Met/HGF receptor modulates bcl-w expression and inhibits apoptosis in human colorectal cancers

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Summary The met proto-oncogene is the tyrosine kinase growth factor receptor for hepatocyte growth factor. In the present study, we investigated the role of met expression on the modulation of apoptosis in colorectal tumours. The gene expressions of c-met and the anti-apoptotic bcl-2 family, including bcl-2, bcl-xL and bcl-w, were analysed in human colorectal adenomas and adenocarcinomas by using a quantitative polymerase chain-reaction combined with reverse transcription. In seven of 12 adenomas and seven of 11 carcinomas, the c-met gene was overexpressed. The bcl-w, bcl-2 and bcl-xL genes were over-expressed in nine, five and six of 12 adenomas and in five, two and seven of 11 carcinomas, respectively. The c-met mRNA level in human colorectal adenomas and carcinomas was correlated with bcl-w but not with bcl-2 or with bcl-xL mRNA level. The administration of c-met-antisense oligonucleotides decreased Met protein levels in the LoVo human colon cancer cell line. In the case of c-met-antisense-treated cells, apoptotic cell death induced by serum deprivation was more prominent, compared to control or c-met-nonsense-treated cells. Treatment with c-met-antisense oligonucleotides inhibits the gene expression of bcl-w in LoVo cells. On the other hand, the gene expression of bcl-2 or bcl-xL was not affected by treatment with c-met-antisense oligonucleotides. These findings suggest that Met expression modulates apoptosis through bcl-w expression in colorectal tumours.

Keywords: c-met; bcl-w; apoptosis; colorectal cancers

The c-met oncogene encodes a cell-surface tyrosine kinase receptor for hepatocyte growth factor (HGF)/scatter factor (Bottaro et al, 1991; Naldini et al, 1991), a potent mitogen for epithelial cells which also promotes cell movement. The HGF-Met signaling pathway has been shown to be involved in tumour development and progression. The coexpression of HGF and Met molecules, which generates an autocrine stimulatory loop, is tumorigenic in NIH3T3 cells (Rong et al, 1992). In addition to tumorigenicity, deregulated HGF-Met signalling has also been shown to enhance in vitro invasiveness (Bhargava et al, 1992; Giordano et al, 1993; Rong et al, 1994; Rosen et al, 1994) and in vivo metastatic potential (Rong et al, 1994; Rosen et al, 1994) of various cell types. The results from several recent studies suggest that the HGF-Met system modulates the process of apoptosis. For example, HGF has been shown to protect canine renal tubular epithelial cells against apoptosis induced by the detachment of cells from their substrate (Frisch and Francis, 1994). An infusion of HGF inhibited apoptosis of rat hepatic ductal cells after the administration of the hepatocarcinogen 2-acetylaminofluorene (Nagy et al, 1996). Furthermore, transgenic expression in the liver of truncated Met, a constitutively active oncogenic form of c-met, blocked staurosporine-induced apoptosis of hepatocytes (Amicine et al, 1997).

It has been reported that the c-met gene is over-expressed in a high percentage of colorectal adenomas and carcinomas (Liu et al, 1992; Di Renzo et al, 1995; Umeki et al, 1999). However, the issue of whether Met expression in colorectal tumours is involved in resistance to apoptosis is presently unclear. The purpose of this study was to investigate the role of Met expression in the modulation of apoptosis in colorectal tumours. In the present study, the gene expressions of c-met and the anti-apoptotic bcl-2 family, including bcl-2, bcl-xL and bcl-w, were analysed in human colorectal adenomas and adenocarcinomas by means of a quantitative polymerase chain reaction combined with reverse transcription (RT-PCR), a technique which we have already employed in other studies (Kondo et al, 1995; 1996). An apparent correlation between c-met and bcl-w mRNA levels in colorectal tumours was found. Furthermore, we showed that the inhibition of Met production by c-met antisense promotes apoptosis induced by serum deprivation and inhibits the gene expression of bcl-w but not bcl-2 or bcl-xL in LoVo human colon cancer cells.

