Nuclear Factor- κ B Regulates Cyclooxygenase-2 Expression and Cell Proliferation in Human Gastric Cancer Cells

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SUMMARY: Nuclear factor- κ B (NF- κ B) is a transcriptional regulator of inducible expression of genes including cyclooxygenase-2 (COX-2), regulating cell proliferation. NF- κ B is kept silent in the cytoplasm via interaction with the inhibitory protein I κ B α and transmigrated into the nucleus upon activation. However, constitutive NF- κ B has been found in the nucleus of some cancer cells. We investigated the role of NF- κ B in COX-2 expression and cell proliferation in human gastric cancer AGS cells. AGS cells were treated with antisense oligodeoxynucleotide (AS ODN) or sense oligodeoxynucleotide (S ODN) for the NF- κ B subunit p50, or they were transfected with a mutated I κ B α gene (MAD-3 mutant) or a control vector, pcDNA-3. AGS cells were treated with COX-2 inhibitors such as indomethacine and NS-398 or prostaglandin E₂. mRNA expression for COX-2, and protein levels for p50, I κ B α , and COX-2 were determined by reverse transcription polymerase chain reaction and Western blot analysis. The NF- κ B levels were examined by electrophoretic mobility shift assay. Thromboxane B₂ (TXB₂) and 6-keto-prostaglandin F_{1 α} (6-keto-PGF_{1 α}) levels were determined by enzyme-linked immunosorbent assay. Cell proliferation was assessed by viable cell counting, [³H] thymidine incorporation, and colony formation. The nuclear level of p50 decreased in AGS cells treated with AS ODN. The I κ B α mutant was observed in cells transfected with the mutated I κ B α gene. NF- κ B was inhibited in cells treated with AS ODN or transfected with the mutated I κ B α gene, compared with the cells treated with S ODN or transfected with control vector. Cell proliferation, mRNA expression and protein level of COX-2, and production of TXB₂ and 6-keto-PGF_{1 α} were inhibited in cells treated with AS ODN or transfected with the mutated I κ B α gene, which had lower NF- κ B levels than cells treated with S ODN or transfected with control vector. COX-2 inhibitors suppressed cell proliferation and production of TXB₂ and 6-keto-PGF_{1 α} in a dose-dependant manner. Prostaglandin E₂ prevented the inhibition of proliferation in cells treated with AS ODN or transfected with the mutated I κ B α gene. In conclusion, NF- κ B mediates COX-2 expression, which may be related to cell proliferation, in human gastric cancer cells. (*Lab Invest* 2001, 81:349–360).

Many cellular genes involved in the early process of immune, acute phase, and inflammatory responses are regulated at the level of transcription by nuclear factor- κ B (NF- κ B) (Baeuerle and Baltimore, 1996; Barnes and Karin, 1997). Several reports have shown a role for NF- κ B gene products in cell proliferation, transformation, and tumor development (Siebenlist et al, 1994; Wulczyn et al, 1996). NF- κ B is an inducible transcription factor that mediates signal transduction between the cytoplasm and nucleus in many cell types (Baeuerle and Henkel, 1994). NF- κ B is a member of the Rel family, which includes p50 (NF- κ B1), p52 (NF- κ B2), Rel A (p65), c-Rel, rel B, and *Drosophila morphogen dorsal* gene product (Siebenlist et al, 1994). In resting cells, NF- κ B is localized in the cytoplasm as a hetero- or homodimer, and is noncovalently associated with cytoplasmic inhibitory proteins, including I κ B. Upon stimulation by a variety

of pathogenic inducers, such as viruses, mitogens, bacteria, agents providing oxygen radicals, and inflammatory cytokines, the NF- κ B complex migrates into the nucleus and binds DNA recognition sites in the regulatory regions of the target genes (Thanos and Maniatis, 1995). In most resting-state mammalian cells, NF- κ B is inactive. However, NF- κ B was aberrantly activated in human breast cancer and in rat mammary tumors from the aromatic hydrocarbon-induced model of breast cancer under resting state, whereas low levels of NF- κ B were found in untransformed human breast epithelial cells and normal rat mammary glands (Sovak et al, 1997; Wu et al, 1996). Furthermore, the inhibition of constitutive NF- κ B in B cell lymphomas and breast cancer cell lines leads to the induction of apoptosis (Sovak et al, 1999; Wu et al, 1996). Thus, NF- κ B proteins could be directly involved in cell proliferation. Our previous studies (Kim et al, 1999, 2000) demonstrated that a constitutive level of NF- κ B was observed in the nucleus of gastric carcinoma AGS cells. NF- κ B dimers (a p50/p65 heterodimer and a p50 homodimer) were detected in these cells and the major NF- κ B band was a p50 homodimer, even in resting-state cells. Upon stimulation by *Helicobacter pylori* infection, a p50/p65 het-

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erodimer was highly elevated and a p50 homodimer was slightly increased in the nucleus of AGS cells.

NF- κ B activation is tightly regulated by its endogenous inhibitor, I κ B, which complexes NF- κ B in the cytoplasm. To date, the most extensively studied I κ B protein is I κ B α (36 kd), which is encoded by the human MAD-3 gene or its homologues in different species (Haskill et al, 1991). The mechanisms that lead to the degradation of I κ B α proteins are poorly understood, but involve changes in the phosphorylation state of I κ B α (Le Bail et al, 1993; Thanos and Maniatis, 1995). Two serines in the N-terminal domain of I κ B α , serine residues 32 and 36, are critical for I κ B α stability (Brown et al, 1995). Substitution of these two serine residues by alanine residues renders I κ B α undegradable by cellular activators (Brown et al, 1995; Traenckner et al, 1995). Among many proteins exhibiting I κ B function, I κ B α is the only inhibitor that dissociates from the NF- κ B complex in response to cell stimulation, with kinetics matching NF- κ B translocation to the nucleus (Sun et al, 1994). Therefore, it was suggested that the activation of NF- κ B is regulated by NF- κ B/I κ B α dissociation (Beg and Baldwin, 1993; Liou and Baltimore, 1993). Mutant I κ B α acts as an NF- κ B super-repressor (Wang et al, 1996).

