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Research Paper

Dysregulation of *Reg* Gene Expression Occurs Early in Gastrointestinal Tumorigenesis and Regulates Anti-Apoptotic Genes

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ABSTRACT

Expression of anti-apoptotic genes is frequently elevated in tumors, where they increase resistance to chemotherapeutic agents and predict poor patient outcomes. However, key cellular factors regulating anti-apoptotic genes in tumors remain unknown. Increased expression of the regenerating (*Reg*) genes is commonly observed in gastrointestinal (GI) malignancies including colorectal cancer (CRC). We therefore examined *Reg* gene expression and associated changes in anti-apoptotic genes in an animal model of GI tumorigenesis. Using real time RT-PCR, we measured expression of *Reg* genes in human colorectal adenocarcinoma specimens, colon adenocarcinoma cell lines and adenomas from multiple intestinal neoplasia (min) mice heterozygous for a germ-line mutation of the adenomatous polyposis coli (*APC*) gene. Expression of *Reg* genes is increased in human colorectal adenocarcinomas and in the intestine of $APC^{min/+}$ mice at four weeks of age, a time preceding the spontaneous second mutation in the *APC* gene. Individual Reg genes exhibited regional expression profiles across the GI tract in mice. Adenomas from 14-week old mice had significant increases in at least one member of the *Reg* gene family, most commonly Reg IV and an associated increase in expression of the anti-apoptotic gene, Bcl-2. Addition of exogenous recombinant human Reg IV to human colon adenocarcinoma cells significantly increased Bcl-2 and Bcl-x_L expression and induced resistance to ionizing radiation. These results show that dysregulation of *Reg* genes occur early in tumorigenesis. Furthermore, increased expression of *Reg* genes, specifically Reg IV contribute to adenoma formation and lead to increased resistance to apoptotic cell death in CRC.

INTRODUCTION

Tumorigenesis is a multistep process involving somatic mutations or epigenetic changes affecting tumor suppressor and oncogenes.[1,2] Additional genetic alterations create a permissive environment for clonal expansion of cells that are resistant to apoptosis. Advanced forms of common malignancies, such as colorectal, gastric, prostate or breast carcinoma are often associated with poor responses to adjuvant chemotherapy (CT) and/or ionizing radiation (IR).[3] Apoptosis is a prominent mechanism for cell death following CT or IR.[4] Accordingly, considerable attention has been given to the Bcl-2 family genes as possible regulators of intrinsic tumor resistance to therapy.[5] Repressors of programmed cell death, such as Bcl-2 and Bcl-x_L, decrease IR- and CT-induced apoptotic cell death in cell culture.[6] However, key cellular factors that regulate expression of anti-apoptotic genes in tumors remain unknown. Defining dominant pathways responsible for modulation of apoptosis-regulating proteins would significantly enhance our understanding of tumor behavior and could broaden current strategies for therapeutic intervention.

The regenerating (*Reg*) genes constitute a family belonging to calcium dependent (C-type) lectin gene superfamily.[7-10] The *Reg* family genes included six members (Reg I, Reg II, Reg IIIα, Reg IIIβ, Reg IIIδ and Reg IIIγ) in the mouse and three members (Reg Iα, Reg Iβ and Reg III) in humans.[11] Human Reg IV, a novel member of the family was identified by high throughput sequencing of a library derived from patients with ulcerative colitis, constituting fourth member of the *Reg* gene family in human.[10] A mouse homologue has also been identified constituting the seventh member in mouse. Expression of *Reg* genes is increased following injury, supporting a potential role in tissue repair and regeneration.[12,13] Expression of Reg proteins by colorectal, gastric, and pancreatic adenocarcinomas have recently been shown to have an adverse association with patient survival.[14-16] Reg IV was among several genes with increased expression in cancer cell lines selected for increased in vitro resistance to the chemotherapeutic agent, 5-FU.[6] Reg IV expression was

associated with intestinal differentiation in gastric adenocarcinoma[17] and highly elevated in colorectal cancer (CRC).[18,19] Reg IV has also been identified as a promising marker of hormone refractory metastatic prostate cancer.[20] Recently we observed the mitogenic effect of Reg IV protein, when added to the cultures of human colon adenocarcinoma cell lines with subsequent changes in expression of genes associated with altered apoptosis and metastasis.[21] This supports the hypothesis that *Reg* gene products are responsible for altered apoptosis associated with a more aggressive tumor phenotype. Here we examined the expression of individual Reg genes in human colorectal adenocarcinoma specimens and adenomas from multiple intestinal neoplasia (Min) mice heterozygous for a germ-line nonsense mutation of the adenomatous polyposis coli (*APC*) gene. These animals spontaneously develop multiple polyps in the small and large intestine at 10–12 weeks of age following spontaneous second mutation in the *APC* gene. This study shows specific regional expression profiles of *Reg* genes along the cranio-caudal axis of the GI tract. Our results identify aberrant expression of the *Reg* genes as one of the earliest events in gastrointestinal tumorigenesis. Reg IV was specifically upregulated at the time of adenoma formation and contributed to the increased resistance to apoptotic cell death.