MATERIALS AND METHODS

Subjects

The study involved five control subjects (three males and two females; age range, 36–58 years) with no colorectal lesions as evidenced by endoscopy, 12 patients with colorectal adenoma (seven males and five females; age range, 46–77 years) and 11 patients with colorectal carcinoma (seven males and four females; age range, 35–71 years). Informed consent was obtained from each subject. Four tissue specimens were obtained from normal control mucosa, adenomas, carcinomas, and surrounding non-neoplastic mucosa in the case of patients with an adenoma or carcinoma for histologic evaluation and extraction of RNA. Formalin-fixed, paraffin-embedded tumours were stained with haematoxylin and eosin and classified histologically by expert pathologists as well-differentiated adenocarcinoma (n = 10),
moderately differentiated adenocarcinoma \((n = 1)\), adenoma without severe dysplasia \((n = 8)\), or adenoma with severe dysplasia \((n = 4)\). This study was approved by ethical committee.

**Cells and cell culture**

Human colon cancer LoVo cells were cultured in Ham’s F-12 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Whittaker Bioproducts, Walkersville, MD, USA), 100 units ml\(^{-1}\) penicillin, and 100 mg ml\(^{-1}\) streptomycin in a water-saturated atmosphere containing 5% CO\(_2\) at 37°C.

**RNA extraction**

Total RNA was extracted from tissue specimens (three of the biopsy specimens) and LoVo cells using the guanidinium thiocyanate/acid phenol method. As an internal standard, total RNA was extracted from mouse and rat colon.

**Oligonucleotides used for amplification**

In order to amplify c-met, bcl-w, bcl-2 and bcl-x\(_L\) mRNA in tissue specimens and LoVo cells by nested PCR, two pairs of primers were synthesized. The primer sequences were as follows: (a) 5'- GATCTGGGACATGAAATG-3' and 5'- TCTTCTGATGATTCTCCCTC-3' for the first-step c-met amplification (Park et al, 1987; Chan et al, 1988); (b) 5'-GTAATGCGCCCCAGTGTAAG-3' and 5'-CAAGGATTCAAGACAGCT-3' for the second-step c-met amplification; (c) 5'-TATAGCGTGGACAAGGG-3' and 5'-TCTGCACTGTCCTCCTACAGT-3' for the first-step bcl-w amplification (Gibson et al, 1996); (d) 5'-TCTGTGGGAGCTTGGCC-3' and 5'-TCTCCAGGTAAGCCACCATC-3' for the second-step bcl-w amplification; (e) 5'-CAGCTGACCTGAGCCCTT-3' (which is also used for the second-step bcl-2 amplification) and 5'-CCCTCGGTATCCTG-GATCC-3' for the first-step bcl-2 amplification (Cleary et al, 1986; Negriini et al, 1987); (f) 5'-CAGCT GCCCTGACCCTCC-3' and 5'-GCCCTGTTAGTGACTCGT-3' for the second-step bcl-2 amplification; (g) 5'-AAGGATACAGCCTG-GAGTC-3' and 5'-ATCCTGCTACCTCAATCGT-3' for the first-step bcl-x\(_L\) amplification (Boise et al, 1993; González-García et al, 1994); (h) 5'-TCATATGCGCCACCATCAGT-3' and 5'-CTAGCCTTTGCACGCACTG-3' for the second-step bcl-x\(_L\) amplification. For the amplification of 28S rRNA, a pair of PCR primers was synthesized. The sequences were 5'-CATGTGGACACAGCTTGAAA-3' and 5'-CCCTGCTTCAACAAAGAAA-3' (Hadjiolov et al, 1984; González et al, 1985). Although both the human and internal standard cDNAs originating from c-met mRNA, bcl-w mRNA, bcl-2 mRNA, bcl-x\(_L\) mRNA, or 28S rRNA are amplified by the same primers, only the human mRNA fragments amplified with these primers contained an, Mva I (c-met), Dpn II (bcl-w), Ban II (bcl-2), Ava I (bcl-x\(_L\)), or Eae I (28S rRNA) restriction endonuclease site.