Antisense oligodeoxynucleotides (AS ODN) are small, synthetic molecules, 15 to 25 base pairs in length, and are usually single-stranded DNA complementary to the mRNA transcribed from the target gene. Formation of a duplex structure occurs through base-pairing between the antisense DNA and its target mRNA, inhibiting gene expression (Milligam et al, 1993). Postulated mechanisms of AS ODN include steric hindrance of gene transcription or gene translation, blockage of mRNA processing or splicing, and degradation of mRNA through the action of RNase H activity (Crooke, 1992). Oligodeoxynucleotides (ODN) can be chemically manipulated by replacement of a nonbridging oxygen with sulfur in a phosphate linkage to form phosphorothioate-modified ODN, making ODN more resistant to degradation by nucleases (Brown et al, 1994). Thus, AS ODN can be made to bind to a single gene or its transcription product and act within the constraints of that gene's expression, minimizing side effects. As mentioned above, two species of NF- κ B dimers (a p50/p65 heterodimer and a p50 homodimer) are present in activated gastric cancer cells (Keates et al, 1997; Kim et al, 1999). Thus, as a choice for targeting by AS ODN, p50 offers the best potential for effecting NF- κ B expression and the genes that NF- κ B regulates.

Cyclooxygenase, the enzyme that catalyzes the oxidation of arachidonic acid, is expressed in two isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is constitutively expressed in most tissues and is thought to be important in maintaining cellular homeostasis (O'Neil and Hutchinson, 1993). In contrast, COX-2 is frequently undetectable at baseline in normal tissues, but readily expressed in gastrointestinal epithelial cells in response to inflammatory cytokines, lipopolysaccharide, mitogens, and oxygen radicals (Williams and Du

Bios, 1996). The role of COX-2 in gastric tissue is uncertain. However, prostaglandins produced via COX-2, including prostaglandin E₂ (PGE₂) (Hida et al, 1998; Tjandrawinata et al, 1997), prostaglandin A₁, A₂, and D₂ (Bhuyan et al, 1986), prostaglandin J₂ and 15-deoxy-delta 12,14-prostaglandin J₂ (Chinery et al, 1999), are believed to be the major contributors to cell proliferation and the inflammatory process (Seibert et al, 1994, 1995). They can be mutagenic (Plummer et al, 1995) and tumorigenic (Boolbol et al, 1996). Therefore, chronic expression of COX-2 may play an important role in gastric carcinogenesis in addition to propagation of gastric inflammation. Zimmermann et al (1999) reported that PGE₂ synthesis was 600 times higher in human esophageal carcinoma cell lines. They showed high COX-2 expression that was directly related to cell proliferation. PGE₂ has been regarded as a primary COX-2 product in smooth muscle cells (Yamamoto et al, 1999), colon carcinoma cells (Kojima et al, 2000), lung cancer cells (Hida et al, 1998), esophageal cancer cells (Zimmermann et al, 1999), and prostatic carcinoma cells (Tjandrawinata et al, 1997). COX-2 is constitutively expressed in certain groups of cancers (Battu et al, 1998; Sheng et al, 1997) and is related to the cell proliferation in those cancers (Higashi et al, 2000; Molina et al, 1999). COX-2 inhibition by specific COX-2 inhibitors suppresses cell proliferation and induces apoptosis in pancreatic cancer cells (Ding et al, 2000) and in a colorectal cancer cell line (Elder et al, 1997; Hara et al, 1997). However, in gastric cancer cells, COX-2 inhibition did not induce apoptosis, it only inhibited cell proliferation (Sawaoka et al, 1998). However, there have been no direct studies of the relation among transcription factors regulating COX-2 expression and cell proliferation or apoptosis in cancer cells. The promoter region of the human COX-2 gene contains two NF- κ B consensus sites, and the expression of COX-2 is largely regulated by NF- κ B in several cells (Hla and Neilson, 1992; Yamamoto et al, 1995). Because some evidence supports a role of NF- κ B in the COX-2 expression that regulates cell proliferation (Gallois et al, 1998; Kojima et al, 2000), we hypothesize that inhibition of constitutive NF- κ B may suppress COX-2 expression and thus, cell proliferation, in human gastric cancer cells.

We conducted the present study to evaluate the role of NF- κ B on COX-2 expression and cell proliferation in gastric cancer AGS cells by treating AS ODN for p50 or transfecting mutated I κ B α genes (MAD-3 double point mutation at positions 32 and 36) to inhibit NF- κ B activation. To evaluate the relationship between COX-2 expression and cell proliferation, AGS cells were treated with COX-2 inhibitors, such as indomethacine and NS-398 or PGE₂, and cell proliferation was determined. After treatment with AS ODN for p50 or transfection with the mutated I κ B α gene, the levels of p50 and I κ B α s and NF- κ B activation were determined by Western blot analysis and electrophoretic mobility shift assay (EMSA). Levels of COX-2 mRNA and protein were determined by reverse transcription polymerase chain reaction (RT-PCR) and Western blot

analysis. Stable COX-2 products, thromboxane B₂ (TXB₂) and 6-keto-prostaglandin F_{1 α} (6-keto-PGF_{1 α}) were determined by enzyme-linked immunosorbent assay (ELISA). Cell proliferation was assessed by viable cell counting, [³H] thymidine incorporation, and colony formation.