MATERIALS AND METHODS

Cell lines and culture. HCT116 and HT29 colon adenocarcinoma cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (Cambrex, Walkersville, MD) containing 10% heat inactivated fetal bovine serum (Sigma, St. Louis, MO). Cells were placed in serum-free media overnight prior to treatment with endotoxin-free recombinant human Reg IV (rhR4).[22]

Human colorectal carcinoma specimens. Five millimeter sections and total RNA isolated from human colorectal adenocarcinoma specimens and paired normal mucosa were obtained from the Tissue Procurement Core of the Siteman Cancer Center, Washington University.

Animals. Breeding pairs of C57Bl/6j *APC*$^{min/+}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained on a 10% fat diet (Harlan Teklad, Madison, WI). Young *APC*$^{min/+}$ mice were genotyped as previously described.[23]

Immunohistochemistry. Immunohistochemical staining of human colorectal adenocarcinoma specimens and adenoma isolates from *APC*$^{min/+}$ mice was performed by using previously characterized antibodies against Reg IV[22] and Bcl-2 (Transduction laboratory, BD Biosciences, Franklin lakes, NJ) in the Digestive Disease Research Center Histopathology Core.

Real time RT-PCR analysis. Total RNA isolated from human colorectal adenocarcinoma isolates and paired normal mucosa, adenomas from *APC*$^{min/+}$ and their wild-type littermates (*APC*$^{+/+}$) and human colon adenocarcinoma cells (HCT116 and HT29) was converted to cDNA using Superscript II reverse transcriptase and random hexanucleotide primers (Invitrogen, Carlsbad, CA). Samples were analyzed by real time RT-PCR using Jumpstart Taq DNA polymerase (Sigma, St. Louis, MO) and SYBR Green nucleic acid stain (Molecular Probes, Eugene, Oregon) or Taqman probes (IDT, Coralville, IA) for individual genes. Crossing threshold values for individual genes were normalized to GAPDH (murine) or β-Actin gene expression. Probe and primer sets used for real time RT-PCR analysis are shown in Table 1.

Western blot analysis. Cell lysates from HCT116 and HT29 cells were subjected to PAGE electrophoresis and blotted on to Immobilin^{TM}-PVDF membranes (Millipore, Bedford, MA).

Table 1 **Probe and primer sets for Real Time RT-PCR**

Gene	Species*	Primer	Probe & dye
GAPDH	M	GGCAAATTCAACGGCACAGT AGATGGTGATGGGCTTCCC	JOE/AGGCCGAGAATGGGAAGCTTGTCAC/BHQ
β Actin	H & M	ATCATTGCTCCTCCTGAGCG (F) GCTGATCCACATCTGCTGGAA (R)	SYBR
Reg I	M	CATCCTGCTCTCATGCCTGAT (F) GCAGATGGCAGGTCTTCTTCA (R)	TET/CCTGTCTCCAAGCCAAGGCCAGG/BHQ
Reg Ia	H	TGATGTTCCTGTCTCTGAGCCA (F) CCTTCTGGGCAGCTGATCC (R)	SYBR
Reg Ib	H	TCTGAGCCAAGGCCAGGA (F) GCCTTCTGGGCAGCTGATT (R)	SYBR
Reg II	M	ACAGCCAAGGCCAGGTAGCT (F) GGGCAGTTGATTTTGGCAGA (R)	FAM/ACTTCCCCTTGGCTGAAAAAGACCTTCC/BHQ
Reg III	H	GTAACAGCTACTCATACGTCTGGA TTG (F) CTCCCAACCTTCTCCATTGG (R)	SYBR
Reg IIIα	M	GGATTGGGCTCCATGATCC (F) TCAGCACATCGGAGTTACTCCA (R)	FAM/CCTCCATTGGGRTGTTGACCCATTGT/BHQ
Reg IIIβ	M	TGCCTTGTTTCAGATACCACAGA (F) GGTGTCCTCCAGGCCTCTTT (R)	TET/TGGTTTGATGCAGAACTGGCCTGC/BHQ
Reg IIIδ	M	GTGTTGCCTGATGTCCCTTTC (F) CAGCTGATGCGTGGAGAAGAC (R)	FAM/TTTCTGGGATTGTTCACCTTGTACCCA/BHQ
Reg IIIγ	M	GGTAACAGTGGCCAATATGTA TGG (F) CCACCTCTGTTGGGTTCATAG (R)	TET/TGGGCTCCATGACCCGACACTG/BHQ
Reg IV	M	CGTGCGGCTACTCTTACTGCT (F) AGCTGGGTCTCAAGATATCGCT (R)	FAM/CTGGGTAGCTGGCCCCGAAGTCC/BHQ
Reg IV	H	TGAGCTGCCTGGCCAAA (F) AAGTAACCATAGCAATTGGACTT GTG (R)	SYBR
Bcl 2	M	TGGATCCAGGATAACGGAAG (F) CAAACAGAGGTCGCATGCTG(R)	SYBR
Bcl 2	H	CGGTCTCCTGGTGCCATTAT (F) GAAACGTCACCTGCCCCC (R)	SYBR
Bcl xL	H	CCCATGCTCCGTTATCCTG (F) TAAGTCGCCATCCAAGCTGC (R)	SYBR

*H, human; M, murine.