**RT-PCR**

The conversion of RNA to cDNA was carried out in a final volume of 5 µl containing 1 µg or less human and mouse RNA mixture of different proportion in the reaction solution of 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl\(_2\), 3 mM dithiothreitol, 10 µM random primer (Takara Shuzo, Kyoto, Japan), 1 mM each dNTPs, 6 units RNase inhibitor (Pharmacia LKB Biotechnology, Tokyo, Japan), and 1 unit RAV-2 reverse transcriptase (Takara Shuzo). The c-met, bcl-w, bcl-2, and bcl-x\(_L\) cDNAs (0.5 µl of the reverse transcription product) were amplified by nested PCR with two pairs of primers in PCR buffer, containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl\(_2\), 0.001% (w/v) gelatin, 400 µM of each dNTP, 1 µM each of the first-step or second-step 5' and 3' primers, and 0.25 units of Thermus aquaticus DNA polymerase (Taq polymerase) (Perkin-Elmer Cetus, Norwalk, CT, USA). Each amplification profile involved denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and extension at 72°C for 3 min. The profile was repeated for 30 cycles. After the second-step PCR amplification, 1 µl from each sample was added to 20 µl of fresh PCR buffer containing the second-step 5' and 3' primers, and subjected to one additional cycle to prevent heterodimeric DNA formation between the human and mouse PCR products (Kondo et al, 1995). For the determination of the 28S rRNA concentration, rat 28S rRNA was used for the internal standard and the cDNA from human and rat 28S rRNA was amplified for 30 cycles and one additional cycle as described previously (Kondo et al, 1995).

**Quantitative analysis**

After the amplification step was completed, the DNA fragments corresponding to the c-met mRNA, bcl-w mRNA, bcl-2 mRNA, bcl-x\(_L\) mRNA and 28S rRNA were digested with Mva I (New England Biolabs, Beverly, MA, USA), Dpn II (Takara Shuzo), Ban II (Takara Shuzo), Ava I (New England Biolabs,) and Eae I (Takara Shuzo), respectively. The digested samples were then injected directly into a high-performance liquid chromatography (HPLC) system with a UV-spectrophotometric detector (Model SPD-6A, Shimadzu, Kyoto, Japan) operated at 260 nm. The analytical column was a TSK gel DEAE-NPR (Tohos, Tokyo, Japan). The digested DNA fragments were chromatographed under a 20 min linear gradient of NaCl from 0.4–0.6 M prepared in 20 mM Tris-HCl, pH 9.0, at a flow rate of 1.0 ml min\(^{-1}\). The HPLC elution profile revealed the baseline separation of three DNA fragments. The resulting fragments were identified as two human fragments (c-met: 145 base pairs (bp) and 242 bp) and an internal standard fragment (c-met: 93 bp) for the determination of the c-met mRNA, bcl-w mRNA, bcl-2 mRNA, bcl-x\(_L\) mRNA and 28S rRNA levels, the peak-height ratio of the 236 bp to the 316 bp fragment, the 208 bp to the 286 bp fragment, the 208 bp to the 217 bp fragment, the 144 bp to the 199 bp fragment, the 236 bp to the 316 bp fragment and the 148 bp to the 242 bp fragment, the 208 bp to the 217 bp fragment, the 144 bp to the 199 bp fragment, the 236 bp to the 316 bp fragment and the 148 bp to the 242 bp fragment were measured, respectively. The coefficient of variation of this assay was 8.5% for c-met mRNA, 9.4% for bcl-w mRNA, 9.2% for bcl-2 mRNA, 7.2% for bcl-x\(_L\) mRNA and 9.0% for 28S rRNA. The c-met, bcl-w, bcl-2 and bcl-x\(_L\) mRNA levels were expressed as a relative c-met mRNA/28S rRNA ratio, a relative bcl-w mRNA/28S rRNA ratio, a relative bcl-2 mRNA/28S rRNA ratio, and a relative bcl-x\(_L\) mRNA/28S rRNA ratio, respectively.

**Antisense oligonucleotides**

HPLC-purified 20-mer c-met antisense oligonucleotides and nonsense (a scramble version) control oligonucleotides were designed based on the oligonucleotide sequence.
purchased from Bex (Tokyo, Japan). The antisense oligonucleotides straddled the predicted translation-initiation site of human c-met mRNA. The sequences used were 5'-ACAGCGGGGCTTCTATTAT-3' (c-met-antisense) and 5'-TCGGCTACAAGCTACGGTTG-3' (c-met-nonsense).

Western blot analysis
Cells were washed in PBS and lysed in lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM EDTA, 100 mM NaF, 1 mM PMSF, 0.25 TIU ml⁻¹ of aprotinin, and 10 µg ml⁻¹ of leupeptin. Aliquots containing 30 µg of total protein were size-fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (5–20% gradient gels). Immunoblot analysis was performed as described previously using a rabbit anti-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Kitamura et al, 1999). Protein concentrations of the homogenates were determined using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA).