Results

Protein Levels of P50, I κ B α , and NF- κ B of AGS Cells Treated with ODN or Transfected with the Mutated I κ B α Gene

To inhibit NF- κ B activation, the cells were treated with AS ODN or sense oligodeoxynucleotide (S ODN) for 72 hours (Figs. 1A and 2A), or transfected with control pcDNA3 vector (pcN-3) or the mutated I κ B α gene (IW-6 and IW-10) (Figs. 1B and 2B). The effect of AS ODN treatment was assessed by measuring the level of p50 protein in the nucleus. Western blot analysis for p50 showed that AS ODN caused a reduction in p50 protein compared with control cells and cells treated with S ODN (Fig. 1A). NF- κ B levels in the nuclear extracts of cells treated with AS ODN were lower than in control cells or cells treated with S ODN (Fig. 2A).

AGS cells were stably transfected with a mutated I κ B α gene, which is not susceptible to phosphorylation at positions 32 and 36, and resists degradation in transient transfection (Le Bail et al, 1993). The stably transfected clones of the control vector (pcN-3) and of the mutated I κ B α gene (IW-6, IW-10), were selected and analyzed by Western blot analysis for mutated I κ B α expression (Fig. 1B). In control cells and cells transfected with pcN-3, two bands had an electrophoretic mobility of 36 and 38 kD, corresponding to wild-type I κ B α (I κ B α wt) and phosphorylated I κ B α (I κ B α -P), respectively. In the cells transfected with the mutated I κ B α gene (IW-6 and IW-10), a major band (38 kD) was detected with a specific I κ B α antibody. It was

Figure 1.

Western blot analysis for p50 and I κ B α of AGS cells treated with oligodeoxynucleotide (ODN) or transfected with the mutated I κ B α gene. For p50 analysis, cells were treated with antisense (AS) ODN or sense (S) ODN for 72 hours, and 20 μ g of nuclear protein were subjected to 8% SDS-PAGE and transferred onto membrane. The NF- κ B subunit p50 was visualized with p50-specific antibody (A). For I κ B α analysis, cells were transfected with a control pcDNA3 vector (pcN-3) or the mutated I κ B α gene (IW-6 and IW-10), and cell lysates (100 μ g of protein) were subjected to 8% SDS-PAGE and transferred onto membranes. Wild-type I κ B α (I κ B α wt), phosphorylated I κ B α (I κ B α -P), and mutant-type I κ B α (I κ B α mt) were visualized with specific antibodies. Arrows indicate the two bands with electrophoretic mobilities of 36 and 38 kD (B). Control cells received neither treatment nor transfection (Control).

reported that transfection with a mutated I κ B α gene generated a reduced electrophoretic mobility product corresponding to a 38 kD band (Cai et al, 1997; Jobin et al, 1998). Thus, the slower migration band (38 kD) probably corresponded to the product of the mutated I κ B α gene (mutant-type I κ B α ; I κ B α mt). To investigate whether the introduction of an exogenous mutated I κ B α gene leads to a suppression of constitutive NF- κ B, we performed an EMSA with nuclear extracts from the transfected cells. As shown in Figure 2B, NF- κ B levels of the cells transfected with the mutated I κ B α gene (IW-6 and IW-10) were significantly reduced compared with control cells or with the cells transfected with pcN-3. Additionally, the IW-10 clone had more I κ B α mutant and less NF- κ B than the IW-6 clone. These results show that mutated I κ B α expression is directly related to inhibition of NF- κ B activation.

Cell Proliferation of AGS Cells Treated with ODN or Transfected with the Mutated I κ B α Gene

To evaluate the relationship between NF- κ B inhibition and cell proliferation, the cells treated with ODN (Fig. 3A) or transfected with the mutated I κ B α gene (Fig. 3B) were cultured for 96 hours, and the cell number was counted after trypan blue exclusion. Cell proliferation decreased in the cells treated with AS ODN or transfected with the mutated I κ B α gene (IW-6 and IW-10), compared with control cells, the cells treated with S ODN, or the cells transfected with pcN-3. This result was confirmed by other methods: determining the percentage of 3(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) positive cells (Fig.

Figure 2.

NF- κ B levels in the nuclei of AGS cells treated with ODN or transfected with the mutated I κ B α gene. Nuclear extracts (1 μ g) were prepared from the cells treated with AS ODN or S ODN for 72 hours (A), or transfected with control pcDNA3 vector (pcN-3) or the mutated I κ B α gene (IW-6 and IW-10) (B), and subjected to electrophoretic mobility shift assay (EMSA). Control cells received neither treatment nor transfection (Control).

Figure 3.

Cell numbers of AGS cells treated with ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene. Cells were treated with AS ODN or S ODN for 72 hours (A) or transfected with control pcDNA3 vector (*pcN-3*) or the mutated $\text{I}\kappa\text{B}\alpha$ gene (*IW-6* and *IW-10*) (B). Cells, plated at 2×10^4 cells/well, were incubated for the indicated times and cell numbers were counted by trypan blue exclusion. Control cells received neither treatment nor transfection (Control). Each point represents the mean $\pm$ standard deviation of four separate experiments.