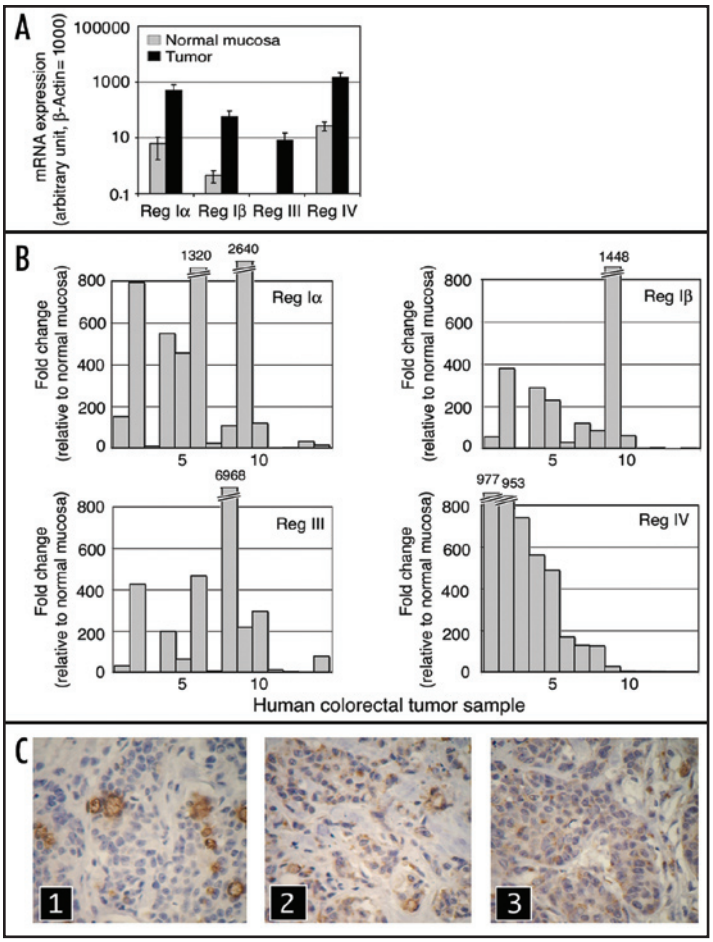

Figure 1. Reg IV is the dominant member of *Reg* gene family upregulated in human colorectal adenocarcinomas. (A) Absolute mRNA expression of Reg Iα, Reg Iβ, Reg III and Reg IV genes was determined in 14 surgically resected human colorectal adenocarcinomas specimens and paired normal mucosa. Expression level of Individual Reg gene was normalized to 1000 arbitrary units of β-actin gene expression and plotted on logarithmic scale. Expression profile shows that Reg IV is the dominant member of *Reg* family expressed at higher level in normal mucosa and further increased in colorectal adenocarcinomas. (B) Relative to normal mucosa, fold change in expression of *Reg* genes was determined for individual colorectal adenocarcinoma specimens. 100%, 86%, 86% and 71% of colorectal adenocarcinoma specimens demonstrated increased Reg IV, III, Iα and Iβ expression respectively greater than 1.5-fold. (C) Immunohistochemical staining of adenocarcinoma isolates demonstrated increased expression of Reg IV protein. Representative sections demonstrate strong expression of Reg IV by a subset of cells with goblet-like morphology (left panel) in normal mucosa and staining by all cells (middle and right panels) in adenocarcinomas.

Antibodies were purchased from Transduction laboratory (BD Biosciences, Franklin Lakes, NJ) and Santa Cruz Biotechnology (Santa Cruz, CA). Specific proteins were detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ).

In vitro radiation-survival colony assay. HCT116 and HT29 cells were plated at 10^4 cells/25 cm^2 flasks and incubated in serum containing media overnight to allow cell adherence. Cultures were then incubated in serum free media with or without 100 nM rhR4. After 18 hours, cells were subjected to either 0 or 4 Gy γ-irradiation (IR) in a Gamacel 40 cesium irradiator at 0.96 cGy/min. Cells were allowed to grow until the development of microscopically visible colonies.

Statistical Analysis. Values were expressed as the mean ± s.e.m. Data were analyzed by 2-tailed t test. A p-value of less than 0.05 was considered as statistically significant.