Number of viable cells and cell viabilities
LoVo cells were seeded at 5 × 10⁴ cells per 60 mm dish and cultured in Ham’s F-12 medium supplemented with 10% heat-inactivated foetal calf serum. After preincubation with 10 µM c-met-antisense or c-met-nonsense oligonucleotides for 4 days, medium was removed and replaced with serum-deprived medium containing 10 µM c-met-antisense or c-met-nonsense oligonucleotides. The serum-deprived medium containing oligonucleotides was not changed until measurement of cell viabilities. The number of viable cells was determined by haemocytometry using trypan blue exclusion.

Measurement of apoptosis by flow cytometry
DNA degradation was assessed by measuring the DNA content of individual cells by flow cytometry. In preparation for flow cytometry, cells were collected after brief trypsinization, washed with PBS, and fixed in 70% cold ethanol. Samples were then treated with RNase, stained with 10 µg ml⁻¹ propidium iodine, and analysed using a Becton Dickinson FACScan. Cell-cycle distributions were quantified using Cellquest software.

Statistical analysis
Statistical analysis was made using Wilcoxon matched-pairs signed-rank test or Mann–Whitney U test. A level of P < 0.05 was accepted as statistically significant. For the measurements of the levels of c-met mRNA, bcl-w mRNA, bcl-2 mRNA, bcl-xL mRNA and 28S rRNA, each data-point represents the mean of three measurements.

RESULTS
Gene expression of c-met, bcl-w, bcl-2 and bcl-xL in human colorectal adenomas and carcinomas
In seven of 12 (58%) adenomas and seven of 11 (64%) carcinomas, the c-met gene was found to be overexpressed (Figure 1), when overexpression is defined as an increase of greater than two-fold over the expression in adjacent normal tissue. The bcl-w, bcl-2 and bcl-xL genes were overexpressed in nine (75%), five (42%) and six (50%) of 12 adenomas and in five (45%), two (18%) and seven (64%) of 11 carcinomas, respectively. The levels of bcl-w mRNA in tumours with c-met-overexpression were apparently higher compared with the levels in tumours without c-met-overexpression (Figure 2). A significant correlation (correlation coefficient (r) = 0.754, P < 0.0001) was observed between the bcl-w mRNA levels and c-met mRNA levels (data not shown). The levels of bcl-2 or bcl-xL mRNA were not correlated with the gene expression of c-met in colorectal adenomas and carcinomas.

Evaluation of c-met protein levels by Western blotting
The c-Met protein in LoVo cells was detected as a single polypeptide with a molecular weight of 190 kDa, finding consistent with previous reports that LoVo cells express constitutively activated Met protein which is uncleaved because of defective post-translational processing (Mondino et al, 1991). When added to cultures of LoVo cells for 4 days, c-met-antisense produced concentration-dependent reductions in the steady state levels of Met protein (Figure 3). In contrast, the Met protein level of 10 µM c-met-nonsense-treated cells was similar to that of untreated cells.

Effect of antisense oligonucleotides to c-met on apoptosis induced by serum deprivation
After preincubation of LoVo cells with 10 µM c-met-antisense or c-met-nonsense oligonucleotides for 4 days, the serum was removed from the medium. Pretreatment with antisense oligonucleotides rapidly decreased the number of viable cells after serum deprivation in a time-dependent manner (Figure 4). Nonsense-
treated cells survived for more than 6 days after serum deprivation. In order to determine if the decrease in the number of viable cells after serum deprivation could be attributed to apoptosis, the number of apoptotic cells was measured by flow cytometry. Fragmented DNA contained in apoptotic cells resulted in an increase of two-fold or more. Vertical bar to the right of each group represents the mean ± standard deviation. NS = not statistically significant.

**Effect of antisense oligonucleotides to c-met on gene expression of bcl-w, bcl-2 and bcl-xL**

After incubation of LoVo cells with 10 µM c-met-antisense or c-met-nonsense oligonucleotides for 4 days, expression levels of bcl-w, bcl-2 and bcl-xL mRNAs were determined by competitive RT-PCR method. In the case of the c-met-antisense treated cells, the bcl-w mRNA level was decreased when compared to either untreated or c-met-nonsense-treated cells (Figure 6). Gene expression of bcl-2 or bcl-xL of LoVo cells was not affected by treatment with c-met-antisense or c-met- nonsense oligonucleotides.

