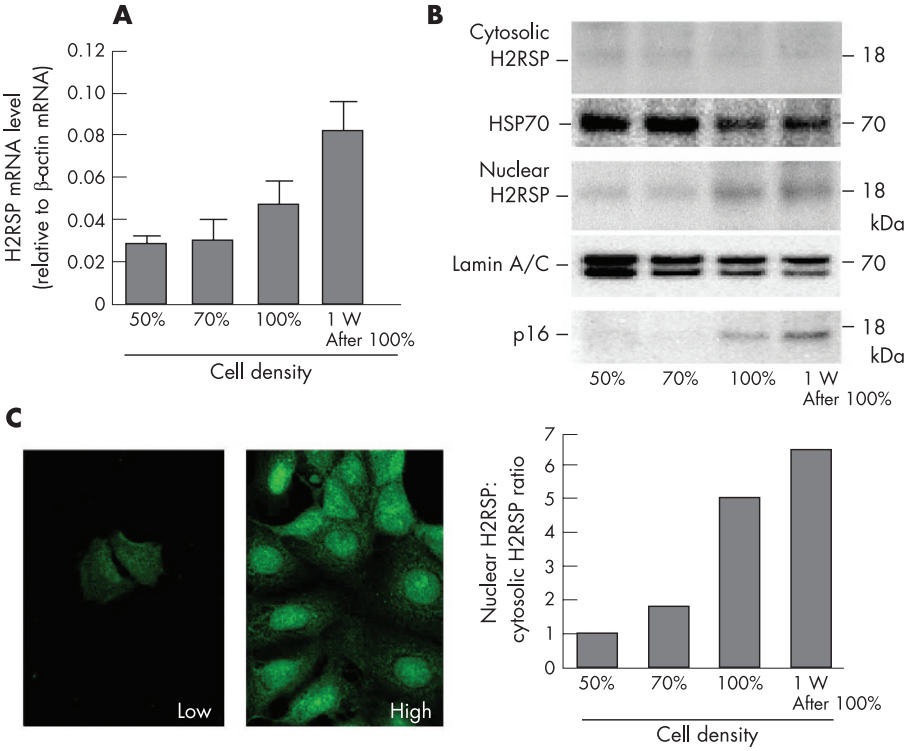

Figure 3 (A) Expression of hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP) in cultured DLD-1 cells at varying culture densities. The level of H2RSP mRNA was measured by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and normalised by the β-actin mRNA level in the same sample. The cells at the saturation density (ie, 1 week after a 100% density) showed a threefold increase H2RSP mRNA level compared with those at a 50% cell density. (B) Subcellular localisation of H2RSP protein in cultured DLD-1 cells at varying culture densities. The signals of H2RSP detected in cytosolic and nuclear fractions at either cell density were normalised by internal loading control (HSP70 and lamin A/C, respectively), and nuclear:cytoplasmic H2RSP ratio was calculated and shown as a bar graph. Values are mean of triplicated experiments. Immunoblotting for p16 was also shown as a marker for cellular proliferation. (C) Immunofluorescence analysis of the subcellular localisation of H2RSP in DLD-1 cells. Predominant nuclear or nucleolar localisation of H2RSP was observed in cells of high density (high) compared with those of low density (low).

Subsequently, the sections were incubated with anti-H2RSP antibody (5 μg/ml in 1% BSA/PBS) or anti-Ki-67 antibody (MIB-1; 1:50 dilution) at 4℃ overnight. Two kinds of anti-H2RSP antibody were tested, and showed similar results. Selected cases were also stained for β-catenin and p16. For the absorption test, the antibodies were pretreated with 100-fold excess amounts of recombinant H2RSP. Negative controls consisted of an omission of the primary antibodies. The sections were rinsed in PBS and incubated with Envision-labelled polymer (DAKO) for 30 min at 37℃. After washing, the sections were visualised with nickel, cobalt-3,3′-diaminobenzidine (Pierce, Rockford, Illinois, USA) and counter stained with haematoxylin. The histology and immunoreactivity were independently evaluated by two pathologists.

For immunofluorescence, cells cultured on chamber slides were fixed with 4% formaldehyde/PBS. They were then washed with PBS, followed by incubation in 0.2% Triton X/3% BSA/PBS for 1 h. The anti-H2RSP peptide antibody (10 μg/ml) was added in 3% BSA/PBS and the slides were incubated at 4℃ overnight. Alexa Fluor 488-conjugated Fab fragment of goat anti-rabbit IgG (Invitrogen, Carlsbad, California, USA) at a dilution of 1:200 was used as detection antibody.

In situ hybridisation, laser-captured microdissection and real-time reverse transcriptase-polymerase chain reaction analysis

For in situ hybridisation (ISH) study, frozen sections (4-μm thick) were fixed in 4% paraformaldehyde/PBS, dehydrated and used for ISH reaction with a fully automated ISH apparatus (Ventana, Yokohama, Japan) as described previously.[19] A 352-bp cDNA fragment corresponding to bases 88–439 of the human H2RSP cDNA sequence[15] was used as a template to generate digoxigenin-labelled probes. The same amount of each antisense or sense probe (1 ng/slide) was used. The reaction was visualised with BlueMap Kit (Roche, GmbH, Penzberg, Germany) and counterstained with nuclear fast red. In some experiments, immunohistochemical staining was also performed in serial frozen sections.

For laser-captured microdissection (LCM), thick-sliced frozen sections (10 μm) were subjected to LCM using a Leica SVS LMD System (Leica Microsystems, Wetzlar, Germany). Cancer cells and adjacent normal epithelium were selectively dissected from samples of six cases of colorectal-differentiated adenocarcinoma, and total cellular RNA was obtained.[19] For real-time reverse transcriptase-polymerase chain reaction (RT-PCR), total RNA (1 μg) was reverse transcribed and the resultant cDNA subjected to real-time PCR on a LightCycler with a master mix of SYBR Green I (Roche).[19] The amount of mRNA per sample was normalised by β-actin mRNA level, and the tumour versus normal (T:N) ratio was calculated. The primer sequences of H2RSP were as follows: sense, 5′-CCGCCATGGAGTTCGA CCTG-3′; antisense, 5′-GAGCTGTGGTGTCCTTGCTT-3′.

All fresh human tissues were obtained from surgical specimens of patients with colorectal adenocarcinoma and ulcerative colitis in the University of Miyazaki Hospital, Miyazaki, Japan. Informed consent was obtained from the patients and the protocol was approved by the ethical board of the Faculty of Medicine, University of Miyazaki.

