Abstract. Early growth response-1 (Egr-1) is implicated in the regulation of cell growth, proliferation, differentiation and apoptosis. Egr-1 is considered to be either a tumor-suppressor or tumor-promoter, depending on the cell type and environment. The aim of the present study was to evaluate the expression of Egr-1 in colorectal cancer and its correlation with tumor cell proliferation, apoptosis and clinicopathological features. The expression of Egr-1 in colorectal cancer tissues was investigated by reverse transcription-polymerase chain reaction (RT-PCR), western blotting and immunohistochemistry. Apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL), and cellular proliferative activity was evaluated by immunohistochemical staining with the Ki-67 antibody. Egr-1 expression was significantly elevated in colorectal cancer tissues, when compared to that in the paired normal mucosa at the mRNA and protein levels. In addition, Egr-1 expression was significantly increased in the metastatic lymph node tissues, when compared to that in the non-metastatic lymph node tissues at the protein level. The mean Ki-67 labeling index (KI) and apoptotic index (AI) values for 158 tumors were 53.6±15.4 and 9.0±1.0, respectively. Higher KI values were significantly associated with distant metastasis. Lower AI values were significantly associated with lymph node metastasis. However, KI or AI values were not associated with patient survival. The mean KI value of Egr-1-positive tumors was significantly higher than that of Egr-1-negative tumors. However, there was no significant difference between Egr-1 expression and AI value. Positive expression of Egr-1 was significantly associated with age, lymphovascular invasion, lymph node and distant metastasis, tumor stage and poor survival. These results indicate that Egr-1 may be associated with colorectal cancer progression via tumor cell proliferation.

Introduction

Cell proliferation and apoptosis must be properly balanced in order to support proper development and maintain healthy homeostasis of mature tissues (1). A highly regulated process to control this balance involves each cell receiving survival signals from its microenvironment and from external stimuli (1).

Cancer cells are characterized by their ability to proliferate in an uncontrolled manner in contrast to normal cells, in which the proliferation is tightly regulated (2). In addition, apoptosis is a continuous physiologic process in tissue homeostasis, and functions as a defense against pathogens and helps with the elimination of unwanted cells (3-5). Dysregulated apoptosis can lead to extended aberrant cell viability or may favor the accumulation of transforming mutations, and it is thought to contribute to carcinogenesis (3-5). Therefore, tumor development and progression are generally regarded as dependent on an increased proliferation rate and an apoptosis rate too low to balance cell growth.

Early growth response-1 (Egr-1) is a Cys2-His2-type zinc-finger transcription factor that is rapidly induced in response to a broad range of extracellular stimuli and plays a critical role in controlling cell growth, proliferation, differentiation and apoptosis (6-12). Interestingly, Egr-1 is considered to be either a tumor-suppressor or tumor-promoter, depending on cell type and external stimuli. The growth suppressing activity of Egr-1 has been observed in certain human cancers such as fibrosarcoma, glioblastoma, lung and breast cancer (13-16). In contrast, Egr-1 has been found to promote tumor cell growth in certain human cancer tissues such as prostate, skin and kidney cancers, in which Egr-1 is found at
elevated levels (17-19). In particular, Egr-1 expression is associated with higher Gleason scores and poor differentiation in human prostatic cancer (20). Accumulating evidence suggests that Egr-1 is involved in the development and progression of human cancers, particularly as a tumor promoter.

Previously, Egr-1 was found to promote tumor cell growth and inhibit apoptosis in human colon cancer cells (21-23). Therefore, Egr-1 expression is believed to be involved in tumor development and progression by affecting cell proliferation and apoptosis in human colorectal cancer. However, there are no data concerning the impact of Egr-1 on tumor cell proliferation and apoptosis in human colorectal cancer tissues.

The aim of the present study was to evaluate the expression of Egr-1 in human colorectal cancer and its correlation with tumor cell proliferation, apoptosis and clinicopathological features including patient survival.