RESULTS

Reg IV is most upregulated gene of Reg family in human colorectal adenocarcinomas. In order to determine *Reg* gene expression in gastrointestinal tumors, we measured the expression of human *Reg* genes in 14 colorectal adenocarcinoma resection specimens and paired normal mucosa. mRNA expression of individual Reg genes was determined by real time RT-PCR and normalized to β-Actin expression. Absolute mRNA level of individual Reg gene was determined based on titrated standard curve using specific primer sets and probes. Reg IV was the dominant member of the *Reg* gene family expressed in normal mucosa (mRNA expression: 6.1 ± 4.5, 0.45 ± 0.2, 0.05 ± 0.03, and 27.2 ± 9.3 of Reg Iα, Reg Iβ, Reg III and Reg IV respectively) (Fig. 1A). While colorectal adenocarcinoma specimens exhibited increased expression of all *Reg* genes, Reg IV, and to a lesser extent Reg Iα constituted the dominant members of the *Reg* gene family expressed by colorectal adenocarcinomas (mRNA expression: 492 ± 304, 56 ± 33, 8 ± 6, and 1456 ± 676 of Reg Iα, Reg Iβ, Reg III and Reg IV respectively) (Fig. 1A). Individual resected tumors showed highly unique patterns of *Reg* gene expression. For example, specimen 3 had increased Reg IV expression only, however, specimen 2 had increases in expression of all members of the *Reg* gene family including Reg IV. 100%, 86%, 86%, and 71% of colorectal adenocarcinoma specimens demonstrated increased Reg IV, III, Iα, and Iβ expression respectively, greater than 1.5-fold relative to adjacent normal mucosa (Fig. 1B). No clear correlation between levels of Reg gene expression and a particular histopathology or tumor stage was observed (data not shown). Consistent with increased Reg IV mRNA, colorectal adenocarcinomas showed prominent expression of Reg IV protein by immunohistochemistry (Fig. 1C).

Expression of *Reg* genes is dysregulated early in tumorigenesis. We first characterized the expression of the *Reg* gene family in the normal murine GI tract. Total RNA was isolated from individual segments of the GI tract, extending from the stomach to the colon of 14-week old mice. *Reg* gene expression was then determined by using Taqman probe and primer sets specific for each of the 7 Reg genes in the murine genome. Individual Reg genes displayed one of three embryologically-derived expression patterns across the cranio-caudal axis of the adult GI tract (Fig. 2A). Reg I, II and IIIδ had maximal expression in the foregut-derived stomach and duodenum; Reg IIIα, IIIβ, and IIIγ in midgut-derived small bowel extending from the jejunum to the ileum, whereas Reg IV was unique with prominent expression in the cecum and colon. We next utilized $APC^{min/+}$ mice to determine at what step expression of individual Reg genes becomes dysregulated during tumorigenesis. We analyzed regional expression of different *Reg* genes in the intestines of four-week old $APC^{min/+}$ mice and wild-type ($APC^{+/+}$) littermate controls. four-week old wild-type $APC^{+/+}$ mice had regional expression patterns mirroring that shown in 14-week normal adults (data not shown). Compared to wild-type $APC^{+/+}$ mice, different intestinal segments of four-week old $APC^{min/+}$ mice showed significant increases in expression of all *Reg* genes, except Reg IV (Fig. 2B). Intestinal regions associated with the greatest predisposition for adenomatous polyp formation had increases of greater than two-fold in Reg I, II, IIIβ and IIIγ expression. Furthermore, segmental increases were seen in both Reg members expressed normally at that site (e.g., Reg IIIβ in the ileum)