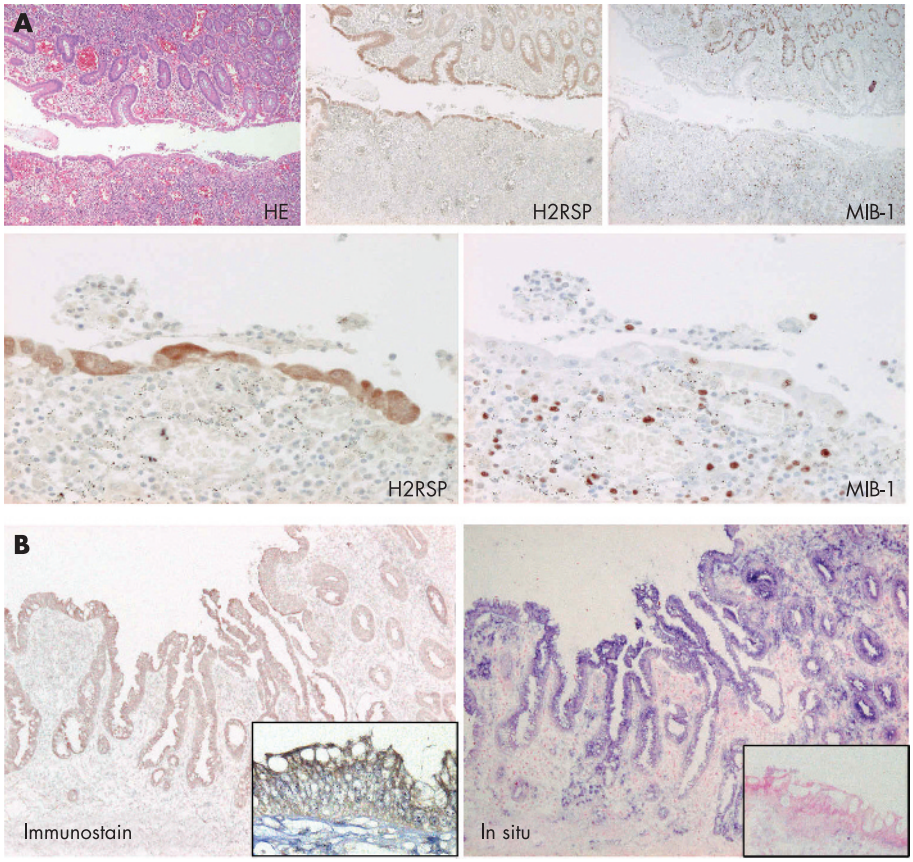

Figure 4 (A) The immunohistochemical localisation of hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP) in inflamed and erosive colonic mucosa (ulcerative colitis), showing strong up regulation of H2RSP in the regenerating epithelial cells. A higher magnification of the regenerating epithelium showing restitution is shown in the lower panel, in which the H2RSP immunoreactivity was observed in the cytoplasm, even though the cells are covering the mucosal surface. Note that these cells are MIB1-negative (non-dividing) migrating cells. (B) Immunohistochemistry (left) and in situ hybridisation (ISH; right) of H2RSP in the inflamed and regenerated colonic mucosa (ulcerative colitis). Strong signals of H2RSP were detected in the regenerated epithelium. A higher magnification of the immunostain (inset) showed cytoplasmic localisation of H2RSP. The inset in the right panel is a negative control of ISH (sense probe). HE, haematoxylin–eosin. Original magnification 40× and 200× (inset).

Cell culture and extraction of cellular proteins and immunoblot analysis

Human colon carcinoma cell line DLD-1 and Chinese hamster ovary (CHO) cell line were obtained from the RIKEN cell bank (Wako, Japan) and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2 at 37℃. To examine the cellular localisation of H2RSP, cellular proteins of DLD-1 cells were extracted at various culture densities and fractionated in cytosolic and nuclear fractions using ProteoExtract Kit (Merck, Darmstadt, Germany). Samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions using a 4–12% gradient gel, and transferred on to an Immobilon membrane (Millipore, Billerica, Massachusetts, USA). After blocking with 5% non-fat dry milk in 50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride and 0.05% Tween 20 (TBS-T), the membrane was probed with anti-recombinant H2RSP antibody with 1% BSA/TBS-T at 4℃ overnight. After washing in TBS-T, the membrane was incubated with peroxidase-conjugated secondary antibody with 1% BSA/TBS-T for 30 min at room temperature. The labelled proteins were visualised with a chemiluminescence reagent (NEN Life Science, Boston, Massachusetts, USA). For the internal control of loading, the cytosolic or nuclear extract was probed with anti-HSP70 or anti-lamin A/C antibody, respectively. As a parameter for cellular growth, whole cellular extracts were subjected to the detection of p16 protein.

Establishment of H2RSP-expressing cells and their growth characteristics in vitro

A mammalian expression plasmid vector, pCIneo (Promega, Madison, Wisconsin, USA), was used to generate stable transfectants. Three expression plasmids, designated F, consisting of human HAI-2 cDNA, FY, consisting of chimeric cDNA of *HAI-2* and *H2RSP* genes, and Y consisting of H2RSP cDNA, were generated by the ligation of *Sal*I/*Not*I-digested PCR products into pCIneo vector. Plasmid without the insert was used for a transfection control (mock). Cultured cells were transfected with the vectors using Tfx-10 reagents (Promega). Stable transfectants were selected by geneticin (0.5 mg/ml) treatment and isolated clones were obtained. The expression levels were verified by northern blot or RT-PCR.[15] F (6 clones), FY (5 clones), Y (6 clones) and mock (2 clones) were isolated using CHO cells, and two clones of each were selected for subsequent study. A clone of DLD-1 overexpressing H2RSP was also prepared. For cell proliferation assay, triplicated 35-mm culture dishes were seeded at 2×10^5 cells. The number of viable cells was counted daily for a week. The experiments were repeated twice.