Materials and methods

Patient and sample selection. The mRNA and protein expression of Egr-1 was evaluated in human colorectal cancer tissues, paired normal colorectal mucosa, metastatic or non-metastatic lymph node tissues of the same patients from colonoscopic biopsies and surgical specimens. For immunohistochemical staining, formalin-fixed and paraffin-embedded tumor specimens from 158 randomly chosen patients who had undergone surgery for colorectal cancer at Chonnam National University, Hwasun Hospital (Jeonnnam, Korea) between July 2004 and June 2005 were studied. None of the patients had received preoperative radiotherapy or chemotherapy. Pathological reports and clinical histories at the time of surgery were reviewed through the medical records. Tumor staging was in accordance with the American Joint Committee on Cancer (AJCC) staging system (24). Survival was measured from the time of surgery until follow-up in December 2010. The study group comprised 94 males and 64 females. The median age was 66.3±12.3 (means ± SD) with a range of 26-90 years. The mean time of surgery until follow-up was 43.1 months with a range of 0.7-56.0 months. This study was performed in accordance with the American Joint Committee on Cancer (AJCC) staging system (24).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cells in 1 ml of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol and subjected to reverse transcriptase polymerase-chain reaction (PCR). For cDNA synthesis, 1 µg of RNA was reverse transcribed with Molony-murine leukemia virus (MMLV) transcription reagents (Invitrogen Life Technologies). PCR amplification of cDNA was performed using gene-specific primers and Go Taq® DNA polymerase (Promega Corporation, Madison, WI, USA). The following primers were used: Egr-1, 5'-CAGTGGCCCTAGTGAGCATGA-3' and 5'-CCGCAAGTGATCTTGGTAT-3'; GAPDH, 5'-ACC ACA GTC GCC ATC AC-3'/5'-TCC ACC ACC CTG TTG CTG TA-3'.

Western blotting. Total cell extracts were prepared in RIPA buffer using the Halt™ protease and phosphatase inhibitor cocktail (both from Thermo Scientific Rockford, IL, USA). Cells were disrupted by sonication and centrifuged at 4°C. Equal amounts of protein were separated on polyacrylamide gels, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Blots were blocked with 5% milk and incubated with primary antibodies against Egr-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunoreactive proteins were visualized by the enhanced chemiluminescence detection system HRP substrate (Millipore). Immunoreactive protein bands were quantified using the luminescent image analyzer LAS-4000 and MultiGauge V3.2 image analyzer software (Fujiﬁlm, Tokyo, Japan).

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue sections (4 µm) with mounted probe on slides, were immunostained with anti-rabbit polyclonal antibody for the Egr-1 antigen (R&D Systems, Inc.) using the avidin-biotin peroxidase complex method. Sections were deparafﬁnized and heated in a microwave oven for 7 min to retrieve the antigens. They were immersed in 0.6% hydrogen peroxide for 10 min to block the endogenous peroxidase activity. The primary antibody, at a concentration of 1:100, was diluted in phosphate-buffered saline supplemented with 5% normal horse serum and 1% bovine serum albumin and then incubated with tissues overnight at room temperature. Anti-mouse immunoglobulin G (Sigma, St. Louis, MO, USA) labeled with biotin was used as a secondary antibody for the detection of primary antibodies and was incubated for 10 min at 45°C. After multiple rinses with universal buffer, the streptavidin-alkaline phosphatase detection system (Biomedex, Foster City, CA, USA) was applied for 8 min. As the final step, the slides were developed for 10 min with the enzyme substrate, 3,3-diaminobenzidine (Sigma). The slides were counterstained with hematoxylin solution for 3 min (Research Genetics, Huntsville, AL, USA). After dehydration, the tissue was sealed with a universal mount (Research Genetics). For negative controls, the primary antibody was omitted and replaced with phosphate-buffered saline.

Assessment of Egr-1 expression. The immunoreactivity was evaluated independently by 2 observers without knowledge of the clinical outcomes, through analysis of intensity, area and pattern of immunostaining. Staining intensity was classified from 0 (no staining) to 3 (strong staining), and the percentage of the staining area was classified as 0 for no positive staining of tumor cells, 1 for positive staining in <10% of the tumor cells, 2 for positive staining in 10-50% of the tumor cells, or 3 for positive staining in >50% of the tumor cells. The staining index was calculated as the product of staining intensity and staining area. Assessment of the staining was evaluated by 2 independent pathologists without knowledge of the clinical outcomes such as tumor stage, grade and survival. Consensus scores were assigned for each case by reviewing the slides with discrepancies in scoring. All sections for which there was disagreement between the 2 observers were re-evaluated.
and discussed. There was total agreement on the classification. The tumors were categorized as having positive expression (staining index ≥4) or negative expression (staining index <4).

Assessment of tumor cell proliferation. Immunohistochemical staining with the polyclonal Ki-67 antibody (MIB-1; diluted 1:150; Dakopatts, Glostrup, Denmark) using the avidin-biotin peroxidase complex method and 3,3-diaminobenzidine (Sigma) as chromogen was performed. A distinct brown staining of the nuclei with strong intratumoral heterogeneity was considered Ki-67-positive. The Ki-67-positive tumor cells were evaluated with a light microscope holding a x100 oil immersion objective by scoring a minimum of 1,000 tumor cells in randomly selected fields. For each case, 3 different counts were performed, and the highest score was chosen as the corresponding index. The Ki-67 labeling index (KI) was presented as the number of Ki-67-positive nuclei/1,000 tumor cell nuclei.