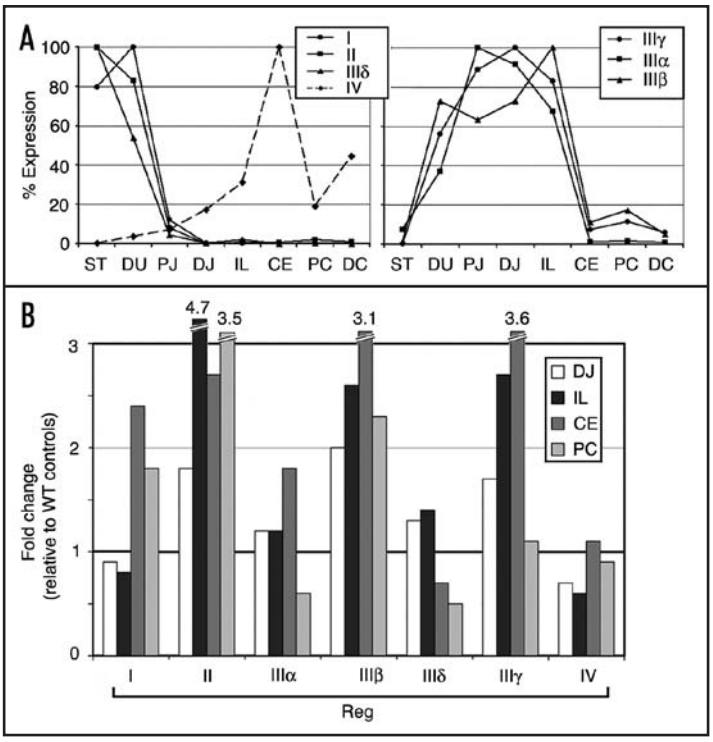

Figure 2. *Reg* gene expression varies regionally in the normal murine GI tract and is dysregulated in 4-week old $APC^{min/+}$ mice. (A) Taqman probe and primer sets specific to individual murine Reg genes were used to determine antero-posterior expression of *Reg* gene family in the GI tract of normal mice. Total RNA isolated from the stomach (ST), duodenum (DU), proximal jejunum (PJ), distal jejunum (DJ), ileum (IL), cecum (CE), proximal colon (PC) and distal colon (DC) were used for reverse transcriptase reactions (n = 5). Expression of *Reg* genes is plotted as relative expression (%) in the gastrointestinal tract by considering highest level as 100. Expression profiles fall into three regionally distinct patterns: foregut (left panel); Reg I, Reg II and Reg IIIδ, midgut (right panel); Reg IIIα, Reg IIIβ, and Reg IIIγ, or hindgut (left panel); Reg IV. (B) 4-week old $APC^{min/+}$ mice and wild-type $APC^{+/+}$ littermates were used to precisely determine when *Reg* gene expression becomes dysregulated in a model of GI tumorigenesis. Total RNA isolated from the adenoma from DJ, IL, CE, and PC segments of four-week old $APC^{min/+}$ mice and wild-type $APC^{+/+}$ littermate was used for reverse transcriptase reactions (n = 6). At four weeks of age, increases in *Reg* gene expression in $APC^{min/+}$ mice precede the second spontaneous mutation in the *APC* gene and histopathologic adenoma formation.

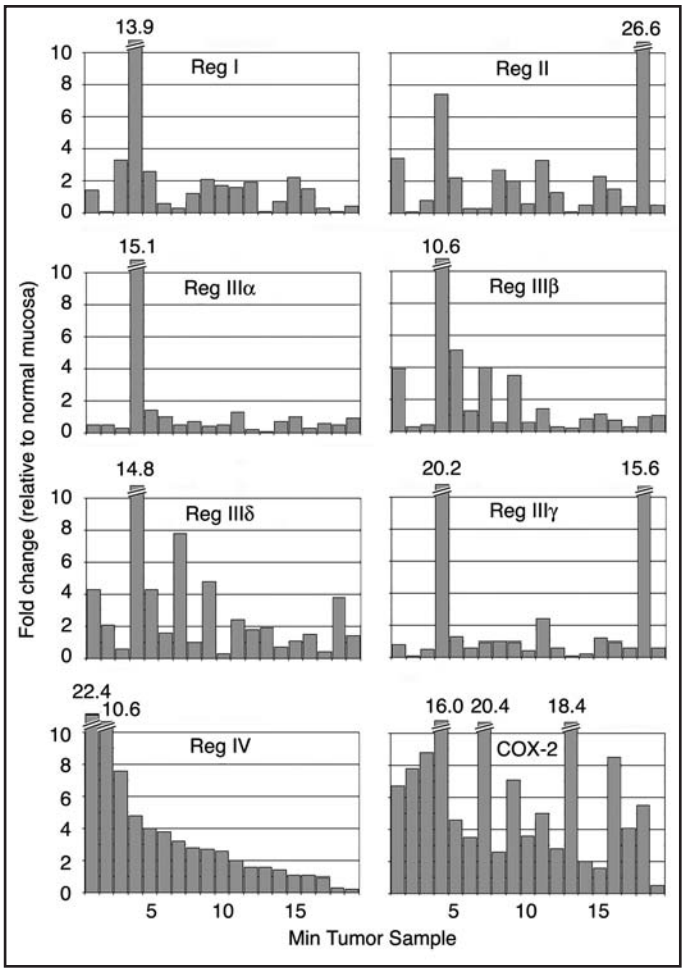

Figure 3. Reg IV is the most frequently upregulated member of *Reg* gene family in microdissected adenomas from 14-week old $APC^{min/+}$ mice. Adenoma development occurs following a spontaneous mutation in the second copy of the *APC* gene. The expression of individual murine Reg genes were determined using RNA isolated from microdissected adenomas and adjacent normal mucosa (n = 19). Cox-2 mRNA was also measured as a marker for effective adenoma isolation. Eighteen of the nineteen adenoma samples demonstrated increased Cox-2 mRNA as expected. Fold increase for individual Reg genes compared to adjacent normal mucosa are indicated. Similar to human colorectal adenocarcinomas, individual adenoma demonstrated increased expression of at least one member of the *Reg* gene family. Reg IV represented the most commonly increased member of the family (≥1.5 fold in 72% of adenomas).

as well as *Reg* genes not expressed at that location (e.g., Reg II and IIIγ). Further, to determine changes in *Reg* gene expression associated with adenoma development, visible adenomas and adjacent normal mucosa were microdissected from 14-week $APC^{min/+}$ mice. Cox-2 expression was used as a marker for successful adenoma isolation. Reg IV represented the most commonly increased member of the family in 19 individual adenomas when compared to their adjacent normal mucosa (≥1.5 fold in 72% of adenomas) (Fig. 3). Similar expression profiles of individual Reg genes were also observed in each adenoma examined, mirroring human colorectal adenocarcinoma specimens.