Knockdown experiment of H2RSP

For the knockdown of *H2RSP* gene, we created an siRNA expression vector for H2RSP using psiRNA-hH1zeo (Invivogen, San Diego, California, USA). Briefly, psiRNA-H2RSP was generated by the ligation of annealed siRNA containing the

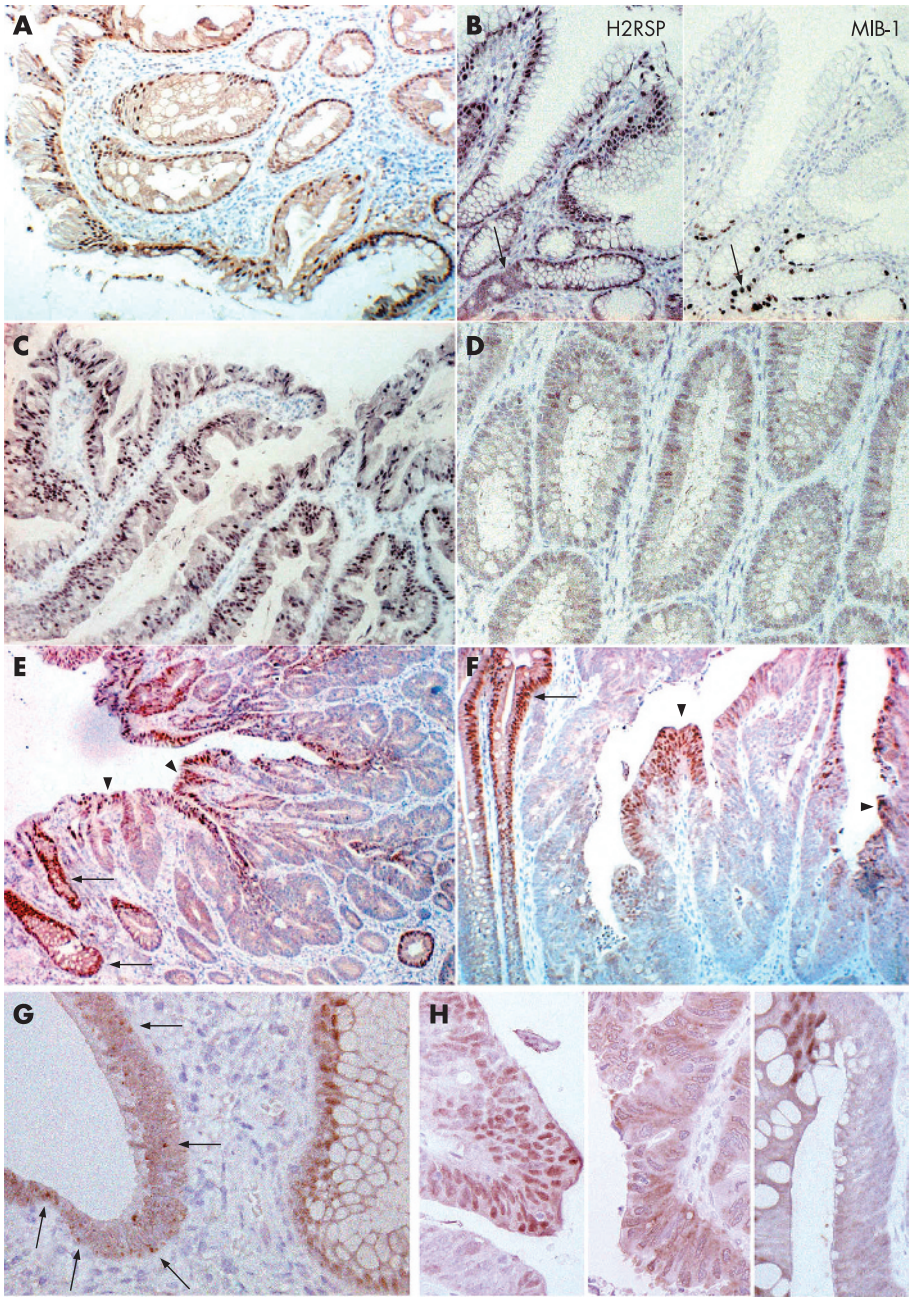

Figure 5 Immunohistochemistry of hyperplastic polyp (A,B); low-grade serrated adenoma (C); low-grade tubular adenoma (D); and high-grade tubular adenomas (E–H). In hyperplastic polyp, the immunoreactivity of hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP) was similar to that of the normal colorectal mucosa (A), and reciprocal immunostain pattern between nuclear H2RSP and MIB-1 was observed (B, arrows). In low-grade adenomas (C,D), similar nuclear staining of H2RSP (C) or reduced immunoreactivity (D) was observed. In high-grade adenomas (E,F), a decreased immunoreactivity was evident in the adenoma cells relative to adjacent normal epithelium (arrows), although the nuclear localisation of H2RSP tended to occur in the adenoma cells covering the surface (arrow heads). Irregular H2RSP immunostain patterns, showing dot-like cytoplasmic localisation (G, arrows), nuclear (H, left), cytoplasmic (H, middle) and decreased immunoreactivity (H, right) were observed.

sequence of 21 nucleotides within the coding region of the *H2RSP* gene into *Bbs*I-digested psiRNA plasmid. The plasmid containing the shuffle sequence was used for a control. Cultured DLD-1 cells were transfected with the vectors using FuGENE 6 transfection reagents (Roche). Stable transfectants were selected by Zeocin (150 μg/ml) and isolated clones were obtained. The annealed sequences for H2RSP siRNA were as follows: sense, 5′-TCCCAACATCCGTGTCCGAATCGCTGTTGA TATCCGCAGCGATTCGGACACGGATGTTT-3′; antisense, 5′-CA AAAAACATCCGTGTCCGAATCGCTGCGGATATCAACAGCGAT TCGGACACGGATGTT-3′.

In vitro invasion assay

Chemotaxicell containing 8-μm pore size polyvinyl-pyrrolidone-free polycarbonate filter (Kurabo, Osaka, Japan) was coated with 12.5 μg/well of Matrigel (BD Bioscience). Cells (4×10^5 cells/0.1 ml FCS-free medium with 0.1% BSA) were placed in the chemotaxicells. As a chemoattractant, 5% FCS was added