Detection of apoptotic cells and bodies. For detection of apoptotic cells, sections of formalin-fixed and paraffin-embedded tissue were processed for in situ immunohistochemical localization of nuclei exhibiting DNA fragmentation, by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) method, using the ApopTag™ Plus In situ Apoptosis Detection kit (Intergen, Purchase, NY, USA). Sections were treated according to the manufacturer's instructions as previously described (25). Briefly, sections were deparaffinized and rehydrated with xylene and ethanol, and permeabilized with 20 µg/ml proteinase K (Sigma) for 15 min at room temperature. Endogenous peroxidase was inactivated by coating the samples with 2% hydrogen peroxide (H₂O₂). Sections were rinsed with phosphate-buffered saline, and then immersed for 120 min in TdT buffer at 37˚C. Afterwards, they were incubated for 60 min with the anti-digoxigenin peroxidase-conjugate, followed by the peroxidase substrate diaminobenzidine. Finally, sections were counterstained with Mayer's hematoxylin. The positive control sections were treated with 0.7 g/ml DNase I (Sigma) in potassium cacodylate buffer (pH 7.2) for 10 min before treatment with TdT buffer. As a negative control, a number of tissue samples were subjected to treatment without TdT. The percentage of apoptotic cells was determined by counting labeled cells at a x400 magnification in randomly selected and homogeneous fields. Apoptotic cells were also identified by their characteristic morphological features in hematoxylin and eosin-stained sections such as cell shrinkage and chromatin margination or chromatin condensation with formation of apoptotic bodies (25). The apoptotic index (AI) was expressed as the number of positive nuclei including apoptotic bodies among 1,000 tumor cell nuclei.

Statistical analysis. All statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS/PC Plus Professional Statistics 15.0; SPSS, Inc., Chicago, IL, USA). The correlation between Egr-1 expression and the clinicopathological parameters was examined by χ² test and Fisher's exact test. The relationship between Egr-1 expression and KI or AI was evaluated by the Student's t-test. Survival curves were calculated according to the Kaplan-Meier method, and the differences were tested with a log-rank test. A p-value of <0.05 was considered to indicate a statistically significant result.

Results

Expression of Egr-1 in human colorectal cancer tissues by reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting. (A) Egr-1 mRNA expression by RT-PCR. (B) Egr-1 protein expression by western blotting. T, colorectal cancer tissue; N, paired normal colorectal mucosa. (C) Expression of Egr-1 was significantly higher in the human colorectal cancer tissue than that in the normal colorectal mucosa at the mRNA and protein levels. Each bar of the histogram represents the means ± SE of 20 cases. *p<0.01 vs. normal gastric mucosa. Egr-1, early growth response-1.
Expression of Ki-67 protein and detection of apoptosis in tissue specimens. Ki-67 immunoreactivity was found in the nuclei of cancer cells (Fig. 3A). Positive cells were commonly observed in the advancing margin of the tumors. The standard morphologic criteria of apoptotic cells using the TUNEL method include cell shrinkage and chromatin margination or chromatin condensation with formation of apoptotic bodies. Almost all of the positively stained cells and bodies were considered to be apoptotic cells as they corresponded morphologically to the standard criteria of apoptotic cells (Fig. 3B). Nonspecific staining in necrotic foci showed a faint and diffuse staining and it was distinguished from apoptotic nuclei by simple morphological examination.

Correlation between the expression of Egr-1 protein and clinicopathological features. The correlation between Egr-1 protein expression and clinicopathological parameters is shown in Table I. Positive expression of Egr-1 was significantly associated with age, lymphovascular invasion, lymph node and distant metastasis and tumor stage (p=0.023, p=0.024, p<0.001, p<0.001 and p<0.001, respectively). Furthermore, positive expression of Egr-1 was associated with poor survival (p=0.007) (Fig. 4).
Correlation between the expression of Egr-1 protein and tumor cell proliferation or apoptosis. The Ki-67 labeling index (KI) for 158 tumors ranged from 21.9 to 85.7 with a mean KI of 53.6±15.4. The mean KI value of Egr-1-positive tumors was 60.1±14.9 which was significantly higher than that of the Egr-1-negative tumors (p=0.031) (Table II). The apoptotic index (AI) for 158 tumors ranged from 0.9 to 30.0 with a mean AI of 9.0±1.0. However, there was no significant difference between Egr-1 expression and AI (p=0.357) (Table III).