Reg IV regulates anti-apoptotic genes. Increased Bcl-2 and Bcl-x_L expression is a frequent occurrence in colon adenocarcinomas and is predictive of poor prognosis.[24] To show an upregulation of Bcl-2 in $APC^{min/+}$ mice, we examined Bcl-2 expression in adenomas by immunohistochemistry (Fig. 4A). In 14-week old wild-type $APC^{+/+}$ mice (WT), staining was largely confined to the lamina propria immune cell populations (left panel). Epithelial staining in macroscopically normal appearing mucosa from $APC^{min/+}$ mice (μAde) was restricted to microadenomas (middle panel). However, the epithelium in microdissected gross adenomas from $APC^{min/+}$ mice (Ade) was globally stained for Bcl-2 (right panel). Bcl-2 mRNA expression was also increased in adenomas and closely paralleled changes in Bcl-2 protein staining (Fig. 4B). The pronounced increase in Bcl-2 mRNA occurred coincides with the development of macroscopic adenomas. In addition, adenomas from 14-week $APC^{min/+}$ mice with increased Reg IV expression generally demonstrated an associated increase in Bcl-2 mRNA (Fig. 4C). To establish a causative association between Reg IV and anti-apoptotic genes, 100 nM rhR4 was added to cultures of human colon adenocarcinoma cell lines (Fig. 5). Bcl-2 and Bcl-x_L mRNA expression in HCT116 (left panel) and HT29 (right panel) cells were determined by real time RT-PCR analysis. Bcl-2 expression

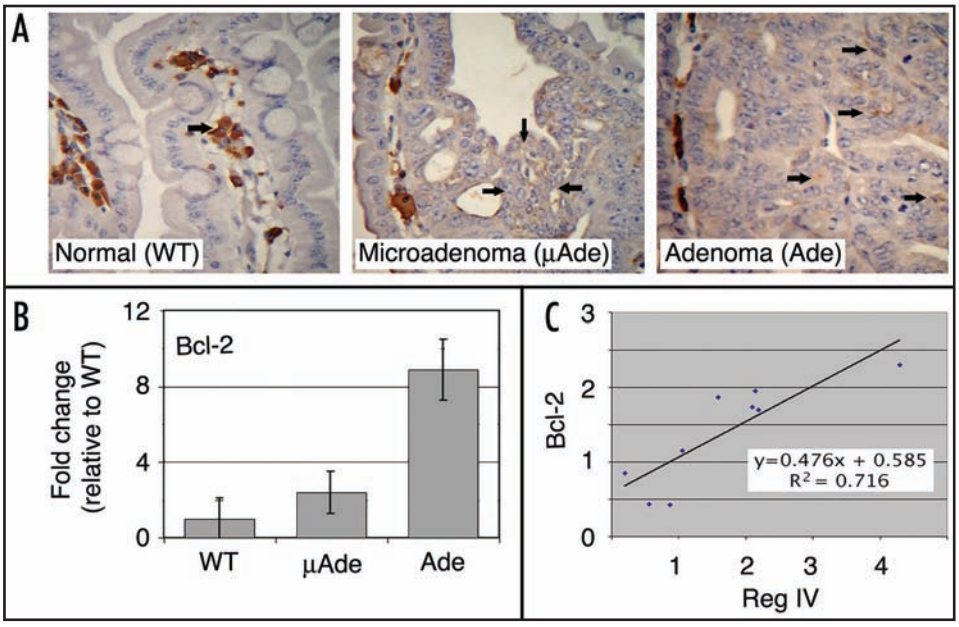

Figure 4. Bcl-2 expression is increased in microdissected adenomas from 14-week $APC^{min/+}$ mice. (A) Representative sections demonstrate normal ileum from 14-week old wild-type $APC^{+/+}$ mice (normal) showing prominent Bcl-2 staining (arrowheads) in lamina propria immune cell populations but not in epithelial cells (left panel), ileac microadenoma from 14-week $APC^{min/+}$ mice (microadenoma) showing strong Bcl-2 staining of the adenoma (arrowheads) but in the normal surrounding epithelium (middle panel), and ileac gross adenomas from 14-week $APC^{min/+}$ mice (adenoma) showing global staining of epithelium cells (right panel). (B) Relative to normal mucosa from wild-type $APC^{+/+}$ mice (WT), Bcl-2 mRNA expression is increased in histologically normal mucosa (μAde) and adenomas (Ade) from 14-week $APC^{min/+}$ mice (n = 5). A pronounced increase in Bcl-2 mRNA occurs coincident with the development of frank adenomas. (C) Relative to histologically normal mucosa, fold increases in Reg IV and Bcl-2 expression in adenomas from 14-week $APC^{min/+}$ mice were used to determine correlation between these two genes. Adenomas from $APC^{min/+}$ mice with increased Reg IV expression demonstrated an associated increase in Bcl-2 mRNA (Correlation coefficient, $R^2 = 0.716$).