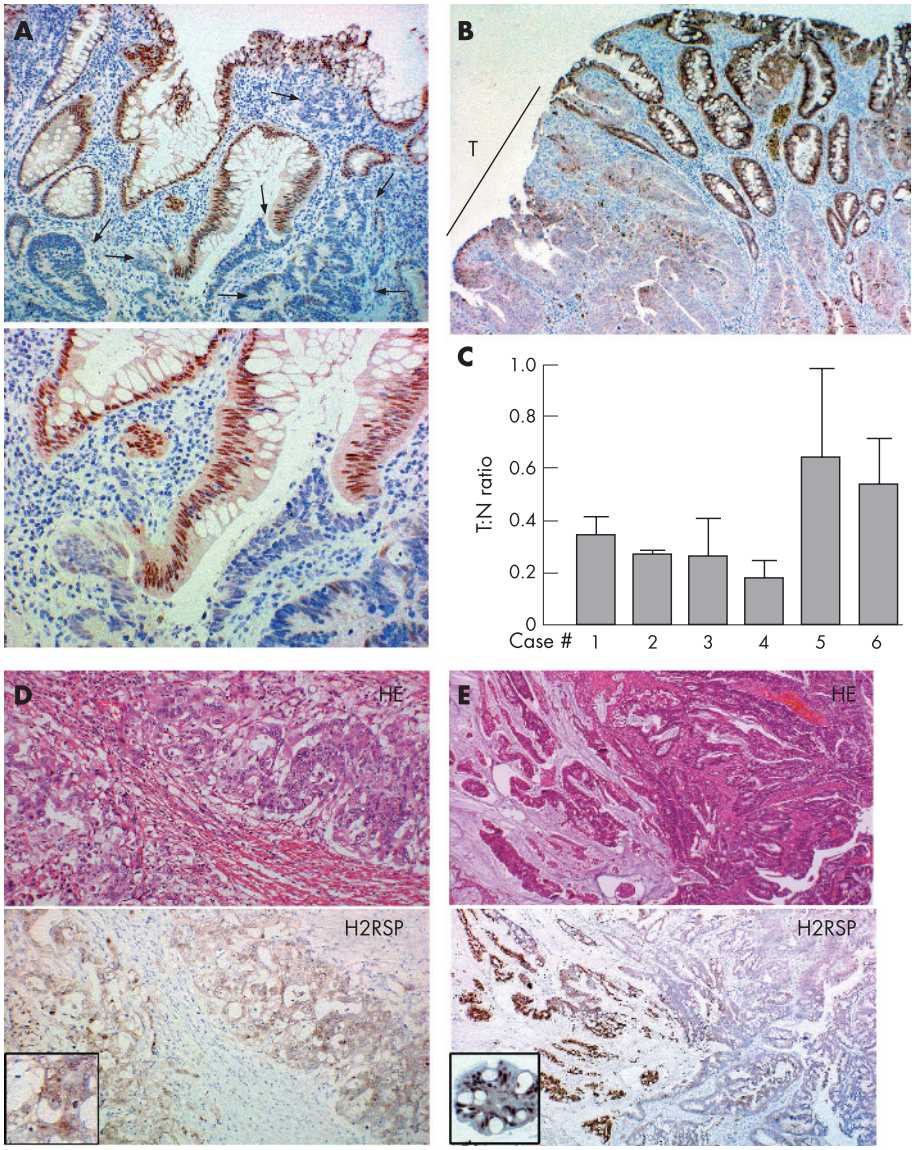

Figure 6 The expression of hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP) in colorectal adenocarcinomas. (A) In well-differentiated adenocarcinoma, a markedly reduced immunoreactivity of H2RSP was observed in the carcinoma cells (arrows). The lower panel shows a high magnification of the boundary between cancer cells and non-cancerous epithelium. (B) Similar findings were observed in well to moderately differentiated adenocarcinoma. Only focally, weak and irregular immunoreactivity was detected at the surface portion. (C) The level of H2RSP mRNA in cancer cells relative to the corresponding normal epithelium was examined by laser-captured microdissection (LCM) followed by real-time reverse transcriptase-polymerase chain reaction. Six cases of well-differentiated adenocarcinoma were examined. The mRNA levels were normalised by β-actin mRNA in the same sample. The results were shown as a T:N ratio. The mean (SE) of three independent experiments is indicated. (D) Immunohistochemistry of H2RSP in poorly differentiated adenocarcinoma. A weak but diffuse cytoplasmic reactivity is observed. A higher magnification is also shown (inset). (E) Immunohistochemistry of H2RSP in mucinous carcinoma. Both moderately differentiated adenocarcinoma portion (right half part) and mucinous carcinoma portion (left part) are shown. Although H2RSP was considerably down regulated in the differentiated adenocarcinoma portion, a strong nuclear immunoreactivity was evident in most mucinous carcinoma cells floating in mucin lakes. A higher magnification of the mucinous carcinoma cells is also shown (inset). Original magnification 40× and 100× (inset).

into the lower compartment. After incubation at 37℃ in 5% CO_2 for 24 h, the filters were fixed with 4% formaldehyde/PBS and stained with haematoxylin. Invasion was quantified by counting the cells in 10 randomly selected fields (100-fold magnification).

Statistical analysis

The statistical parameters were assessed using the Statview 5J software program and significance was determined using either Mann–Whitney U test or one-way analysis of variance. p Values <0.05 were considered to be significant. The values were expressed as the mean or mean (SE).

RESULTS

Expression and localisation of H2RSP in normal intestinal tissues

We first examined the immunohistochemical localisation of H2RSP in normal human intestinal tissue and compared their staining patterns with those of the serial sections stained by MIB-1 recognising proliferating cells. In the normal epithelia of jejunum and colon, H2RSP immunoreactivity was mainly observed in the nuclei of the surface epithelial cells, which were negative for MIB-1 (fig 2A–C). The positive staining disappeared by omission of primary antibodies or addition of excess recombinant H2RSP (data not shown). These nuclear