4A), the amount of [^3H] thymidine incorporation (Fig. 4B), and the degree of colony formation (Fig. 4C). After 96 hours in culture, the percentage of MTT-positive cells decreased in AS ODN-treated cells and in cells transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene. [^3H] thymidine incorporation of the cells treated with AS ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene was significantly lower than in cells treated with S ODN or transfected with a control vector after 24 hours in culture. To examine the effect of NF- κB inhibition on colony formation, the cells were cultured in a soft agar, an ex vivo model of tumor cell growth. After 10 days in culture, control cells formed colonies with efficiency of approximately 3%. Colony formation of the cells treated with AS ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene was significantly lower than those treated with S ODN or control vector. Inhibition of cell proliferation was lower in IW-10 cells than in IW-6 cells, determined by cell numbers, percentage of MTT-positive cells, [^3H] thymidine incorporation, and colony formation. These results showed that inhibition of NF- κB activation caused a strong down-regulation of cell proliferation, which correlated with the expression level of mutated $\text{I}\kappa\text{B}\alpha$ (Figs. 1B and 2B).

mRNA Expression and Protein Level of COX-2 and Production of TXB_2 and 6-Keto-PGF $_{1\alpha}$ in AGS Cells Treated with ODN or Transfected with a Mutated $\text{I}\kappa\text{B}\alpha$ Gene

RT-PCR analysis for mRNA expression of COX-2, standardized by co-amplifying this gene with the housekeeping gene β -actin, showed that AGS cells constitutively expressed COX-2 in resting cells. COX-2 expression was lower in the cells treated with AS ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene (IW-6 and IW-10) than in control cells, cells treated with S ODN, or cells transfected with pcN-3 (Fig. 5A).

Figure 4.

Cell proliferation of AGS cells treated with ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene, as determined by the percentage of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) positive cells (A), thymidine incorporation (B), and colony formation (C). Cells were treated with AS ODN or S ODN for 72 hours or transfected with control pcDNA3 vector (*pcN-3*) or the mutated $\text{I}\kappa\text{B}\alpha$ gene (*IW-6* and *IW-10*). For the analysis of MTT-positive cells, cells were plated at 1×10^3 cells/well and cultured for 96 hours. Viable cells were assessed by MTT assay and MTT-positive cells were expressed as a percentage of control (A). For the experiment on [^3H] thymidine incorporation, cells were plated at 5×10^4 /well and cultured for 24 hours. After addition of 1 $\mu\text{Ci}/\text{ml}$ of [^3H] thymidine, the cells were cultured for 6 hours. [^3H] thymidine incorporation of control cells was regarded as 100% and relative [^3H] thymidine incorporation was expressed as a percentage of control (B). For the colony formation assay, 1 ml of basal layer culture medium with a final 0.5% agarose concentration was prepared in 35 mm culture dishes. The cells, at 2×10^3 cells/ml, in 1 ml of upper layer medium (0.2% agarose) were overlaid onto the preformed basal layer. After a 10 day incubation, colonies were stained with crystal violet and colonies with more than 100 cells were counted. Cell proliferation was determined as the mean colony numbers, compared with those of control (100%) (C). Control cells received neither treatment nor transfection (Control). Each bar represents the mean $\pm$ standard deviation of four separate experiments. * $p < 0.05$ vs S ODN; + $p < 0.05$ vs pcN-3.

Western blot analysis for COX-2 protein in the AGS cells showed similar inhibition with AS ODN treatment or transfection of the mutated $\text{I}\kappa\text{B}\alpha$ gene (Fig. 5B). This led to low levels of TXB_2 and 6-keto-PGF $_{1\alpha}$, metabolic products of COX-2 in the AGS cells (Fig. 6), which had relatively low levels of NF- κB in the nucleus (Fig. 2). From these results, we postulate that NF- κB seems to be activated to reach nuclear levels even in resting cells, and NF- κB mediates COX-2 expression and cell proliferation in AGS cells. Thus, inhibition of NF- κB activation either by suppressing translation of the NF- κB subunit p50 (AS ODN) or by suppressing the phosphorylation of $\text{I}\kappa\text{B}\alpha$ (IW-6 and IW-10) induces low COX-2 gene expression, which results in a low rate of cell proliferation in AGS cells.

Figure 5.

Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis for COX-2 on cells treated with ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene. Cells were treated with AS ODN or S ODN for 72 hours or transfected with control pcDNA3 vector (*pcN-3*) or the mutated $\text{I}\kappa\text{B}\alpha$ gene (*IW-6* and *IW-10*). For RT-PCR analysis, total RNA was isolated from the cells by the guanidine thiocyanate extraction method. mRNA expression of COX-2 was assessed using RT-PCR standardized by coamplifying this gene with the housekeeping gene β -actin (A). For Western blot analysis, cell lysates (100 μg of protein) were subjected to 8% SDS-PAGE and transferred onto membranes. COX-2 protein was visualized with a COX-2-specific antibody (B). Control cells received neither treatment nor transfection (Control).

Figure 6.

Levels of thromboxane B_2 (TXB $_2$) and 6-keto-prostaglandin $\text{F}_{1\alpha}$ (6-keto-PGF $_{1\alpha}$) of the cells treated with ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene. Cells were treated with AS ODN or S ODN for 72 hours or transfected with control pcDNA-3 vector (*pcN-3*) or the mutated $\text{I}\kappa\text{B}\alpha$ gene (*IW-6* and *IW-10*). The cells were plated at 5×10^4 cells/well and cultured for 24 hours. TXB $_2$ and 6-keto-PGF $_{1\alpha}$ were determined in the medium by ELISA. Control cells received neither treatment nor transfection (Control). Each bar represents the mean $\pm$ standard deviation of four separate experiments. * $p < 0.05$ vs S ODN; + $p < 0.05$ vs pcN-3.

Effects of COX-2 Inhibitors on Proliferation of AGS Cells

We examined whether inhibition of COX-2 would reduce the proliferation of AGS cells (Fig. 7). Treatment of a nonspecific COX-2 inhibitor, indomethacine, resulted in a dosage-dependant suppression of cell

Figure 7.