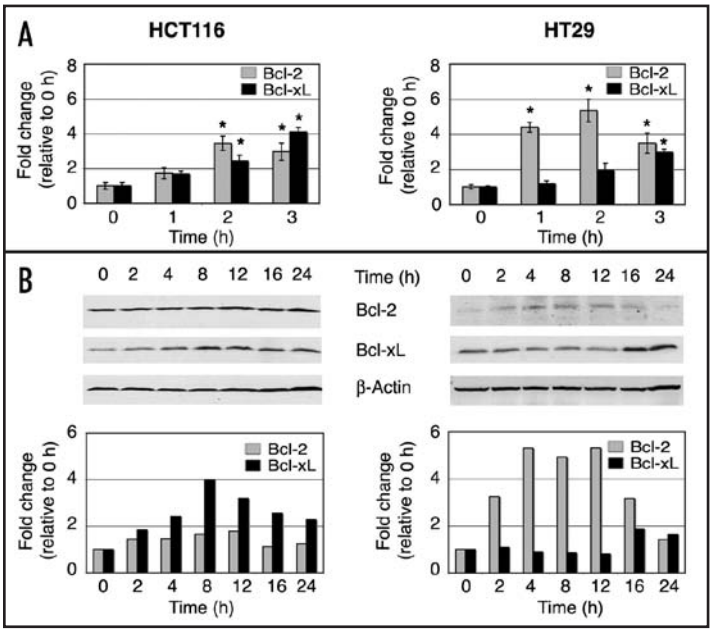

Figure 5. Reg IV treatments increase anti-apoptotic genes, Bcl-2 and Bcl-x_L in human colon adenocarcinoma cells. (A) HCT116 and HT29 human colon adenocarcinoma cell monolayer were treated for various times (h) with 100 nM rhR4. SYBR green nucleic acid dye and specific primer sets were used to determine mRNA expression of Bcl-2 and Bcl-x_L. Addition of rhR4 to HCT116 cells (left panel) and HT29 cells (right panel) significantly increased Bcl-2 and Bcl-xL gene expression (*$p < 0.05$). B) Western blot showing Bcl-2 and Bcl-x_L protein expression at various times (h) following addition of 100 nM rhR4 to HCT116 (left panel) and HT29 cells (right panel). Lower panel indicates the intensity of bands by densitometry scanning. Treatments of rhR4 to HCT116 and HT29 cells led to increased expression of Bcl-2 and Bcl-x_L proteins and demonstrated parallel changes to their respective mRNA expression.

was increased significantly following rhR4-treatment for 2 h in HCT116 and 1 h in HT29 cells (Fig. 5A). In addition, significant increases in Bcl-x_L expression were observed, when rhR4 was added to the culture medium of HCT116 cells for 2 h and HT29 cells for 3 h (Fig. 5A). To determine if the increases in Bcl-2 and Bcl-x_L mRNA were associated with changes in protein expression, western blotting was performed on cell lysates isolated following rhR4 treatment. Corresponding increases in Bcl-2 and Bcl-x_L protein were observed in HCT116 and HT29 cells (Fig. 5B). In response to rhR4-treatments, increases in Bcl-2 expression occurred earlier than that of Bcl-x_L. These data show that exogenous Reg IV regulates expression of Bcl-2 and Bcl-x_L genes and supported our previous finding of increased expression of anti-apoptotic genes in response to elevated level of Reg IV protein in colorectal adenocarcinoma cells.[21]

Reg IV treatment induces cell survival against radiation-induced apoptosis. Repressors of programmed cell death may directly increase resistance to therapy-induced cell death. We therefore investigated a possible protective role of Reg IV in human colon adenocarcinoma cells using an in vitro radiation-survival colony assay (Fig. 6). HCT116 and HT29 cells grown on culture plates containing media with or without 100nM of rhR4 were exposed to 4 Gy IR. The microscopically visible colonies in this model are reflective of a single surviving and proliferating cell. Exogenous Reg IV treatment significantly increased the number of colonies. Following 4 Gy IR, colony counts increased from 34.8 ± 5.1 to 50.4 ± 3.5 (45% increase, $p < 0.05$) in HCT116 (left panel) and

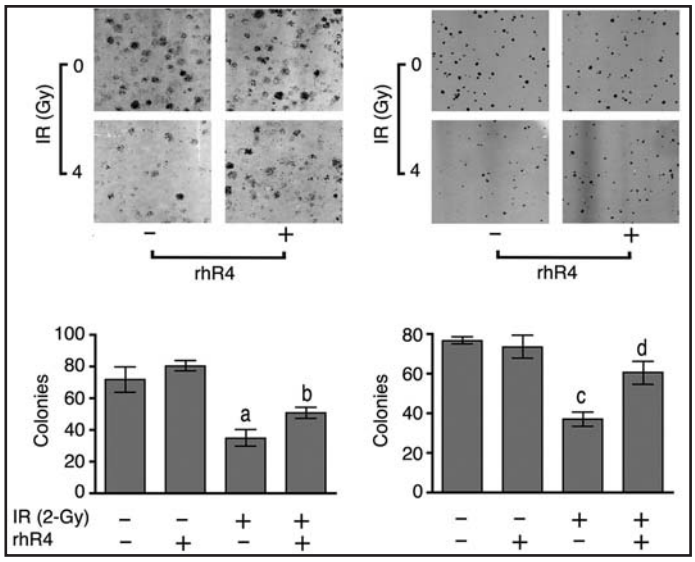

Figure 6. In vitro radiation-survival colony assay showing decreases in radiation-induced apoptosis following rhR4 treatment in human colon adenocarcinoma cells. HCT116 (left panel) and HT29 cells (right panel) were plated at 10^4cells/25 cm^2 flask and incubated overnight to allow cell adherence. Cultures were then treated with complete media with or without 100 nM rhR4. After 18 hours cells were subjected to either 0 or 4 Gy IR and allowed to grow until the development of visible colonies. Colonies counted reflects survival and proliferation of individual cells (lower panel). Addition of rhR4 to HCT116 and HT29 cells significantly increased the number of surviving cells following 4 Gy IR (A and C, $p < 0.05$, control vs. 4 Gy IR; B and D, $p < 0.05$, 4 Gy IR vs. 4 Gy IR + rhR4 with HCT116 and HT29 cells respectively).