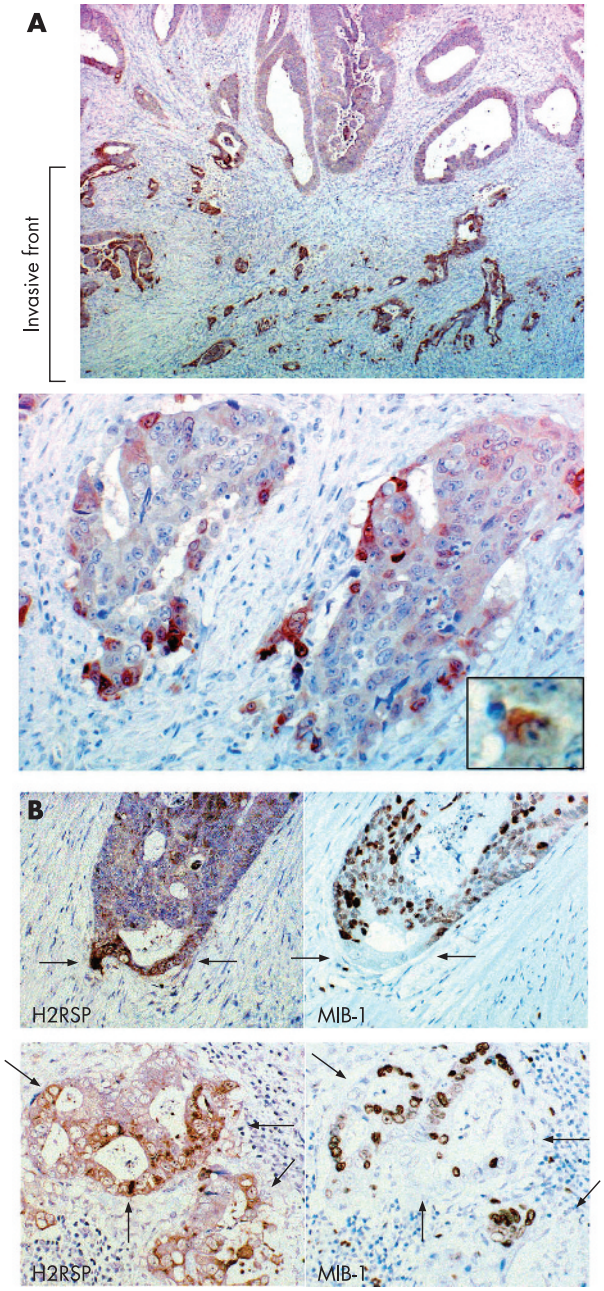

Figure 7 (A) The paradoxical up regulation of hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP) at the invasive front of well (upper panel) and moderately (lower panel) differentiated adenocarcinomas. Strong immunoreactivity of H2RSP was observed in the sprouting tumour cells showing active stromal invasion. Note that these cells show a cytoplasmic immunolocalisation of H2RSP (lower panel, inset) as observed in the regenerating intestinal epithelial cells. (B) The reciprocal immunostain pattern of H2RSP and MIB-1 at the invasive front. Note that MIB-1-negative cells show a strong H2RSP immunoreactivity (arrows).

H2RSP-positive surface epithelial cells were considered to be matured and differentiated intestinal epithelial cells. Goblet cells of the colon also showed nuclear immunoreactivity of H2RSP as long as the cells were present on or near the mucosal surface. On the other hand, the cells at the proliferating zones, which were strongly positive for MIB-1, did not show nuclear localisation of H2RSP with a cytoplasmic staining pattern (fig 2C). Therefore, the nuclear staining of H2RSP showed a pattern opposite to that of MIB-1. These staining patterns were essentially similar to those observed in murine intestinal tissues.[19] A normal human gastric mucosa also showed similar immunostaining pattern, with nuclear localisation of H2RSP in the cells covering the mucosal surface (data not shown). These results of immunohistochemical studies suggested that H2RSP is localised in the cytoplasm of mucosal epithelial cells at the proliferating zone and then translocated and accumulated in the nuclei possibly along with the cellular migration towards the mucosal surface and after cellular differentiation. It was therefore assumed that the nuclear translocation of H2RSP may be somehow involved in the maturation and differentiation of surface epithelial cells of gastrointestinal mucosa.

Localisation and nuclear translocation of H2RSP in cultured epithelial cells and the effects of an H2RSP overexpression on cellular proliferation

To assess the possible role of H2RSP in cellular proliferation or differentiation, we examined the nuclear translocation of H2RSP and its relationship to cellular proliferating activity in vitro. In this study, a human intestinal adenocarcinoma cell line, DLD-1, was cultured, and fractionated samples (nuclear and cytosolic fractions) were extracted at various cell densities—namely, 50%, 70%, 100% cell density and overconfluency (maintained for 1 week after 100% cell density). Then the expression and localisation of H2RSP were analysed. A low level of H2RSP mRNA was observed in DLD-1 cells and the level increased in parallel to the increased cell density. By a RT-PCR analysis, approximately a threefold increase in the level of H2RSP mRNA was observed in the overconfluent cells compared with cells at 50% density (fig 3A). H2RSP was detected in both the cytosolic and nucleic fractions at each density, but the proportion of nuclear H2RSP increased significantly in response to the increased cellular density (fig 3B). Indeed, the ratio of nuclear H2RSP to cytosolic H2RSP increased 6.57-fold at the saturation density relative to that at 50% density, thus indicating the accumulation of H2RSP in the nuclei of cells at high cell density (fig 3B). The predominant nuclear (and nucleolar) localisation of H2RSP in cells of high density was further confirmed by immunofluorescence (fig 3C). Therefore, H2RSP may be translocated from the cytoplasm to the nuclei in parallel with the down regulation of the epithelial cell proliferation.

We next wished to examine the effect of engineered expression of H2RSP on cellular proliferation. For this purpose, we prepared stable CHO clones with an overexpression of H2RSP (designated as Y), HAI-2 (F) or HAI-2/H2RSP chimeric mRNA (FY). Two stable clones were selected for each mRNA (fig 1A). The in vitro doubling time was prolonged in Y clones relative to mock, F, or FY clones, with mean doubling times of 26.8 (Y), 22.1 (mock), 21.9 (F) and 20.4 (FY) hours (fig 1A). A similar effect of H2RSP was also observed in DLD-1 cells (fig 1B). These results indicated that the stable overexpression of H2RSP resulted in reduced cellular proliferation in vitro. Figure 1 shows the schematic representation of these constructs, and *HAI-2* and *H2RSP* gene organisation.

Altered expression pattern of H2RSP in inflamed intestinal tissues

We next investigated the H2RSP immunoreactivity in inflamed intestinal tissue specimens taken from patients with ulcerative colitis (fig 4A). An increased immunoreactivity of H2RSP was observed in the regenerating epithelial cells of the injured mucosal tissues. Notably, the up regulated H2RSP immunoreactivity was mainly observed in the cytoplasm of the regenerating epithelial cells, even on the mucosal surface (fig 4A, lower panel). These cells were considered to be migrating epithelial cells showing restitution, but not proliferating cells,