Correlation between KI or AI and clinicopathological features. The correlation between KI or AI and clinicopathological parameters associated with Egr-1 expression is shown in Table IV. The mean KI value of the positive distant metastatic tumors was 64.2±13.7 which was significantly higher than that of the negative tumors (p=0.042). The mean AI value of the negative lymph node metastatic tumors was 11.5±7.1 and was significantly higher than that of the positive tumors (p=0.024). However, there was no association between KI or AI and age, tumor stage or lymphovascular invasion. In addition, KI or AI was not associated with patient survival (data not shown).

Discussion

Egr-1 is a member of the zinc-finger transcription factor family that is rapidly and transiently induced by a number of external stimuli including growth factors, cytokines and radiation injury. It regulates a wide spectrum of biological processes including cell growth, proliferation, apoptosis, cell cycle arrest, senescence, differentiation and cancer progression (6-12). Notably, Egr-1 expression has been found to be elevated in human prostatic (20) and gastric cancer tissues (26,27) and it has been suggested that Egr-1 plays an important role in carcinogenesis and cancer progression in the prostate and stomach (20,26,27). Our study showed that expression of Egr-1 mRNA and protein was increased in colorectal cancer tissue when compared with the barely detectable levels that were present in the matched normal colorectal mucosa. This result suggests that Egr-1 expression is implicated in colorectal carcinogenesis.

Although Egr-1 is considered to be a tumor-suppressor gene in several types of cancers (13-16), Egr-1 expression has been reported to be associated with cancer progression in prostatic and gastric cancers (20,26,27). In our study, increased expression of Egr-1 was significantly associated with age,
lymphovascular invasion, lymph node and distant metastasis, tumor stage and poor survival. Moreover, Egr-1 expression at the protein level was significantly increased in metastatic lymph node tissues, when compared to that in the non-metastatic lymph node tissues. These results strongly suggest that Egr-1 is involved in colorectal cancer progression and may have prognostic significance for colorectal cancer patients.

The continuous balance between cell proliferation and apoptosis is essential for the optimal function of nearly all tissues and organ systems, and must be properly coordinated. However, if cell proliferation exceeds apoptosis, neoplastic diseases and cancer may occur. Previous studies have shown that Egr-1 is involved in tumor development and progression by promotion of tumor cell growth and inhibition of apoptosis in human colon cancer cells (21-23). Therefore, we investigated the impact of Egr-1 on cell proliferation and apoptosis in colorectal cancer tissues.

Ki-67 is a nuclear antigen that is expressed in all stages of the cell cycle except for G0 and early G1, and it is an established proliferation marker and has been extensively used to estimate the growth fraction of tumors (28,29). Previous reports showed that Ki-67 expression was associated with tumor progression and patient survival in various human cancers including colorectal cancer (30-32). Recently, Egr-1 was found to promote tumor cell proliferation in gastric cancer cells (33). In the present study, higher KI values were significantly associated with distant metastasis, and the mean KI value of Egr-1-positive tumors was significantly higher than that of Egr-1-negative tumors. These results suggest that Egr-1 may be involved in tumor development and progression of colorectal cancer by affecting tumor cell proliferation.

The TUNEL method has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis (25,34,35). Previously, apoptosis-related genes have been identified as independent prognostic factor, and they have proven to be useful therapeutic targets in cancer therapy (36-38). Egr-1 did exert an effect as an inhibitor of the apoptotic pathway in colorectal cancer cells (23). In our study, a lower AI value was significantly associated with lymph node metastasis, but the AI value did not correlate with patient survival. Furthermore, no significant difference was noted between Egr-1 expression and AI in colorectal cancer tissues. These results suggest that the steps in apoptosis are not dependent on Egr-1 alone, and are controlled by numerous regulators including pro-apoptotic and anti-apoptotic genes (3-5).

In summary, Egr-1 expression was significantly elevated in colorectal cancer tissues, when compared to that in paired normal mucosa. In addition, Egr-1 expression was significantly increased in metastatic lymph node tissues, when compared to that in non-metastatic lymph node tissues. Increased expression of Egr-1 was significantly associated with age, lymphovascular invasion, lymph node and distant metastasis, tumor stage and poor survival. KI was significantly associated with distant metastasis. The mean KI value of Egr-1-positive tumors was significantly higher than that of Egr-1-negative tumors. However, there was no significant difference between Egr-1 expression and the AI value. These results indicate that Egr-1 may be associated with colorectal cancer progression via tumor cell proliferation.

Acknowledgements

This study was supported by a grant (0720570) from the National R&D Program for Cancer Control, Ministry of Health and Welfare, Republic of Korea, and partly by a research funds for the Research Institute of Clinical Medicine, Chonnam National University Hospital in 2012 (CRI 12035-1), Republic of Korea.
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