Effect of COX-2 inhibitors on cell proliferation of AGS cells, determined by the percentage of MTT-positive cells and thymidine incorporation. For the analysis of MTT-positive cells, 1×10^3 cells/well were treated with indomethacine or NS-198 for 96 hours. Viable cells were assessed by MTT assay and MTT positive cells were expressed as a percentage of control (A). For [^3H] thymidine incorporation, cells were plated at 5×10^4 cells/well and cultured for 24 hours. After addition of 1 $\mu\text{Ci}/\text{ml}$ of [^3H] thymidine, the cells were cultured for 6 hours. [^3H] thymidine incorporation of control cells was regarded as 100% and relative [^3H] thymidine incorporation was expressed as a percentage of control (B). Each bar represents the mean $\pm$ standard deviation of four separate experiments. * $p < 0.05$ vs control.

proliferation, as determined by percentage of MTT-positive cells after 96 hours in culture and [^3H] thymidine incorporation after 24 hours in culture. Similar results were obtained by treatment with a specific COX-2 inhibitor, NS-398. The inhibitory effect of NS-398 on cell proliferation was more potent than that of indomethacine. An approximately 50% inhibition of cell proliferation was reached by treatment with 200 μM of indomethacine and by 100 μM of NS-398. Similar inhibitory effects by indomethacine and NS-398 on COX-2 activity were observed, as determined by the levels of TXB $_2$ and 6-keto-PGF $_{1\alpha}$ in the medium after 24 hours in culture (Fig. 8). Thus, COX-2 expression may be one of the major pathways leading to cell proliferation in AGS cells.

Effect of PGE $_2$ on Proliferation of AGS Cells

After 24 hours in culture, the effect of PGE $_2$ treatment on cell proliferation was observed by [^3H] thymidine incorporation in AGS cells that were either treated with AS ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene (IW-6 and IW-10) (Fig. 9). Effects were also measured by the percentage of MTT-positive cells after 96 hours of culture (Fig. 10). After 24 hours of culture, PGE $_2$ had no effect on [^3H] thymidine incorporation of control cells, cells treated with S ODN, or cells transfected with pcN-3. However, PGE $_2$ stimulated [^3H] thymidine incorporation of the cells treated with AS ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene (IW-6 and IW-10), in a dose-dependant manner. This suggests that addition of PGE $_2$ prevented inhibition of cell

Figure 8.

Levels of TXB₂ and 6-keto-PGF_{1α} of the cells treated with COX-2 inhibitors. The cells received neither treatment nor transfection and were plated at 5×10^4 cells/well, treated with indomethacine or NS-398, and cultured for 24 hours. TXB₂ and 6-keto-PGF_{1α} were determined in the medium by ELISA. Control cells were not treated with COX-2 inhibitors (Control). Each bar represents the mean $\pm$ standard deviation of four separate experiments. * $p < 0.05$ vs corresponding control.

Figure 9.

Effect of prostaglandin E₂ (PGE₂) on proliferation of AGS cells treated with ODN or transfected with the mutated I κ B α gene, determined by thymidine incorporation. Cells were plated at 5×10^4 cells/well, treated with PGE₂ and cultured for 24 hours. After addition of 1 μ Ci/ml of [³H] thymidine, the cells were cultured for 6 hours. [³H] thymidine incorporation of control cells was regarded as 100% and relative [³H] thymidine incorporation was expressed as a percentage of control. Each bar represents the mean $\pm$ standard deviation of four separate experiments. * $p < 0.05$ vs the corresponding none.

proliferation in cells with low levels of NF- κ B in the nucleus. The stimulatory effect of PGE₂ on cell proliferation was observed in control cells, cells treated with S ODN, and in cells transfected with the control vector, as determined by the percentage of MTT-positive cells after 96 hours of culture (Fig. 10). The effect of PGE₂ on cell proliferation was greater in the

Figure 10.

Effect of PGE₂ on cell proliferation of AGS cells treated with ODN or transfected with the mutated I κ B α gene, determined by percentage of MTT-positive cells. 1×10^3 cells/well were treated with PGE₂ for 96 hours. Viable cells were assessed by MTT assay and MTT-positive cells were expressed as a percentage of control. Each bar represents the mean $\pm$ standard deviation of four separate experiments. * $p < 0.05$ vs the corresponding none.

cells treated with AS ODN or transfected with the mutated I κ B α gene than in control cells, cells treated with S ODN, or cells transfected with control vector.

Discussion

Histopathologic and epidemiologic studies showed that gastric cancer may be associated with hyperproliferation of gastric cells (Kemppainen et al, 1997; Persing and Prendergast, 1999) and this hypothesis was proven in studies on *Helicobacter pylori* infection (Correa, 1997; Guarner et al, 1993; Lynch and Axon, 1995; Moss, 1999). Increased proliferation of gastric cells by *Helicobacter pylori* was inhibited by supplementation of antioxidants and NF- κ B inhibitors (Bukin et al, 1993; Sharma et al, 1995). Although there is no direct evidence, these studies suggest that NF- κ B activation in gastric cells may be associated with hyperproliferation and gastric carcinogenesis. NF- κ B participates in cell proliferation, transformation, and tumor development (Siebenlist et al, 1994; Wulczyn et al, 1996). Several studies have demonstrated that inhibition of constitutive NF- κ B in B-cell lymphoma and breast cancer cells led to cell apoptosis, and that ectopic c-Rel expression promoted cell survival (Ar-sura et al, 1996; Sovak et al, 1997; Thanos and Maniatis, 1995). Furthermore, NF- κ B activation was linked to the protection of multiple types of cells from apoptosis (Wang et al, 1996).