36.8 ± 3.5 to 60.3 ± 5.7 (64% increase, $p<0.005$) in HT29 cells (right panel). In the absence of IR, rhR4 treatment did not result in a difference in the number of colonies. This data indicates that Reg IV promotes tumor cell survival following a potent apoptotic stimulus.

DISCUSSION

Most adenocarcinomas are relatively resistant to CT and IR.[25-27] Efforts to overcome this resistance by increasing concentration of cytotoxic drugs or dosage of irradiation has been failed to significantly improve the therapeutic response. Apoptosis is a prominent mechanism for death of cancer cells following CT or IR.[4,26] Novel cancer treatment strategies targeting the genes that promote apoptosis or blocking factors that inhibit apoptosis may prove adjuvant in the treatment of many malignancies. The expression of the *Reg* gene family is increased in common malignancies including colorectal, gastric, hepatocellular and pancreatic adenocarcinomas and may have an adverse association with patient outcomes or survival.[14-17,28] We observed increased expression of all genes of *Reg* family in a series of human colorectal adenocarcinoma specimens. Reg IV was the most upregulated member of the family in these specimens. This was in accordance with previously reported data showing elevated expression of Reg IV in colorectal carcinomas.[18,19]

The multistep tumorigenesis model proposed by Vogelstein and Fearon describes step-wise transformation from the benign polyp to the malignant phenotype of colorectal cancer.[1,2] A similar multistep process was also observed in a hepatocarcinogenesis model in rats.[29] In each of these models, it appears that tumorigenesis is associated with an increased cell proliferation and an associated decrease in apoptotic cell death. It is likely that a variety of cellular pathways may contribute to decreased apoptosis at specific stages of tumorigenesis. This may also be required for the development of a malignant phenotype. The $APC^{min/+}$ mouse model was chosen, because it mimics the developmental process of human GI tumorigenesis. These mice spontaneously develop multiple adenomas in small and large intestine at around 10–12 weeks of age following a second spontaneous mutation in the *APC* gene. Compared to wild-type littermate controls ($APC^{+/+}$), four-week old $APC^{min/+}$ mice already had significant increases in expression of *Reg* genes, preceding the second spontaneous mutation in the *APC* gene. However, increased Reg IV expression was not detected prior to the polyp formation or adenomatous changes by histology. Significant increases in Reg IV expression were noted in a series of adenomas microdissected from 14-week old $APC^{min/+}$ mice. This data is first of its kind to demonstrate that the Reg IV might have a potential role during adenoma formation following second spontaneous mutation in *APC* gene. These data also demonstrated that adenoma formation was associated with increased expression of at least one Reg gene, mirroring the results observed in human colorectal adenocarcinomas. Our results in the $APC^{min/+}$ mouse model are in agreement with findings of a 2–3 fold increase in Reg IV expression in flat colonic mucosa containing microscopic adenomatous changes in three patients with familial adenomatous polyposis (data not shown).

Increases in Reg IV expression in adenomas of 14-week old $APC^{min/+}$ mice had an associated increase in Bcl-2 expression. Human colorectal adenocarcinoma specimens with increased Reg IV expression exhibited increased expression of Bcl-2 and Bcl-x_L mRNA (data not shown). A causative role for Reg IV in regulation of the Bcl-2 family genes has been confirmed with increased expression of Bcl-2 and Bcl-x_L at mRNA and prtotein levels following addition of rhR4 to culture media of human colon adenocarcinoma cell lines. This result supported our previous finding of increased expression of anti-apoptotic genes via Reg IV-mediated EGFR/Akt signaling cascades.[21] Furthermore, addition of rhR4 to human colon adenocarcinoma cells led to significantly greater resistance to cell death following IR. Functional blocking of Reg IV protein increased cell susceptibility to IR-induced death (data not shown). Collectively, these data suggest that up-regulation of Reg IV and corresponding changes in cellular predisposition to apoptosis may play a requisite early role in adenoma formation.

In summary, individual Reg genes show a specific expression profile along the canio-caudal axis of the GI tract. The expression of *Reg* genes is increased in colorectal adenocarcinoma and constitutes an early event in intestinal tumorigenesis in $APC^{min/+}$ mice. While the $APC^{min/+}$ mouse model does not completely recapitulate human colon cancer given the expression of polyps in both the small bowel and colon, the model offers the unique opportunity to study genetic changes occurring at a premalignant stage, before more profound genetic derangements and clonal selection pressures. Increased Reg IV expression might lead to a tumor phenotype displaying increased resistance to apoptotic cell death. These results identify Reg proteins as previously unappreciated regulators of anti-apoptotic proteins in early tumorigenesis and may contribute to increased resistance to apoptotic death during therapy. Strategies designed to reduce endogenous Reg expression or block downstream signaling warrant further investigation for use in the prevention or treatment of established gastrointestinal adenocarcinomas.

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