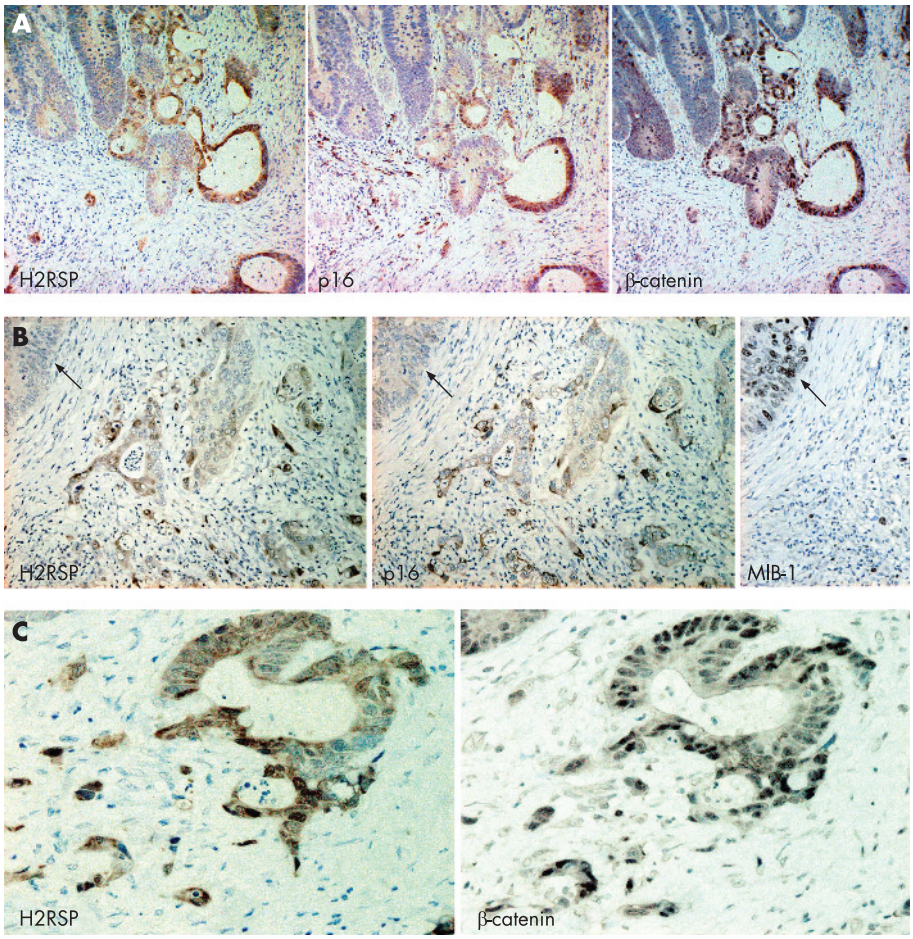

Figure 8 (A) The expression of hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP), p16 and nuclear β-catenin at the invasive front of differentiated adenocarcinoma. Serial sections were immunostained and compared. The H2RSP-positive cancer cells at the invasive front were also positive for p16 and nuclear β-catenin. (B) The coexpression of H2RSP and p16 in sprouting cancer cells at the invasive front of differentiated adenocarcinoma. Note that MIB-1 immunoreactivity is reciprocal to H2RSP and p16 (indicated by arrow). (C) The coexpression of H2RSP and nuclear β-catenin in sprouting cancer cells at the invasive front.

because only a few cells were positive for MIB1 (fig 4A). The inflamed regenerated mucosa also showed an enhanced cytoplasmic H2RSP expression in the epithelial cells (fig 4B). Low but distinct immunoreactivity of H2RSP was occasionally observed in the interstitial cells, which were infiltrating inflammatory and myofibroblastic cells, in the injured mucosa. The ISH for H2RSP mRNA also confirmed the expression in regenerated surface epithelium and increased number of H2RSP-positive interstitial cells in the injured mucosa (fig 4B). Therefore, regenerating epithelial cells of injured intestinal mucosa expressed H2RSP, and the nuclear translocation of H2RSP was impaired in these cells. These findings are compatible with our previous observations using a mouse experimental colitis model.[19]

H2RSP expression in hyperplastic polyps and adenomas

In colorectal hyperplastic polyps, immunoreactivity of H2RSP was closely similar to that of the normal mucosa, thus showing nuclear staining in the surface hyperplastic epithelium (fig 5A, B). These cells were negative for MIB-1, and the MIB-1-positive cells at the crypt base showed cytoplasmic H2RSP immunoreactivity (fig 5B, arrow). However, these patterns of H2RSP immunolocalisation were altered in neoplastic polyps such as adenomas. Generally, the H2RSP immunoreactivity was decreased in adenoma cells. However, nuclear localisation of H2RSP was often seen in low-grade adenomas (fig 5C, D). In high-grade adenomas, the decreased immunoreactivity of H2RSP was evident (fig 5E, F). Nuclear and cytoplasmic staining patterns of H2RSP were irregularly observed in both surface and deep portions (fig 5G, H), although there was still a tendency of nuclear localisation of H2RSP in cells covering the surface (fig 5E, F).

H2RSP expression in colorectal adenocarcinomas

In colorectal adenocarcinomas, a decreased immunoreactivity of H2RSP was evident relative to the adjacent normal epithelium (fig 6A, B). A marked reduction of immunoreactivity was noted in 23 cases among 34 cases of differentiated adenocarcinomas examined. The decreased expression of H2RSP was also confirmed in the carcinoma cells by LCM followed by RT-PCR analysis (fig 6C). Although H2RSP-positive cancer cells were still observed in part, no consistent findings for the subcellular localisation of H2RSP could be identified, thus showing irregular combinations of predominantly cytoplasmic, mixed cytoplasmic and nuclear, and predominantly nuclear immunoreactivities (data not shown). On the other hand, some carcinoma cases showed a preserved and diffuse immunoreactivity of H2RSP. This was particularly evident in mucinous carcinomas, and also in poorly differentiated adenocarcinoma cases (fig 6D, E). In four of five cases of poorly differentiated adenocarcinoma, weak but diffuse immunoreactivity of H2RSP was noted, and the

cytoplasmic localisation of the immunoreactivity was predominantly seen (fig 6D). On the other hand, in mucinous carcinomas, strong nuclear or nucleolar immunoreactivity of H2RSP was commonly observed in the tumour cells floating in mucin lakes (fig 6E).