In the present study, we found that unstimulated gastric cancer AGS cells had constitutive NF- κ B in the nucleus, and that inhibition of constitutive NF- κ B suppressed cell proliferation. These data suggest that NF- κ B may have an important role in human gastric cancer cell proliferation. In up to 96 hours of culture, inhibition of constitutive NF- κ B did not induce apo-

ptosis of AGS cells, as determined by Hoechst staining and DNA fragmentation (data not shown). This is in agreement with the report by Sawaoka et al (1998), which demonstrated that both NS-398 and indomethacine inhibited cell proliferation in gastric cancer MKN 45 cells in which COX-2 was overexpressed. However, neither NS-398 nor indomethacine induced apoptosis, as determined by DNA fragmentation. Some cancer cells, pancreatic cancer cells (Ding et al, 2000), and colorectal cancer cells (Chinery et al, 1998; Elder et al, 1997; Hara et al, 1997) showed growth inhibition and apoptosis by COX-2 inhibition. The differential effects on apoptosis by COX-2 inhibitors may be explained by different culture conditions (serum content in the medium, incubation time, etc.) and cell-type specificity.

In transformed cell lines, AS ODN to Rel A (p65) inhibited both cell proliferation and the ability of the cells to adhere to an extracellular matrix, and ablated tumor growth in mice (Higgins et al, 1993; Kitajima et al, 1992). NF- κ B is involved in the expression of adhesion molecules (Collins et al, 1995) and various enzymes including COX-2. Thus, autocrine actions of enzymes and mediators induced by NF- κ B could lock cells into permanently activated states, mimicking continuous growth-promoting signaling, and allowing easier adherence to extracellular matrix for carcinogenesis.

Of the possible target genes of NF- κ B, we focused on COX-2 expression because the human and murine COX-2 gene promoters contain a regulatory DNA sequence to which NF- κ B binds (Boolbol et al, 1996), and COX-2 expression is regulated by NF- κ B in several cell lines (Boolbol et al, 1996; Hla and Neilson, 1992). In some cancer cells, COX-2 is involved in the regulation of cell proliferation (Martinez et al, 1997; Sheng et al, 1997). In the present study, we demonstrated that NF- κ B regulated COX-2 expression and prostaglandin synthesis in AGS cells, as determined by the levels of TXB₂ and 6-keto-PGF_{1 α} in the medium. COX-2 expression was necessary for mitogen- or endotoxin-induced production of prostaglandins in murine fibroblasts and macrophages, whereas the COX-1 present in these cells cannot utilize arachidonic acid released in response to mitogen or endotoxin stimulation (Shimakura and Boland, 1992). Consequently, prostaglandins induced by COX-2 may exert different effects from those induced by COX-1. COX-2 inhibitors suppressed prostaglandins and cell proliferation in several gastrointestinal cancer cells (Nishikawa et al, 1997), pancreatic cancer cells (Ding et al, 2000) and colorectal cancer cell lines (Elder et al, 1997; Hara et al, 1997). This suggests that prostaglandins induced by COX-2, not by COX-1, affect cell proliferation in those cells. PGE₂ production by COX-2-stimulated cell proliferation in some cancer cells (Battu et al, 1998; Higashi et al, 2000; Molina et al, 1999; Sheng et al, 1997) was confirmed in present study. We used nonspecific (indomethacine) and specific (NS-398) COX-2 inhibitors to suppress COX-2 expression and demonstrated that NS-398 was more potent than indomethacine in inhibiting cell proliferation

and COX-2 activity, as determined by the levels of TXB₂ and 6-keto-PGF_{1 α} . NS-398 has been as effective as AS ODN for COX-2 in lowering prostaglandin production and cell proliferation in gastric epithelial cells (Sawaoka et al, 1999). Therefore, we could postulate that suppression of COX-2 expression by NS-398 might effectively inhibit cell proliferation, similar to the effect of AS ODN for COX-2. Although we did not use AS ODN for COX-2 in present study, the results obtained from NS-398 demonstrate that cell proliferation is related to COX-2 expression in AGS cells. Clearly, the present study shows that NF- κ B regulates COX-2 expression, and that inhibition of COX-2 suppresses proliferation of gastric cancer AGS cells.

Although the present results demonstrate that NF- κ B and COX-2 are involved in gastric cancer cell proliferation, the role of NF- κ B and COX-2 in gastric carcinogenesis remained to be determined. The members of the NF- κ B family have been identified as protooncogenes (Wulczyn et al, 1996). Several investigators indicated that NF- κ B was aberrantly activated in B-cell lymphoma, in human breast cancer, and in rat mammary tumors from the aromatic hydrocarbon-induced model of breast cancer. Antioxidants such as pentoxifylline and N-acetyl cysteine, which suppress NF- κ B activation, are in clinical use for treating some cancers (Biswas et al, 1993; Chinery et al, 1998). Ristimaki and colleagues (1997) reported that mRNA expression of COX-2 in human gastric carcinoma tissue was significantly higher than that in noncancerous adjacent tissue. Additionally, current users of aspirin, a nonspecific COX-2 inhibitor, had a decreased risk for gastric cancer in a case control study (Farrow et al, 1998).

The present study demonstrates that inhibition of constitutive NF- κ B results in inhibition of COX-2 expression and proliferation of gastric cancer cells. Thus, NF- κ B may have a novel role in gastric cancer cell proliferation via COX-2 expression. NF- κ B inhibitors and COX-2 inhibitors may be potentially useful for the treatment of gastric cancer. However, further study should be performed to elucidate the precise role of constitutive NF- κ B and COX-2 in initiation and promotion of gastric carcinogenesis.

Materials and Methods

Cell Culture

Human gastric cancer AGS cells (gastric adenocarcinoma, ATCC CRL 1739) were obtained from the American Type Culture Collection (Rockville, Maryland) and cultured in RPMI-1640 medium (pH 7.4) (Sigma, St. Louis, Missouri), supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, New York) and antibiotics (100 U/ml of penicillin and 100 μ g/ml of streptomycin).