**DISCUSSION**

The c-met oncogene encodes the receptor for HGF, a potent mitogen for epithelial cells that also promotes cell motility and invasiveness. It has recently been suggested that HGF protects...
epithelial cells against apoptosis induced by various stimuli (Frisch and Francis, 1994; Fan et al, 1998). Previous studies have shown that the c-met gene is overexpressed in colorectal adenomas and carcinomas (Liu et al, 1992; Di Renzo et al, 1995; Umecki et al, 1999). In the present study, we investigated the role of c-met expression in colorectal tumours in the modulation of apoptosis. The relationship between the mRNA expression level of c-met and those of the anti-apoptotic bcl-2 family including bcl-w, bcl-2, bcl-xL was examined in colorectal adenomas and carcinomas. The overexpression of the c-met gene was detectable in more than 50% of the lesions at both stages of adenomas and carcinomas. Our observations are in agreement with previous reports (Liu et al, 1992; Di Renzo et al, 1995). Up-regulation of the bcl-xL gene was also observed in adenomas and carcinomas. In contrast to bcl-2 gene expression, the gene expression of bcl-2 seemed to decline during the progression of adenomas to carcinomas. In some colorectal carcinomas, the expression of bcl-2 gene was decreased compared with that in adjacent normal tissues. These results are in agreement with earlier immunohistochemical study in which Bcl-2 and Bcl-xL protein expression was examined (Krajewska et al, 1996). In this study, we demonstrated, for the first time, the overexpression of the bcl-w gene in many colorectal adenomas and carcinomas. Furthermore, the expression level of the bcl-w gene was correlated with the c-met expression level.

In order to clarify the role of Met expression on cell survival and gene expression of the anti-apoptotic bcl-2-related gene, we treated LoVo human colon cancer cells with c-met-antisense oligonucleotides. In LoVo cells, although no mutations were present in the Met-coding region, the tyrosine kinase encoded by the c-met proto-oncogene is constitutively activated via a defective post-translational processing of the precursor protein (Mondino et al, 1991). c-met-antisense oligonucleotides effectively inhibited Met protein production and increased the level of apoptotic cell death after serum deprivation, suggesting that Met expression in colon cancer cells plays an important role in preventing apoptosis. Furthermore, as shown in Figure 6, the inhibition of Met production resulted in a decrease in gene expression of bcl-w but not bcl-2 or bcl-xL in LoVo cells.

bcl-w has been cloned as a bcl-2-related gene and has been shown to be expressed in many tissues, especially the colon (Gibson et al, 1996). It has been shown that the enforced expression of bcl-w enhances survival of several cell types exposed to a variety of cytotoxic agents. High levels of Bcl-w enables a myeloid cell line to resist apoptosis induced by IL-3 withdrawal or γ-irradiation and a T hybridoma cell line is rendered refractory to γ-irradiation and dexamethasone (Gibson et al, 1996). These facts, along with our results that the decrease in Met protein content inhibited bcl-w expression and resulted in increased cell death induced by serum deprivation, suggest that Met enhances cell survival through the modulation of Bcl-w expression. In a recent study using MDA-MB-453 human breast cancer cells, it has been reported that pretreatment of MDA-MB-453 cells with HGF inhibited the adriamycin-induced decrease in the levels of Bcl-xL (Fan et al, 1998), suggesting that Bcl-xL may have a role in HGF-mediated cell survival in some cancer cells. However, in colorectal tumours, no relationship between c-met and bcl-xL gene expression was observed. Furthermore, the inhibition of Met protein production by c-met-antisense had no effect on the expression level of bcl-xL gene.

In conclusion, we demonstrated that the expression of c-met gene is up-regulated and correlated with bcl-w expression in human colorectal adenomas and carcinomas. The inhibition of Met protein expression resulted in a decrease in bcl-w gene expression and an increase in apoptotic cell death induced by serum deprivation in LoVo human colon cancer cells. These observations suggest that Met expression plays a role in the protection of cells against apoptosis by targeting Bcl-w in colorectal tumours.

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Figure 6 Effect of c-met-antisense oligonucleotides on gene expression of bcl-w (A), bcl-2 (B) and bcl-xL (C) in LoVo cells. After incubation with 10 μM c-met-antisense or c-met-nonsense oligonucleotides for 4 days, the expression levels of bcl-w, bcl-2 and bcl-xL mRNAs were determined by competitive RT-PCR method. Data are expressed as mean ± standard error (n = 4)

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