An enhanced expression of the H2RSP at invasive front of differentiated colorectal adenocarcinomas

Although H2RSP immunoreactivity was markedly decreased in the centre areas in most cases of differentiated colorectal adenocarcinomas, marked up regulation of H2RSP immunoreactivity was paradoxically observed in the cancer cells at the invasive front of the tumour (fig 7A). This phenotype was apparent in 62% (21/34 cases) of the differentiated adenocarcinomas. This paradoxical up regulation of H2RSP was particularly observed in actively invading cancer cells that showed budding from the cellular nests or sheets, and also in the cells infiltrating singly or as a tiny cluster consisting of a few cells (fig 7A). Indeed 80.9% (4.1%) of these sprouting cancer cells showed H2RSP immunoreactivity. The subcellular localisation of H2RSP in these sprouting cells was variable, and both nuclear and cytoplasmic immunoreactivities were observed. Interestingly, there existed a reciprocal pattern of MIB-1 labelling and H2RSP immunoreactivity at the invasive front (fig 7B). Coexpression of p16 was also often observed in these paradoxically H2RSP-positive cells (fig 8A, B). Notably, these cells also showed nuclear β-catenin consistently (fig 8A, C). These findings suggested that the cells showing paradoxical up regulation of H2RSP at the invasive front were a low-proliferating and invasive subpopulation.[12 13] We next wished to determine the significance of H2RSP immunoreactivity in the metastatic capability of the tumour. Analyses of 29 informative cases of differentiated adenocarcinomas showed that the ratio of H2RSP-positive cells at the invasive front was considerably higher in the cases with lymph node metastases than in those without metastasis (fig 9A). Consequently, Dukes' C/D cancers showed higher ratio of H2RSP-positive cells at the invasion front than Dukes' A/B at a significant level (fig 9A), and the positive ratio increased along with the progression of disease stages (stage I+II, 14.5% (3.4%); stage III, 19.9% (4.2%); stage IV, 27.7% (6.8%)).

To test a possible direct role of H2RSP in the invasive capability of colon cancer cells, the effect of H2RSP overexpression on the in vitro invasiveness of human colon carcinoma cells was examined. Figure 9B, C shows that the overexpression of H2RSP in DLD-1 cells resulted in enhanced Matrigel invasion. As DLD-1 cells expressed low but distinct levels of endogenous H2RSP, we then examined the effect of knockdown of H2RSP on the Matrigel invasion. The siRNA-mediated knockdown of H2RSP reduced the invasion of DLD-1 modestly (fig 9D). However, the knockdown of H2RSP did not alter the in vitro growth of DLD-1 cells (data not shown).

DISCUSSION

In this study, we showed the unique cellular distribution of H2RSP in the human intestinal tissues. H2RSP was expressed in the intestinal epithelium, and its subcellular localisation was changed from the cytoplasm to the nuclei of the epithelial cells along with cellular differentiation. This nuclear translocation of H2RSP was impaired in regenerating epithelial cells showing restitution. We thus extended the study to examine the expression and localisation of H2RSP in neoplastic intestinal epithelium, and found the expression of H2RSP to be down regulated in most cases of differentiated adenocarcinoma cells. However, actively invading cancer cells at the invasive front paradoxically showed a considerably up regulated H2RSP expression, and the ratio of H2RSP-positive tumour cells at the invasive front was considerably high in node-positive cases in differentiated adenocarcinomas.

The function of H2RSP in the gastrointestinal mucosa still remains to be elucidated. H2RSP may not be expressed in the intestinal tissue as a chimeric form with HAI-2,[15] because this transcript was not detected in the gastrointestinal mucosa.[18] Therefore, the intestinal expression of H2RSP is not linked to the activation of hepatocyte growth factor and the subsequent MET signalling pathway, both of which have important roles in the proliferation and differentiation of gastrointestinal mucosa.[20 21] As nuclear H2RSP was observed in the surface epithelial cells, but not in proliferating cells at the crypt base of normal intestinal mucosa, it is interesting to hypothesise that the nuclear translocation of H2RSP is involved, in some fashion, in the transition process from the proliferation phase to terminal differentiation of the intestinal epithelial cells. The results of in vitro experiments seem to be compatible with this hypothesis. Using the cell culture density model of DLD-1 cells, dense cell cultures were found to have a much higher level of nuclear H2RSP than a sparse culture. Moreover, the overexpression of H2RSP resulted in a reduced cellular growth in vitro. There has been a growing number of papers regarding the initiation of cellular proliferation or differentiation of gastrointestinal epithelium.[10 11 22] A recent study suggests that most of the genes involved in cellular proliferation, RNA splicing and transport, and protein translation are down regulated along with maturation of the colon epithelial cells by gene expression profiling.[23] However, the candidates for the molecules regulating termination of the proliferation phase of intestinal epithelial cells are still limited in number,[24–26] and H2RSP may be an additional candidate molecule involved in this termination mechanism.

The question then is how do H2RSP and its nuclear translocation influence the cellular proliferation. H2RSP is a small peptide that consists of 106 amino acids and contains two unique domains—namely, the serine-rich region (exon 3) and the lysine-rich region (exon 4).[15 18] A transfection analysis showed the effective bipartite basic type nuclear localisation signal in the lysine-rich region,[15 18] but the database search of the protein motif showed that no apparent homologous motif of known nuclear proteins interacting with DNA was observed in H2RSP. In our preliminary study, the recombinant H2RSP bound to poly (rG) via its lysine-rich domain, but not to other polynucleotides, including either single-stranded or double-stranded DNA, and no associated nuclear proteins were detectable by an in vitro pull-down assay (data not shown). These observations are compatible with those reported in IMUP-1, an identical peptide with H2RSP.[13] Several nuclear proteins have been reported to bind to poly (rG) or G-rich single-strand DNA, and involved in ribosomal DNA (rDNA) transcription, replication or recombination at the nucleolus.[16 27 28] One possibility is that H2RSP may function as a regulator of rDNA at nucleolus. However, we do not have any direct evidence regarding this possibility at present. Clearly, further studies for the molecular interactions of H2RSP in the nucleus and the mechanism of nuclear translocation of H2RSP are thus required.