ODN Preparation

Single-stranded ODN were produced commercially (GIBCO BRL). Phosphothioate-modified ODN were used to reduce intracellular nuclease digestion. AS

ODN and S ODN targeted the ATG start codon of the p50 mRNA. The sequence of the p50 AS ODN was 5' GGA TCA TCT TCT GCC ATT CTG 3'. The sequence of p50 S ODN was 5' CAG AAT GGC AGA AGA TGA TCC 3'.

Treatment with ODN Using Cationic Liposome

AGS cells were treated with ODN using a cationic liposome, a commercially available transfection reagent, DOTAP (N-[1-(2, 3-dioleoyloxy) propyl]-N, N,N-trimethyl ammonium methylsulfate) (Boehringer-Mannheim, Mannheim, Germany), to improve stability and intracellular delivery of ODN (Lappalainen et al, 1994). When DOTAP was used, the appropriate amount of ODN was incubated with DOTAP (15 μ l/ml) and a final concentration of the ODN of 0.5 μ M at 37° C for 15 minutes. The mixture was added directly to the cells, plated at 2×10^5 cells/ml in 12-well plates, and incubated for 55 hours. Cells were trypsinized and plated again at a density of 5×10^5 cells/ml in 6-well plates. A final concentration of 0.5 μ M of ODN was added to the cells and incubated for an additional 17 hours. Western blot analysis for p50 (Fig. 1A) and EMSA for NF- κ B (Fig. 2A) were performed on the nuclear extract of the cells treated with AS ODN or S ODN and control cells, which received no treatment.

Transfection with a Mutated I κ B α Gene

As a mutated I κ B α gene, the MAD-3 double point mutant (substitution of two serine residues by alanine residues at positions 32 and 36) construct was prepared as described previously (Traenckner et al, 1995). The control vector pcDNA3 (Invitrogen, Carlsbad, California) was transfected into the cells instead of the mutated I κ B α gene. These cells were regarded as a relative control and named pcN-3. Subconfluent AGS cells, plated in 10 cm culture plates, were transfected with 10 μ g of expression construct using DOTAP for 16 hours. After transfection, the cells were trypsinized and plated at 1×10^3 cells per 10 cm tissue culture plate. The cells were cultured in medium containing 400 μ g/ml of G-418 (GIBCO BRL) for 15 to 17 days. Three to four resistant clones were isolated from each plate and examined for mutated I κ B α expression by Western blot analysis. The positive clones for mutated I κ B α were maintained in culture medium containing 200 μ g/ml of G418 for more than 2 months and named IW-6 and IW-10. Western blot analysis for wild-type I κ B α , phosphorylated I κ B α , and mutated I κ B α (Fig. 1A) and EMSA for NF- κ B (Fig. 2A) were performed on cells transfected with the control vector (pcDNA3) or the mutated I κ B α gene and on control cells that received no transfection.

Western Blot Analysis for P50, I κ B α , and COX-2

Cells treated with ODN or transfected with the mutated I κ B α gene were homogenized in Tris-HCl (pH 7.4) buffer containing 0.5% Triton X-100 and a protease inhibitor cocktail (Boehringer-Mannheim, Indianapolis, Indiana) for determination of I κ B α and COX-2.

Nuclear extract was prepared as described in the next paragraph for the determination of p50. The protein concentration of each sample was measured by the method used in Bradford (1976). One hundred micrograms of cellular protein (for I κ B α and COX-2) or 20 μ g of nuclear protein (for p50) was loaded per lane, separated by 8% SDS-polyacrylamide gel electrophoresis under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Inc., Arlington Heights, Illinois) by electroblotting. The transfer of protein and equality of loading in all lanes was verified using reversible staining with Ponceau S. Membranes were blocked using 5% nonfat dry milk. Wild-type I κ B α , phosphorylated I κ B α , mutant-type I κ B α , COX-2 protein, and p50 protein were detected by incubation of the blots with the corresponding monoclonal antibodies (Cayman Chemical, Ann Arbor, Michigan) at a dilution of 1:1000 overnight at 4° C. Blots were incubated with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham) using exposure to BioMax MR film (Kodak, Rochester, New York) was determined.

Extraction of Nuclei

The cells, treated with ODN or transfected with the mutated I κ B α gene, were rinsed with ice-cold phosphate buffered saline (PBS), harvested by scraping into PBS, and pelleted by centrifugation at 1500 $\times$ g for 5 minutes. The cells were lysed in buffer containing 10 mM Hepes, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1.5 mM MgCl₂, 0.2% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonylfluoride (PMSF). The nuclear pellet was resuspended on ice in nuclear extraction buffer containing 20 mM Hepes, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 25% glycerol, 1 mM DTT, and 0.5 mM PMSF (Li et al, 1997), and the nuclear protein concentration was determined by the method used in Bradford (1976).

EMSA

The NF- κ B gel shift oligonucleotide, 5'AGT TGA GGG GAC TTT CCC AGG C-3' (Promega Corporation, Madison, Wisconsin) was labeled with [³²P] dATP (Amersham) using a T4 polynucleotide kinase (GIBCO BRL). An end-labeled probe was purified from unincorporated [³²P] dATP using a Bio-Rad purification column (Bio-Rad Laboratories, Hercules, California) and recovered in Tris-EDTA buffer. Nuclear extracts (1 μ g) were preincubated in buffer containing 12% glycerol; 12 mM Hepes, pH 7.9; 4 mM Tris-HCl, pH 7.9; 1 mM EDTA; 1 mM DTT; 25 mM KCl; 5 mM MgCl₂; 0.04 μ l/ml of poly[d(I-C)] (Boehringer Mannheim); 0.4 mM PMSF; and Tris-EDTA buffer. The labeled probe was added and samples were incubated on ice for 10 minutes. Samples were subjected to electrophoresis at room temperature on a nondenaturing 5% acrylamide gel at 30 mA using 0.5x Tris borate EDTA buffer. The gels were dried at 80° C for 1 hour and exposed to radiog-

raphy film for 6 to 18 hours at -70°C with intensifying screens (Li et al, 1997).

RT-PCR Analysis for COX-2

Gene expression of COX-2 mRNA was assessed using RT-PCR standardized by coamplifying COX-2 with the housekeeping gene β -actin, which served as an internal control. Total RNA was isolated from the cells, treated with ODN, or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene by the guanidine thiocyanate extraction method (Chomczynski and Sacchi, 1987). Total RNA was reverse transcribed into cDNA and used for PCR with human specific primers for COX-2 and β -actin. Sequences of COX-2 primers were 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' (forward primer) and 5'-AGATCATCTCTGCCTGAGTATCTT-3' (reverse primer), giving a 305 bp PCR product (Hla and Neilson, 1992). For β -actin, the forward primer was 5'-ACCAACTGGGACGACATGGAG-3' and the reverse primer was 5'-GTGAGGATCTTCATGAGGTA-GTC-3', giving a 349 bp PCR product (Nakajima-Iijima et al, 1985). Briefly, the PCR was amplified by 32 repeat denaturation cycles at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. During the first cycle, the 95°C step was extended to 2 minutes, and on the final cycle the 72°C step was extended to 5 minutes. PCR products were separated on 1.5% agarose gels containing 0.5 $\mu\text{g/ml}$ of ethidium bromide and visualized by UV transillumination.

Determination of Cell Proliferation

Proliferation of the cells treated with ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene was determined by four methods: trypan blue exclusion, MTT assay, [^3H] thymidine incorporation, and colony formation. Control cells received neither treatment nor transfection. For the trypan blue exclusion test, the cells were plated at 2×10^4 cells/well in a 24-well culture plate and incubated for 24, 48, 72, and 96 hours. Cell number was counted on a hemocytometer using 0.2% trypan blue. For the MTT assay, the cells (1×10^3 cells/well) were plated into a 96-well culture plate and cultured for 96 hours. MTT (0.5 mg/ml) was added and the reaction mixture was incubated for 4 hours at 37°C . Cellular formazan was extracted with acid propan-2-ol and the absorbance was measured with a dual wavelength automatic plate reader at 570 nm/630 nm (Yang et al, 1998). Viable cell number was expressed as the percentage of MTT-positive cells; control cells were regarded as 100%. For [^3H] thymidine incorporation, the cells were plated at 5×10^4 cells/well in a 24-well culture plate. After 24 hours of incubation, 1 $\mu\text{Ci/ml}$ of [^3H] thymidine (Amersham) was added to the cells, which were then incubated for 6 hours. After washing with PBS, the cells were incubated with 10% trichloroacetic acid for 30 minutes. The cells were washed, incubated with a solution containing 0.3 M NaOH and 1% sodium dodecyl sulfate for 1 hour, and the radioactivity of the cell

lysate was counted as cpm by a liquid scintillation counter. [^3H] thymidine incorporation, reflecting DNA synthesis (Chinery et al, 1998), of control cells was regarded as 100% and relative [^3H] thymidine incorporation was expressed as a percentage of the control value. For colony formation, a soft agar assay was performed using a double-layer agar system (Sevignani et al, 1998). One milliliter of basal layer culture medium (RPMI medium with 10% FBS) with a final 0.5% agarose (GIBCO-BRL) concentration was prepared in 35-mm culture dishes. The cells, at 2×10^3 cells/ml, in 1 ml of upper layer medium (0.2% agarose) were overlaid onto the preformed basal layer. After 10 days of incubation, colonies were stained with crystal violet and colonies with greater than 100 cells were counted. Cell proliferation was determined as mean colony numbers, compared with those of control (100%). In addition, control cells that had neither treatment with ODN nor transfection with the mutated $\text{I}\kappa\text{B}\alpha$ gene, were treated with 10, 20, 50, 100, and 200 μM of COX-2 inhibitors, such as indomethacin (Sigma) and NS-398 (Alexis Biochemicals, San Diego, California), or 14, 140, and 1400 nm of PGE₂ (Sigma). The drug concentrations used in the present study were adapted from previous studies for COX-2 inhibitors (Ding et al, 2000; Elder et al, 1997; Hara et al, 1997) and PGE₂ (Millet et al, 1998; Monteleone et al, 1999; Yamamoto et al, 1999). Cell proliferation was determined by MTT assay and [^3H] thymidine incorporation as described above.

Determination of TXB₂ and 6-Keto-PGF_{1 α}

The cells, treated with ODN or transfected with the mutated $\text{I}\kappa\text{B}\alpha$ gene, were plated at 5×10^4 cells/well in 24-well culture plates and cultured for 24 hours. Levels of TXB₂ and 6-keto-PGF_{1 α} were determined in the medium by ELISA kits (R&D System, Minneapolis, Minnesota). Additionally, control cells that received neither treatment with ODN nor transfection with the mutated $\text{I}\kappa\text{B}\alpha$ gene, were treated with 10, 20, 50, 100, and 200 μM of COX-2 inhibitors and levels of TXB₂ and 6-keto-PGF_{1 α} were determined in the medium. Purified human recombinant TXB₂ and 6-keto-PGF_{1 α} were used as standards. Levels of TXB₂ and 6-keto-PGF_{1 α} were expressed as picograms per 10^6 cells.

Statistical Analysis

Results are expressed as means $\pm$ standard deviation of four separate experiments. ANOVA followed by Newman-Keul's test was used for statistical analysis (Zar, 1984). $p < 0.05$ was regarded as statistically significant.

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