To date, little is known regarding the expression of H2RSP in cancer tissue. Recently, Kim *et al*[29] reported that IMUP-1 is up regulated in ovarian cancers. By contrast, the current study showed the H2RSP/IMUP-1 expression to be down regulated in differentiated colorectal adenocarcinomas compared with adjacent normal intestinal epithelium, both in protein and mRNA levels. This discrepancy may be due to the fact that the normal intestinal mucosa expresses a much higher level of H2RSP/IMUP-1 than the normal ovary.[15] On the other hand, mucinous carcinomas showed a preserved H2RSP immunoreactivity, and the nuclear or nucleolar localisation of H2RSP

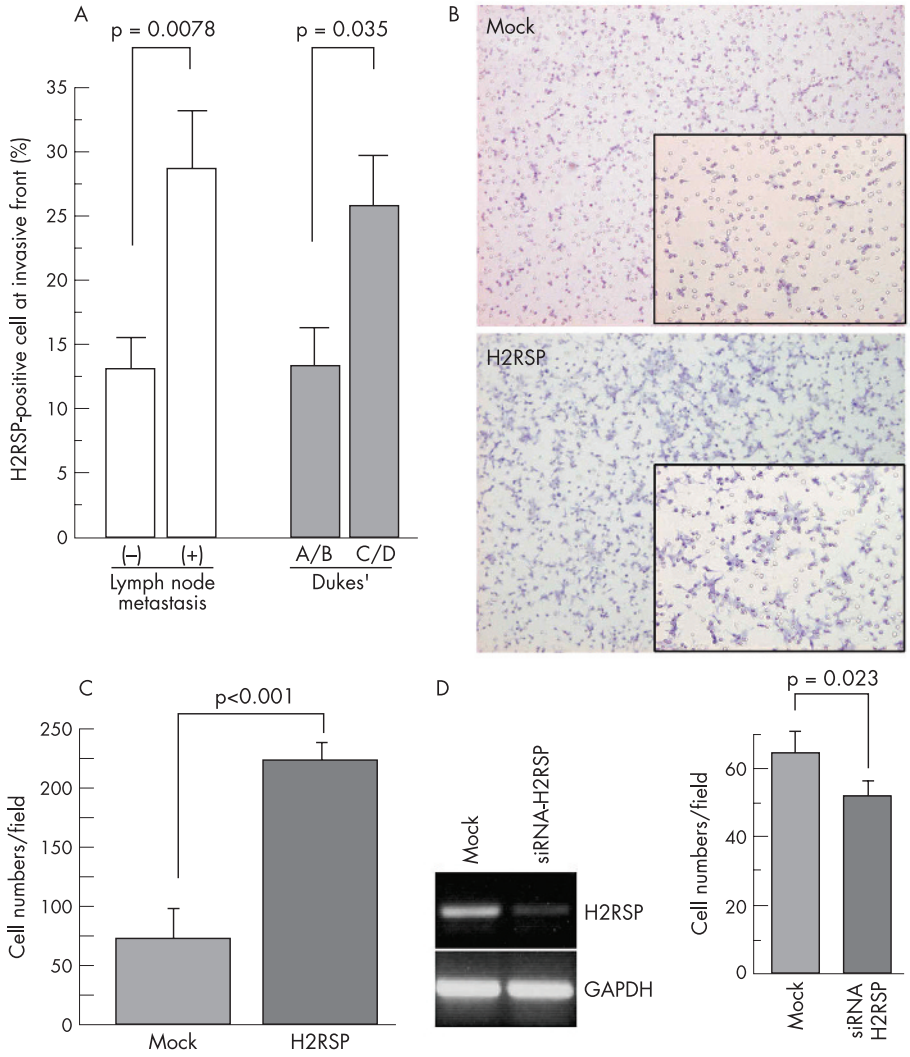

Figure 9 (A) An increase in number of hepatocyte growth factor activator inhibitor type 2-related small peptide (H2RSP)-positive cells at the invasive front in well (to moderately)-differentiated adenocarcinomas with metastases. Ten high-power fields of invasive front were randomly selected and the ratio of H2RSP-positive cancer cells to total cancer cells was counted by two independent pathologists. The values are the mean (SE). (B,D) Effect of H2RSP overexpression on Matrigel invasion of DLD-1 cells in vitro. Invaded cells on the lower surface of Matrigel-coated filter were stained with haematoxylin (B) and were counted (C). (D) The effect of H2RSP knockdown on Matrigel invasion of DLD-1 cells. The reduced expression of H2RSP in siRNA-H2RSP-transfected cells was confirmed by reverse transcriptase-polymerase chain reaction.

was evident in the cancer cells floating in mucin lakes. Poorly differentiated adenocarcinomas also showed a diffuse immunoreactivity of H2RSP in 4 of 5 cases examined. However, these H2RSP-positive poorly differentiated cancer cells showed a predominantly cytoplasmic localisation of this protein.

Although H2RSP immunoreactivity was considerably decreased in the central, differentiated area of adenocarcinomas, the invasive front of the tumours displayed a strong H2RSP expression particularly in budding tumour cells. Of interest was the observation that H2RSP-positive cells were concomitantly observed to show nuclear β-catenin and p16 immunoreactivities. These cells displayed a low proliferative activity as identified by MIB-1 labelling. Therefore, H2RSP-positive tumour cells at the invasive front are an invasive and low-proliferating subpopulation that has been reported in the invasive front of well-differentiated colorectal adenocarcinomas.[12 13 30 31] Consequently, H2RSP immunoreactivity at the invasive front may be a novel marker for metastatic phenotype in differentiated colorectal adenocarcinomas, as the ratio of H2RSP-positive cells at the invasive front was significantly high in node-positive cases and in stage IV cases. In addition, cancer cells at the invasive front showed not only nuclear but also cytoplasmic localisation of H2RSP. This observation may be compatible with the finding that non-neoplastic epithelial cells showing restitution of injured mucosal surface displayed cytoplasmic localisation of H2RSP, as these cells are also migrating and non-proliferating cells. Although it remains to be determined as to whether the paradoxical up regulation of H2RSP is simply an epiphenomenon or is one of the causative factors in invasive behaviour of carcinoma cells, in vitro experiments using engineered H2RSP expression in DLD-1 indicated that H2RSP might be directly involved in the regulation of cellular invasiveness. On the other hand, there may be a discrepancy regarding the H2RSP immunoreactivity between differentiated adenocarcinomas and poorly differentiated adenocarcinomas. In the poorly differentiated case,

weak, but diffuse cytoplasmic immunoreactivity was commonly observed in the cancer cells and no inverse relationship to MIB-1 labelling was present. It is thus suggested that the regulation of H2RSP expression and function may be severely deranged in poorly differentiated adenocarcinoma cells.

In conclusion, nuclear accumulation of H2RSP may somehow be involved in a signal related to growth arrest and differentiation of intestinal epithelial cells. The H2RSP immunoreactivity was found to be decreased in adenomas and more markedly in differentiated adenocarcinomas. However, a paradoxical up regulation of H2RSP was observed in cancer cells at the invasive front of these differentiated adenocarcinomas. H2RSP immunoreactivity at the invasive front of differentiated colorectal adenocarcinoma may serve as a novel marker of actively invading tumour cells. To clarify the precise functions of H2RSP and its nuclear translocation in the intestinal epithelium and tumours, further detailed experiments are called for.

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